# Pesticide residues in food — 2023

## Joint FAO/WHO Meeting on Pesticide Residues

# EVALUATIONS 2023

## Part II – Toxicological

Food and Agriculture Organization of the United Nations

World Health Organization

# Pesticide residues in food – 2023

## Toxicological evaluations

**Sponsored jointly by FAO and WHO**

**Joint Meeting of the**
**FAO Panel of Experts on Pesticide Residues**
**in Food and the Environment**
**and the**
**WHO Core Assessment Group on Pesticide Residues**

**Washington D.C., United States of America, 19–28 September 2023**

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* First full evaluation

** Evaluated within the periodic review programme of the Codex Committee on Pesticide Residues

## Abbreviations and acronyms

| | |
|---|---|
| ACh | acetylcholine |
| AChE | acetylcholinesterase |
| ACTH | adrenocorticotropic hormone |
| AD | administered dose |
| ADI | acceptable daily intake |
| ADME | absorption, distribution, metabolism and excretion |
| A/G, A:G | albumin:globulin ratio |
| ALP | alkaline phosphatase |
| ALT | alanine transaminase |
| APTT | activated partial thromboplastin time |
| AR | Administered (applied) radioactivity |
| AR | Androgen receptor (context related) |
| ARfD | acute reference dose |
| AST | aspartate transaminase |
| AUC | Area under the concentration-time curve |
| BMD | benchmark dose |
| $BMDL_{10}$ | Lower confidence limit on the benchmark dose for a 10% response |
| BrdU | 5-bromo-2′-deoxyuridine |
| BROD | benzoxyresorufin-*O*-dealkylase |
| BUN | blood urea nitrogen |
| bw | body weight |
| ca | circa (approximately) |
| CAR | constitutive androstane receptor |
| CAS | Chemical Abstracts Service (No.) |
| CCPR | Codex Committee on Pesticide Residues |
| ChE | cholinesterase |
| CHO | Chinese Hamster ovary |
| $C_{max}$ | maximum concentration |
| CMC | carboxymethyl cellulose |
| CPA | cyclophophamide |
| CPN | chronic progressive nephropathy |
| DNT | dihydrotestosterone |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| EC3 | the amount of test substance needed to induce a 3-fold increase in cell proliferation in a LLNA |
| $EC_{50}$ | the half-effective concentration value |
| ELISA | enzyme-linked immunosorbent assay |
| EPA | Environmental Protection Agency |
| ER | estrogen receptor |
| EROD | ethoxyresorufin-O-deethylase |
| FAO | Food and Agriculture Organization of the United Nations |
| FOB | functional observational battery |
| FSH | follicle-stimulating hormone |
| $GABA_A$ | γ-aminobutyric acid, type A |
| GC | gas chromatography |
| GC–MS | gas chromatography–mass spectrometry |
| GD | gestation day |
| GGTP | γ-glutamyl transpeptidase/transferase |
| GI | gastrointestinal |
| GLP | good laboratory practice |
| GSD | geometric standard deviation |
| GST-P | glutathione-S-transferase placental form |
| HBGV | health-based guidance values |
| Hb | haemoglobin |
| HCA | α-hexylcinnamaldehyde |
| HCD | historical control data |
| hCG | human chorionic gonadotropin |
| (U)HPLC | (ultra-)high-performance liquid chromatography |
| HPLC-MS/MS | tandem high-performance liquid chromatography/mass spectrometry |
| HPRT | hypoxanthine–guanine phosphoribosyl transferase |
| Ht | haematocrit |
| $IC_{50}$ | half-maximal inhibitory concentration |
| i.p. | intraperitonial, intraperitonially |
| ISO | International Organization for Standardization |
| IUPAC | International Union of Pure and Applied Chemistry |
| i.v. | intravenous/intravenously |
| JECFA | Joint FAO/WHO Expert Committee on Food Additives |
| JMPR | Joint FAO/WHO Meeting on Pesticide Residues |
| LAH | lactic acid hydroxylase |
| LC | liquid chromatography |
| LC-MS | liquid chromatography–mass spectrometry |
| LC-MS/MS | Liquid chromatography with tandem mass spectrometry |
| $LC_{50}$ | median lethal concentration |
| LD | lactation day |
| $LD_{50}$ | median lethal dose |

| | |
|---|---|
| LDH | lactate dehydrogenase |
| LH | luteinizing hormone |
| LLNA | local lymph node assay |
| LOAEL | lowest-observed-adverse-effect level |
| LOAEC | lowest-observed-adverse-effect concentration |
| LOQ | limit of quantitation |
| LSC | liquid scintillation counting |
| MA | motor activity |
| mADI | microbiological ADI |
| mARfD | microbiological ARfD |
| MAC | mean acieved concentation |
| MCH | mean corpuscular/cell haemoglobin |
| MCHC | mean corpuscular haemoglobin concentration |
| MCV | mean corpuscular (cell) volume |
| MF | mutation frequency |
| MLA | mouse lymphoma assay |
| MMAD | mass median aerodynamic diameter |
| MOA | mode of action |
| MOEC | maximum-observed-effect concentration |
| MRL | maximum residue limits |
| MS | mass spectroscopy/spectrometry |
| MTD | maximum tolerated dose |
| MTT | 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide, or methylthiazole tetrazolium |
| NCE | normochromic erythrocyte |
| NMR | nuclear magnetic resonance |
| NOAEC | no-observed-adverse-effect conc. |
| NOAEL | no-observed-adverse-effect level |
| NOEL | no-observed-effect level |
| NTE | neuropathy target esterase |
| OECD | Organisation for Economic Co-operation and Development |
| PB | phenobarbital (sodium) |
| PCE | polychromatic erythrocyte |
| PCE:NCE ratio | Ratio of polychromatic erythrocytes to normochromic erythrocyte |
| PCNA | proliferating cell muclear antigen |
| PCA | polymerase chain reaction |
| PCV | packed cell volume |
| PEG | polyethylene glycol |
| PND | postnatal day |
| PP(D) | postpartum (day) |
| PROD | pentoxyresorufin-*O*-deethylase |
| PT | prothrombin time |
| PXR | pregnane X receptor |
| QSAR | quantitative structure–activity relationship |
| RBC | red blood cell (erythrocyte) |
| RTG | relative total growth |
| S9 | Rat liver supernatant obtained by centrifuging at 9000 *g* |
| SCE | sister chromatid exchange |
| SD | standard deviation |
| SDH(I) | succinate dehydrogenase (inhibitor) |
| SI | stimulation index |
| SMILES | simplified molecular input line entry system |
| sRBC | sheep red blood cell |
| $t_{½}$ | half-life |
| T3 | triiodothyronine |
| T4 | thyroxine |
| TFT | 5-trifluorothymidine |
| TLC | thin-layer chromatography |
| $T_{max}$ | time to reach maximum concentration |
| TPO | thyroid peroxidase |
| TRR | total recovered radioactivity |
| TSH | thyroid-stimulating hormone |
| TTC | threshold of toxological concern |
| UDP-GT | uridine diphosphate glucuronosyltransferase |
| UDS | unscheduled DNA synthesis |
| UL | uniformly labelled |
| WBC | white blood cell/leucocyte count |
| WHO | World Health Organization |
| v/v | volume/volume |
| w/v | weight/volume |
| w/w | weight/weight |

# Introduction

The Meeting was opened by Mr Soren Madsen, WHO JMPR secretary. On behalf of FAO and WHO, Mr Madsen welcome and thanked the participants for providing their expertise and for devoting significant time and efforts to the work of JMPR, noting that this was the first physical JMPR meeting in the United States. Mr Madsen welcomed the new FAO secretariat, new panel members, as well as the new FAO editors. He further expressed his gratitude to the USEPA for hosting the 2023 JMPR meeting and invited Dr Edward Messina, director of the Office of Pesticide Programme of the US Environmental Protection Agency to address the Meeting. Dr Messina welcome the participants to USEPA and emphasized the invaluable contribution of JMPR to the scientific assessment of pesticide residues, He noted that the JMPR evaluations are well-known and recognized not only by governmental regulatory bodies but also by industry, and the public. The JMPR efforts to ensure that pesticides are used safely and effectively are a beacon for regulatory authorities around the world. The director highlighted the role of JMPR in protecting human health and fostering international trade by ensuring the safety of food and agricultural products.

Dr Guibiao Ye, the new FAO JMPR secretariat, took the opportunity to thank WHO for taking a leading role in organizing the meeting. He highlighted the importance of JMPR in food safety and food security. Dr Ye thanked experts for their hard work and great contribution to JMPR and looked forward to working together with them to achieve more for food safety in future.

Prior to the Meeting, the FAO and WHO JMPR Secretariats received declarations of conflicts of interest from their respective panels of experts. The WHO JMPR Secretary reported that all 36 WHO experts had completed the necessary identification and confidentiality forms, with 16 of them declaring interests. However, a review by the WHO Secretariat of these declarations did not identify any conflicts of interest that would impair the full participation of the 16 experts in any of the agenda items for JMPR 2023. The FAO JMPR Secretariat received declarations from all 17 of its experts, and upon review, identified no conflicts of interest that would affect their full participation in JMPR 2023 agenda items. The FAO secretariats reported the reviewing process and conclusions to the Meeting. Additionally, a list of participants and their professional profiles was published on the websites of FAO and WHO two weeks before the meeting. No objections to the selected experts were received from the public, thus both groups of experts from FAO and WHO were cleared for full participation without any identified conflicts of interest.

During the Meeting, the FAO Panel of Experts was responsible for reviewing residue and analytical aspects of the pesticides under consideration, including data on their metabolism, fate in the environment and use patterns, and for estimating the maximum levels of residues that might occur as a result of use of the pesticides according to good agricultural practice (GAP). Maximum residue levels and supervised trials median residue (STMR) values were estimated for commodities of animal origin. The WHO Core Assessment Group was responsible for reviewing toxicological and related data in order to establish acceptable daily intakes (ADIs) and acute reference doses (ARfDs), where necessary.

The Meeting evaluated 35 pesticides, including seven new compounds and four compounds that were re-evaluated within the periodic review programme of the CCPR, for toxicity or residues, or both.

The Meeting established ADIs and ARfDs, estimated maximum residue levels and recommended them for use by CCPR, and estimated STMR and highest residue (HR) levels as a basis for estimating dietary intake.

The Meeting also estimated the dietary exposures (both short-term and long-term) of the pesticides reviewed and, on this basis, performed a dietary risk assessment in relation to the relevant ADI and where necessary ARfD. Cases in which ADIs or ARfDs may be exceeded were clearly indicated in order to facilitate the decision-making process by CCPR.

The Meeting considered a number of current issues related to the risk assessment of chemicals, the evaluation of pesticide residues and the procedures used to recommend maximum residue levels.

# TOXICOLOGICAL MONOGRAPHS AND MONOGRAPH ADDENDA

# 1,4-Dimethylnaphthalene

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# Explanation

1,4-Dimethylnaphthalene (1,4-DMN) is the common and systematic (IUPAC) name for this compound, Chemical Abstracts Service No. 571-58-4. It belongs to the chemical family of alkylated naphthalenes.

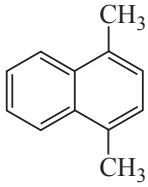
1,4-Dimethylnaphthalene acts as a plant growth regulator preventing sprouting of potato tubers. Besides being synthetically produced, it is also occurs naturally in different plant species (for example potato, rhubarb, poppy). 1,4-Dimethylnaphthalene acts by enhancing dormancy, a natural event triggered by biochemical substances found within the potato (*Solanum tuberosum*).

1,4-Dimethylnaphthalene has not previously been evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed by the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR).

1,4-Dimethylnaphthalene (see Fig. 1) occurs naturally in food with background values up to 60 µg/kg in potato, 0.4 µg/kg in poppy and up to 12 µg/kg in poppy tops. Consistent with other regulatory authorities' reduced data requirements for naturally occurring substances, a reduced data package was submitted to JMPR. Toxicological studies in animals provided in the dossier for 1,4-DMN were limited, especially absorption, distribution, excretion and metabolism (ADME) studies in mammals, and studies of short-term toxicity in second species, long-term toxicity and carcinogenicity, neurotoxicity and reproductive effects. Although background concentrations of 1,4-DMN are usually low, they will lead to constant dietary exposure in humans. Indeed, a much higher exposure of human beings to 1,4-DMN is of anthropogenic origin. The compound is a constituent of mineral oil and gas and as such, continuous exposure can hardly be avoided in modern society.

All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with current Organisation for Economic Co-operation and Development (OECD) test guidelines unless otherwise specified. A number of additional papers were identified from a literature search that complemented the toxicological information submitted for the current assessment. Based on its evaluation of the data, the current Meeting considered it sufficient for risk assessment.

***Figure 1. Chemical structure of 1,4-dimethylnaphthalene (1,4-DMN)***




# Evaluation for acceptable daily intake

## 1. Biochemical aspects

### 1.1 Absorption, distribution and excretion

***(a) Oral route***

***Rat***

Three male rats (age 12–16 weeks, body weights 385–389 g) received ring-labelled [$^{14}$C]1,4-DMN (specific activity $3.34 \times 10^6$ dpm/mg) as a solution in corn oil, by oral gavage. The mean dose delivered was 28.6 mg/kg body weight (bw).

The animals were housed in glass metabolism cages that allowed separate collection of urine and faeces. All test animals had unrestricted access to food and water throughout the study. Urine, cage wash and faeces were collected at 24 and 48 hours after administration and were stored frozen until analysis. All samples were dispensed by weight. The $^{14}$C contents of urine and cage wash were measured by liquid scintillation counting (LSC) without further sample preparation. The radiocarbon contents of faeces were measured by combustion analysis and LSC.

Urine samples were extracted by liquid partition into dichloromethane. Metabolites were analyzed by high-performance liquid chromatography with tandem mass spectrometry (HPLC–MS/MS) or gas chromatography-mass spectrometry (GC–MS) and identified by co-injection and comparison with reference substances.

All test animals appeared normal and healthy throughout the study. Animals were sacrificed 48 hours after dose administration.

The mean recovery of administered radiocarbon was 99.1% (range 94.1%–102.8%). On average, 71.6% of the administered radiocarbon was eliminated in the urine (range 69.7%–73.4%). The mean faecal excretion of radiocarbon was 27.5% (range 20.7%–31.1%) of administered dose (AD) as shown in Table 1. The amount of radioactivity remaining in the total body at sacrifice was not analysed, since 99.1% of radioactivity (mean) was found in urine and faeces within 48 h. The highest amounts of radioactive residues were found within the first 24 h after administration and were mostly found in urine (Table .1).

***Table 1. Recovery of radioactivity in excreta of male rats after a single oral administration of 28.6 mg/kg bw of $^{14}$C-labelled 1,4-dimethylnaphthalene***

| | Mean % of radioactive dose recovered | |
|---|---|---|
| | **0–24 hours** | **0–48 hours** |
| Expired air | Not analyzed | Not analyzed |
| Tissues and carcass | Not analyzed | Not analyzed |
| Cage wash | 5 | 5.6 |
| Urine | 59.3 | 66 |
| Faeces | 13.4 | 27.5 |
| Sum of excreta | 77.7 | 99.1 |
| Total recovery | 77.7 | 99.1 |

Source: Dohn, Ostermann, & Woodbury, 2012

The parent molecule (1,4-DMN) was not detected in urine by HPLC, but trace amounts were confirmed to be present by GC–MS. This study only partially fulfils the data requirements of OECD 417 (22 July 2010) (Dohn, Ostermann, & Woodbury, 2012).

**(*b*) *Intraperitoneal route***

***Rat***

In a published study (not GLP), the distribution, excretion and metabolism of 1,4-DMN was investigated following intraperitoneal administration.

Male rats (weight 200–220 g) received a single intraperitoneal dose of 28 mg/kg bw (approximately 555 kBq per animal) of ring-labelled [U-$^{3}$H]1,4-DMN (specific activity 751 MBq/g). Immediately after administration the rats were placed in individual metabolism cages. Urine and faeces were collected. Blood samples were collected from the tail vein. Rats were decapitated under light-ether narcosis at 4, 8, 24, 48 and 72 hours after administration, with five rats killed at each time point. Several tissues were removed for measurement of the tritium radiolabel. The total balance of tritium was reported for the periods 0–24 hours, 0–48 hours and 0–72 hours in urine, faeces, blood cells plus plasma, liver, adipose tissue, muscles, and the remaining tissues.

After 72 hours about 100% of AD had been found if recovery from urine, faeces, blood cells and plasma, liver, adipose tissue, muscles, and remaining tissues is totalled. The level of tritium in plasma and in all examined tissues reached a maximum four hours after compound administration (that is, at the first measurement). Tritium was widely distributed; after around four hours the highest concentrations of tritium were observed in adipose tissue, followed by liver, kidneys, spleen and adrenals (Table 2).

***Table 2. Distribution of radioactivity in male rat tissues/organs after a single intraperitoneal administration of 28 mg/kg bw of ring-labelled [$^3H$]1,4-dimethylnaphthalene***

| Distribution of radioactivity | % of administered dose (mean ± SD for five animals) | | |
|---|---|---|---|
| | 0–24 h | 0–48 h | 0–72 h |
| Urine | 41.3 ± 10.5 | 52.8 ± 5.4 | 56.5 ± 4.0 |
| Faeces | 21.9 ± 7.3 | 36.3 ± 4.4 | 40.8 ± 3.2 |
| Blood cells and plasma [a] | 1.3 ± 0.0 | 0.9 ± 0.0 | 0.8 ± 0.0 |
| Liver | 0.7 ± 0.0 | 0.5 ± 0.0 | 0.2 ± 0.0 |
| Adipose tissue [b] | 13.9 ± 0.2 | 2.7 ± 0.1 | 0.6 ± 0.0 |
| Muscles [c] | 3.8 ± 0.0 | 3.5 ± 0.0 | 1.4 ± 0.0 |
| Remaining tissues | 0.3 | 0.2 | 0.2 |
| Balance | 83.1 | 97.0 | 100.5 |

SD: Standard deviation; Source: Kilanowicz, Sapota, & Daragó, 2000

Values were rounded to one decimal place.

[a] Blood was assumed to be 7 mL/100 g bw;

[b] Fat tissues were assumed to be 12% of the whole body weight;

[c] Muscles were assumed to be 40% of the whole body weight

### *Excretion*

The time dependent concentration curve of tritium in blood plasma and red blood cells (RBC) during the 72 hours following administration of [$^3$H]1,4-DMN at a dose of 28 mg/kg is presented in Fig. 2. The maximum level ($C_{max}$) of tritium in plasma (0.53 kBq/mL) was reached during the fourth hour after administration of the compound , as shown in Table 3. Following this maximum 1,4-DMN concentration rapidly declined.

***Table. 3 Kinetic parameters extractable from the original paper***

| Sex | Dose (intraperitoneal) | $C_{max}$ (plasma) | $T_{max}$ | Half-life |
|---|---|---|---|---|
| Male | 28 mg/kg bw | *0.53 kBq/mL* [a] | *4.2 hours* [b] | 8 hours |

$C_{max}$: Maximum concentration; $T_{max}$: Time to $C_{max}$;

Derived from Kilanowicz, Sapota, & Daragó, 2000;
Information in *italics* is not given in the report, but included by the applicant

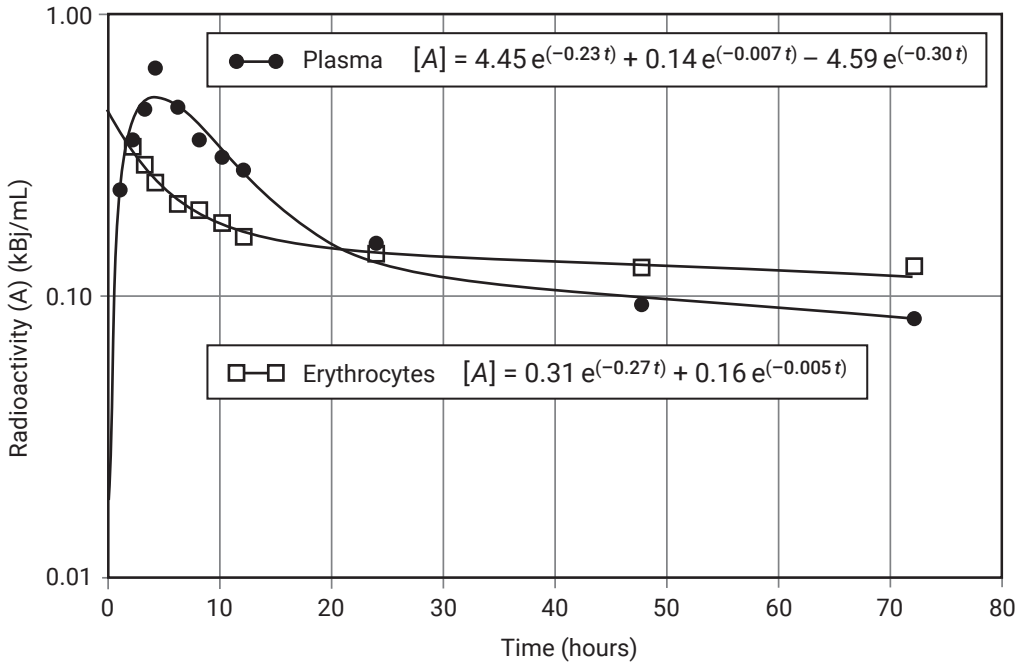
For all calculations, the reported equation for time-dependent tritium concentration in blood plasma was used as follows:
$A(t) = 4.45 \times e^{(-0.23 \times t)} + 0.14 \times e^{(-0.007 \times t)} - 4.59 \times e^{(-0.30 \times t)}$

[a] $C_{max}$ is not given in the report, but was calculated based on $T_{max}$= 4.2 and the kinetics of $^3$H distribution in plasma:
$A(4.2) = 4.45 \times e^{(-0.23 \times 4.2)} + 0.14 \times e^{(-0.007 \times 4.2)} - 4.59 \times e^{(-0.30 \times 4.2)}$

[b] $T_{max}$ was determined by calculating the maximal turning point of $^3$H distribution in plasma:
$A = 4.45 \times e^{(-0.23 \times t)} + 0.14 \times e^{(-0.007 \times t)} - 4.59 \times e^{(-0.30 \times t)}$

According to the study report, the most rapid decrease in excreted radioactivity activity was observed in all examined tissues in the periods 4–8 hours and 8–24 hours after administration. The half-life in blood plasma was about eight hours. After 72 hours more than 97% of the administered tritium had been excreted via urine (56.5%) and faeces (40.8%). Since administration was by the intraperitoneal route, faecal excretion corresponds to the excretion via bile. At 72 hours after intraperitoneal administration, tissue levels (including adipose tissue) of tritium were very low due to nearly complete excretion, indicating that 1,4-DMN and its metabolites do not accumulate in the male rat (Table 2).

***Figure 2. Kinetics of $^{3}H$ distribution in erythrocytes and plasma following a single, intraperitoneal administration of 1,4-DMN-[$^{3}H$] at a dose of 28 mg/kg bw in rats***




The above study was not in compliance with the current OECD guideline 417 (July 2010), nor was the study conducted under GLP. Though this is a published study, no suitable justification was given for the administration of a single dose via intraperitoneal route only, nor as to why there was only the identification of urinary metabolites, not faecal ones. (Kilanowicz, Sapota, & Daragó,2000).

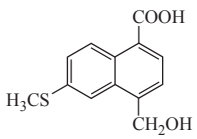
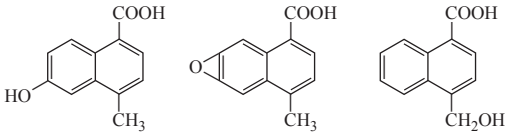
## 1.2 Biotransformation

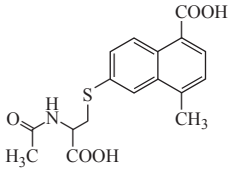
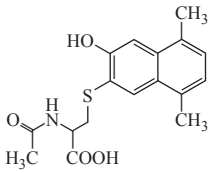
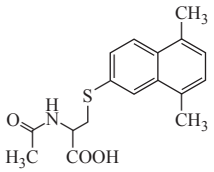
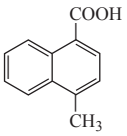
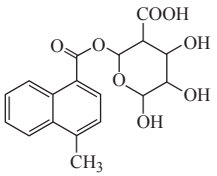
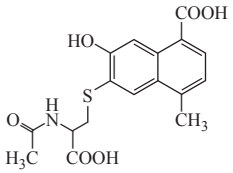
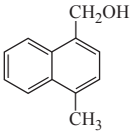
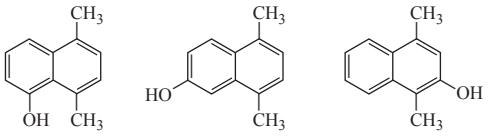
### (a) Oral route

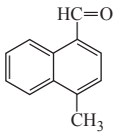
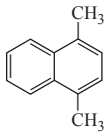
Metabolites were studied in the oral study described above (Dohn, Ostermann & Woodbury, 2012).

The highest levels of radioactive residue were found within the first 24 hours following administration and were mostly found in urine. The nature of urinary $^{14}C$ was examined by HPLC–MS and GC–MS and metabolites identified and the percentage they represent of administrated radioactivity are shown in Table 4. The parent molecule (1,4-DMN) was not detected in urine by HPLC, but trace amounts were confirmed to be present by GC–MS.

***Table 4. Metabolites of 1,4-dimethylnaphthalene identified in urine***

| Chemical name | Suggested chemical structure | % of dose |
|---|---|---|
| Thiomethyl-hydroxymethyl-1-naphthoic acid (isomers) |  (Only one possible structure shown) | 4.6% |
| Hydroxylated 4-methyl-1-naphthoic acid (isomers) containing some thiomethyl-hydroxymethyl-1-naphthoic acid (isomers) |  (Three proposed structures of hydroxylated 4-methyl-1-naphthoic acid are shown) | 13.2% |

| Chemical name | Suggested chemical structure | % of dose |
|---|---|---|
| *N*-acetylcysteine-4-methyl-1-naphthoic acid (one isomer)<br>**and**<br>Hydroxy-*N*-acetylcysteine-dihydro-1,4-dimethylnaphthalene (one isomer) [a] |  | 2.5% |
| *N*-Acetylcysteine-4-methyl-1-naphthoic acid (sum of three other isomers) | (Only one possible isomeric structure of *N*-acetylcysteine-4-methyl-1-naphthoic acid is shown above) | 4% [c] |
| Hydroxy-*N*-acetylcysteine-dihydro-1,4-dimethylnaphthalene (other two isomers from one above) [a] | <br>(Only one possible isomeric structure of hydroxy-*N*-acetylcysteine-dihydro-1,4-dimethylnaphthalene is shown above) | 5% [d] |
| *N*-Acetylcysteine-1,4-dimethylnaphthalene |  | 2.6% |
| 4-Methyl-1-naphthoic acid<br>*Metabolite M23* |  | 6.0% |
| 4-Methyl-1-naphthoic acid glucuronide (sum of two isomers) | <br>(Only one possible structure shown) | 6.1% [d] |
| Hydroxy *N*-acetylcysteine-dihydro-4-methyl-1-naphthoic acid |  | Traces [b] |
| 1-Hydroxymethyl-4-methylnaphthalene<br>*Metabolite M21* |  | Traces [b] |
| Ring-hydroxylated 1,4-dimethylnaphthalene | <br>(Three possible structures shown) | Traces of two isomers found [b] |

| Chemical name | Suggested chemical structure | % of dose |
|---|---|---|
| 4-Methylnaphthoic aldehyde |  | Traces [b] |
| 1,4-Dimethylnaphthalene (parent) |  | Traces [b] |
| **Total identified** | | **44.0%** |

Based on Dohn, Ostermann & Woodbury, 2012

[a] Four broad peaks were identified in the HPLC, most likely regio-isomers;

[b] Not detected by HPLC, but trace amount found by GC–MS;

[c] Sum of three peaks (isomers);

[d] Sum of two peaks (isomers);

Information in *italics* is not given in the report, but included by the applicant;

### *Metabolic pathway*

1,4-Dimethylnaphthalene metabolism can be described by two primary metabolic pathways.

One primary metabolic pathway is initiated by hydroxylation on a methyl group, to form 1-hydroxymethyl-4-methylnaphthalene (metabolite M21). This metabolic intermediate undergoes extensive further metabolism, which is why only trace amounts were detected in urine by GC–MS.

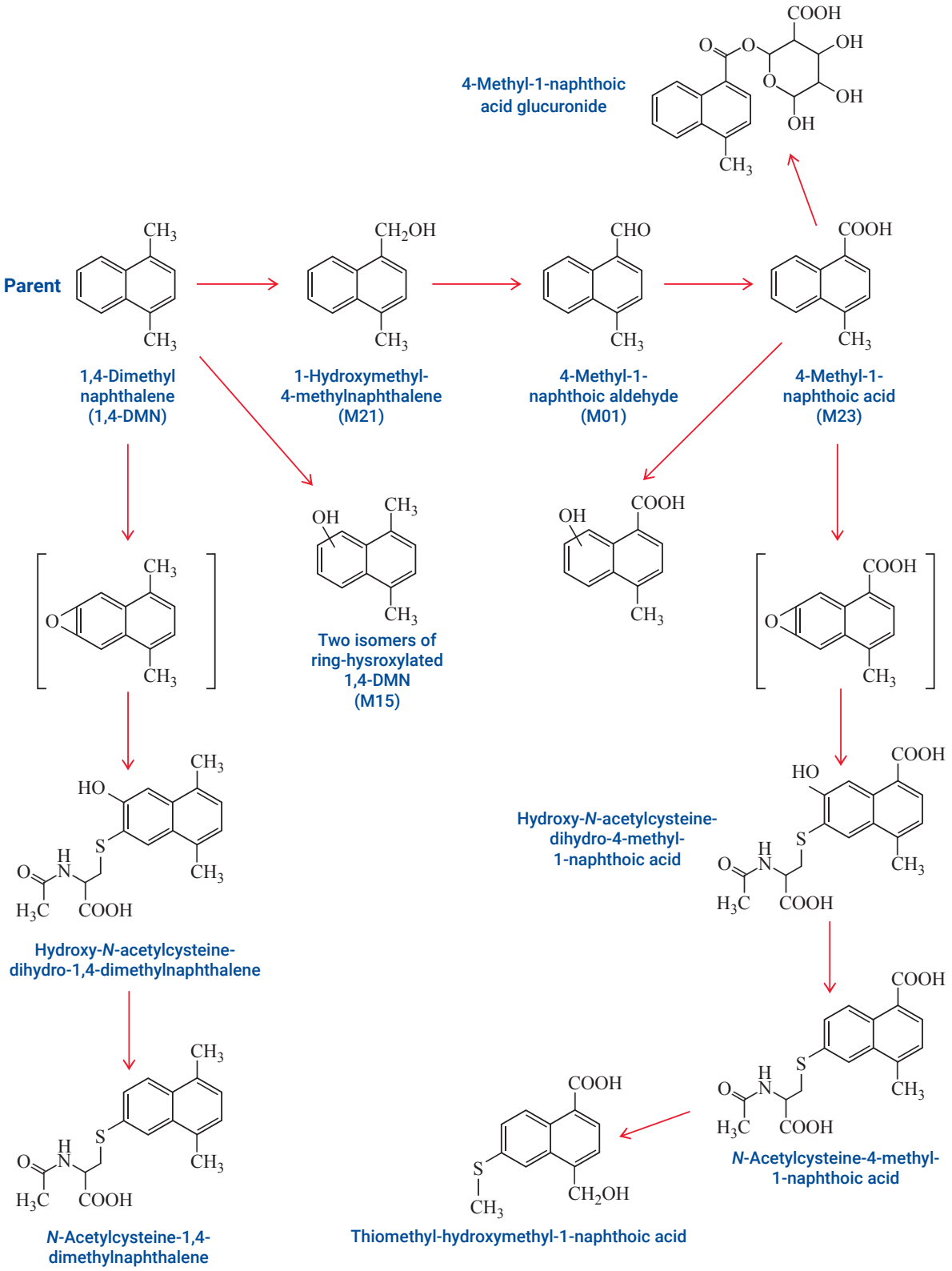
1-Hydroxymethyl-4-methylnaphthalene (M21) is oxidized to 4-methyl-1-naphthoic aldehyde (trace amounts detected in urine by GC–MS), then to 4-methyl-1-naphthoic acid (M23) which was the most abundant metabolite detected in urine, present at 6.0% of AD. Another portion (equivalent to 6.1% of AD) of the 4-methyl-1-naphthoic acid was conjugated with glucuronic acid. Thus, a total of 12.1% of AD was accounted for by 4-methyl-1-naphthoic acid and its glucuronide conjugate. Some of the 4-methyl-1-naphthoic acid (M23) was further metabolized to a hydroxylated 4-methyl-1-naphthoic acid, but the data do not allow identification of the position of hydroxylation.

The second principal metabolic pathway involves formation of an epoxide from either 1,4-DMN or from 4-methyl-1-naphthoic acid. These epoxides react with glutathione to form a vicinal hydroxy thioether. This was further metabolized as follows:

1. The glutathione conjugates are hydrolyzed (loss of the glutamyl and glycyl moieties of the glutathione tripeptide) to form the corresponding cysteine conjugates. These intermediate conjugates were not detected in the urine, due to rapid conversion of the original glutathione conjugates to their *N*-acetyl derivatives (Step 2 below).
2. The cysteine conjugates are *N*-acetylated. Two isomers of this metabolite were detected, accounting for 6.4% of AD.
3. Dehydration to re-form the aromatic naphthalene system, produces *N*-acetylcysteine-1,4-dimethylnaphthalene derivatives (detected at 2.6% of AD).

A detailed proposed metabolic pathway of 1,4-DMN in the rat is shown in Fig. 3.

*Figure 3. Proposed metabolic pathways for 1,4-dimethylnaphthalene in the rat*

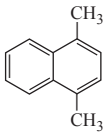
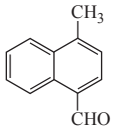
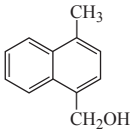
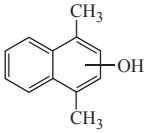
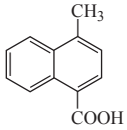
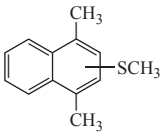



*(Based on Dohn, Ostermann & Woodbury, 2012)*

**(b) Intraperitoneal route**

In the study described above (Kilanowicz, Sapota, & Daragó, 2000) the metabolism of 1,4-DMN following intraperitoneal administration of a single dose of 28 mg/kg bw to rats, was investigated using a tritium radiotracer and gas chromatography–mass spectrometry (GC–MS). Metabolites of 1,4-DMN isolated from urine were identified**.** The yield by ether extraction of [$^3$H]1,4-DMN from urine at pH = 1 was about 70%. The following urinary substances presented in Table 5 were identified by gas chromatography.

***Table 5. Contribution of identified urinary substances following intraperitoneal administration of radiolabelled 1,4-DMN; percentages calculated from peak area of chromatogram***

| Chemical name | Suggested chemical structure | Percentage of total label excreted in urine *(Percentage of administered dose)* [a] |
|---|---|---|
| 1,4-Dimethylnaphthalene |  | 34.84 *(19.68)* |
| 4-Methyl-1-naphthoic aldehyde |  | 3.75 *(2.12)* |
| 1-Hydroxymethyl-4-methylnaphthalene (M21) |  | 29.43 *(16.63)* |
| 1,4-Dimethyl-naphthol (several isomers) |  | Peak 1: 5.35 *(3.02)* Peak 2: 5.24 *(2.96)* |
| 4-Methyl-1-naphthoic acid (M23) |  | 20.00 *(11.3)* |
| 1,4-Dimethyl-methylthionaphthalene |  | 1.39 *(0.79)* |

[a] Information in *italics* is not given in the report, but included by the applicant based on 56.5% radioactivity of administered dose found in urine

***Metabolic pathway***

Of the compounds found in urine only 35% was unchanged 1,4-DMN; almost 64% of the administered compound was metabolized before excretion. The radioactive labelled compounds found in faeces were not identified in this study. From the yield of ether-extracted urine products, the presence of free (that is, unconjugated) compounds and labile conjugates may be deduced prior to hydrolysis in urine at a pH of 1.

Apart from the unchanged 1,4-DMN the rat urine contained metabolites formed via three different pathways.

- Hydroxylation followed by oxygenation of alkyl derivatives of aromatic compounds to appropriate carboxylic acids (4-methyl-l-naphthoic acid, *M21,* labelled **4** in Fig. 4).
- Ring hydroxylation yielding 1,4-dimethylnaphthols (two isomers of the metabolite labelled **8** in Fig. 4) which could proceed either directly, or possibly through epoxide formation (see Fig. 3).
- Formation of reactive epoxide intermediates, followed by their conjugation with glutathione or other organic xenobiotics that can be cleaved by enzymatic hydrolysis to naphthothiophenols, resulting in cysteine-*S*-conjugates. These in turn are enzymatically converted to the corresponding thionaphthol by the release of pyruvate and ammonia. Finally such metabolites, with accessible sulfhydryl groups, can be subjected to methylation, yielding 1,4-dimethyl-methylthionaphthalene (labelled **7** in Fig. 4).

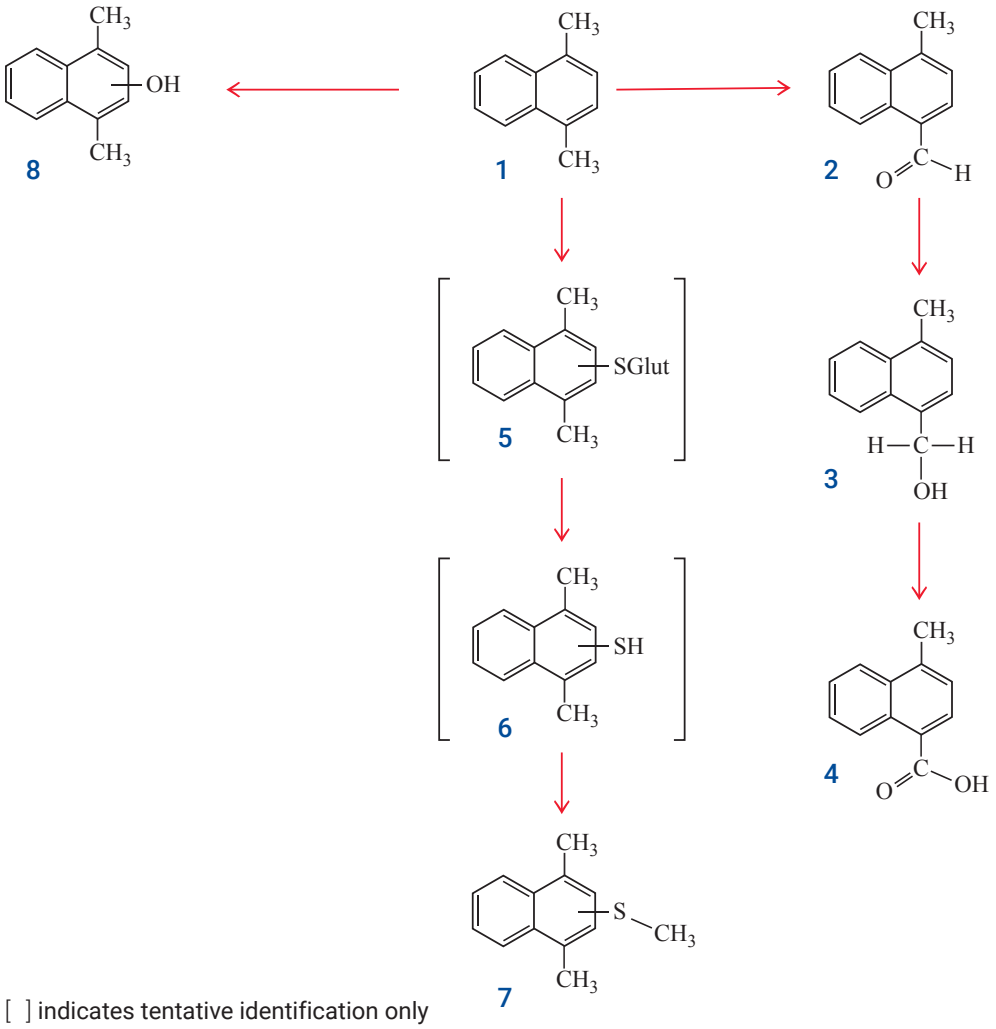
Metabolites labelled **5** and **6** in Fig. 4 were not identified in the urine, however, it must be noted that metabolites of 1,4-DMN are also excreted via bile and faeces, although biliary and faecal metabolites have not been identified. It is likely, but not proven, that there occur metabolites in faeces that have not been identified in urine.

In view of the above it can be concluded that after intraperitoneal administration, 1,4-DMN is rapidly excreted via urine and faeces. After 72 hours approximately 57% of AD had been excreted in the urine and almost 41% in faeces. Since the compound was administered intraperitoneally, this 41% faecal excretion constituted entirely excretion via the bile. There is no evidence of accumulation of 1,4-DMN or its metabolites in fat tissues.

About 35% of the compound found in urine was unchanged 1,4-DMN. Apart from the unchanged substance, the rat urine contains metabolites formed by three different pathways: hydroxylation of the alkyl side chains followed by oxygenation to corresponding carboxylic acids, ring hydroxylation (direct or via epoxide formation) and binding of epoxide intermediates to glutathione leading to thionaphthols.

The study was not conducted under GLP, and with no justification only one dose group was tested. Administration was intraperitoneal only, with no oral route. Metabolites were identified only in urine with no analysis of faeces products (Kilanowicz, Sapota, & Daragó, 2000).

***Figure 4. proposed metabolic pathway from results following intraperitoneal injection of 1,4-DMN in adult male rats. Parent 1,4-DMN (1) and metabolites 2, 3, 4, 7 and 8 were found in urine***




[ ] indicates tentative identification only

**(c) In vitro studies**

The metabolism of the plant growth regulator [$^{14}$C]1,4-DMN was investigated in mixed gender liver microsomes of human, rat, mouse and dog origin. The extent of metabolism was analyzed with nominal test item concentrations of 2.0 µM and 10.0 µM using cryopreserved mixed gender liver microsomes at a protein concentration of 1.0 mg/mL. For each species, duplicate samples were taken for analysis at three time intervals (0, 30 and 60 minutes). As negative controls, test item 1,4-DMN was incubated in medium with various other components: test item only; test item plus NADPH; test item plus microsomes; test item plus inactivated microsomes. The metabolic activity of all microsomes used was confirmed by transformation of testosterone to 6β-hydroxytestosterone and its metabolites.

Samples were quantitatively analyzed LSC and HPLC. Radioactivity was determined by LSC.

After 60 minutes the mean process recoveries for all species and concentrations were 90%–110% of the applied radioactivity (AR).

The metabolism of 1,4-DMN with rat microsomes was significantly slower than with human, mouse or dog microsomes.

In total, 15 metabolites were detected after the incubation of [$^{14}$C]1,4-DMN with mixed gender microsomes obtained from the human, rat, mouse and dog.

At the low concentration the major metabolites detected in human liver microsomes preparations were described as Unknown 4, Unknown 5, Unknown 14 and Unknown 15. At the higher concentration,

Unknown 5 was the main major metabolite (50.2% of AR), followed by Unknown 14 (31.5% of AR), Unknown 4 (8.2% of AR), and Unknown 15 (5.2% of AR). Unknown 5 was also detected in similar amounts with microsomes from the mouse (55.1% of AR) and dog (56.6% of AR) and in lower proportions with rat preparations (12% of AR). Unknown 14 was detected in rat and mouse incubations. At the low concentration Unknown 4 and Unknown 5 were detected in rat, mouse and dog incubations. Unknown 14 was observed in higher proportions with microsomes from the rat and mouse, and likewise Unknown 15 with dog liver microsomes. From this it was concluded that no unique metabolite was formed by human liver microsomes.

All human metabolites present at levels of 5% of dose and greater were also detected in other species (dog, rat or mouse), indicating comparable metabolic pathways in the species tested.

In view of the above it can be concluded that when metabolism of 1,4-DMN by human microsomes was compared with metabolism by microsomes from rats, dogs and mice, no unique metabolite was found for humans. Based on the study results, it seems that rat metabolism of 1,4-DMN is slower than that in humans, dogs or mice. This suggests rats are likely to be exposed for longer to the various metabolites than are humans. 1,4-Dimethylnaththalene was fully metabolized within 60 minutes with human microsomes, whereas 12.9%–43.5% unchanged parent was found after the same incubation time for rats (Erk, 2022).

### 1.3 Effects on enzymes and other biochemical parameters

No data were submitted on this topic.

## 2. Toxicological studies

### 2.1 Acute toxicity

#### (a) Lethal doses

***Table 6. Summary of acute toxicity studies with 1,4-dimethylnaphthalene***

| Species | Strain | Sex | Route | Purity | $LD_{50}$ or $LC_{50}$ | Reference |
|---|---|---|---|---|---|---|
| Rat | Sprague Dawley (Crl: CD®BR) | M + F | Oral [a] | 96.4% | > 2000 mg/kg bw | Johnson, 1993a |
| Rabbit | New Zealand White | M + F | Dermal [b] | 96.4% | > 2000 mg/kg bw | Johnson, 1993b |
| Rat | Sprague Dawley (Crl: CD®BR) | M + F | Inhalation [c] | 96.4% | > 4.15 mg/L [d] | Rajendran, 1993 |

M: Males; F: Females; $LC_{50}$: Median lethal concentration; $LD_{50}$: Median lethal dose;

[a] ***Observations up to 14 days.*** Clinical signs of toxicity were observed in all dose groups. Generally, by the one week post dose observation and until the end of the 14-day observation, all surviving rats appeared recovered from clinical signs. No body weight losses were observed. No gross pathological observations were noted. Based on the combined mortality data of males and females (including that of a previously conducted limit test at an oral dose of 5000 mg/kg bw), the $LD_{50}$ was calculated to be 2730 mg/kg bw with 95% confidence limits of 2346–3178 mg/kg bw. It was concluded that under the conditions of this test that the oral $LD_{50}$ for 1,4-dimethylnaphthalene was greater than 2000 mg/kg bw when administered to fasted male and female rats.

[b] ***Observations up to 14 days.*** No mortality was observed. One male rabbit did not gain weight. No signs of systemic toxicity were observed in any rabbit during the study. Signs of dermal irritation (oedema or erythema) were observed at the application site in all rabbits immediately following removal of the wrappings. Gross necropsy examination showed no test substance-related changes. The current OECD 402 (2017) guideline was fulfilled.

[c] Animals were exposed by inhalation route for four hours to whole body at the limit aerosol mean concentration of 4.15 ± 0.052 mg/L; they were then observed for 14 days. Clinical signs observed during the study included red nasal and ocular discharge, prostration, hypoactivity, ptosis, wet inguinal fur and discolouration around the nose and mouth. Tremors were seen in one female rat the day after exposure. One female rat showed dyspnoea and died within the one day following exposure. All surviving rats recovered from all clinical signs within six days of exposure. All surviving rats gained weight during the study. Necropsy findings for the dead and the surviving rats were within normal limits. The current guideline, OECD 403, (2009) was fulfilled.

[d] *Mass median aerodynamic diameter (MMAD) 2.28 µm;*

### (b) Dermal irritation, ocular irritation and dermal sensitization

*Table 7. Summary of dermal and ocular irritation, and skin sensitization studies on 1,4-DMN*

| Species | Strain | Sex | End-points | Purity | Result | Reference |
|---|---|---|---|---|---|---|
| Rabbit | New Zealand White | M + F | Primary dermal irritation [a] | 96.4% | Slightly to moderately irritating to skin | Johnson, 1993c |
| Rabbit | New Zealand White | M + F | Eye irritation [b] | 96.4% | Irritating to eyes, but reversible | Johnson, 1993d |
| Guinea pig | Hartley | M | Dermal sensitization (Buehler method) | 96.4% | Study not appropriate for the skin sensitization end-point. | Johnson, 1993e |
| Mouse | CBA/J | F | Skin sensitization (LLNA) [c] | 98.4% | Not a dermal sensitizer | Van Huygevoort, (2011) |

M: Males; F: Females; LLNA: Local lymph node assay;

[a] Rabbits (three per sex) were dermally exposed to 0.5 mL of undiluted 1,4-DMN for four hours on the fur-clipped backs (240 $cm^2$). Test sites were covered with an occlusive dressing. Animals were observed for mortality and morbidity for 14 days. Irritation was scored by the method of Draize. Signs of dermal irritation (very slight to moderate oedema and very slight to well-defined erythema) were observed in all six rabbits 30–60 minutes following unwrapping. By the 14-day observation all animals appeared completely recovered from any dermal irritation, although scaliness was observed at the application site of two rabbits at the final observation. As a conclusion, 1,4-DMN induced slight to moderate skin irritation in albino rabbits. The current test guideline was fulfilled.

[b] In a primary eye irritation study, 0.1 mL of undiluted 1,4-DMN was instilled into the conjunctival sac of three rabbits per sex. The untreated left eye of each rabbit served as a control. Animals were observed for 21 days. Irritation was scored by the method of Draize. Signs of ocular irritation corresponding to conjunctivitis were seen. Circumocular alopecia was observed in three animals at 14 days and in two animals at 21 days after application. Corneal opacity and iridial effects were not observed. It was concluded that 1,4-DMN was an eye irritant, but reversibly so. The current test guideline was fulfilled.

[c] Five female mice per dose were exposed to 1,4-DMN using the LLNA procedure according to OECD 429.Three experimental groups were treated with test substance concentrations of 25%, 50% or 100% (w/w) test article on three consecutive days by open application to the ears. Five vehicle control animals were similarly treated but with vehicle alone (acetone:olive oil at 4:1 by volume). The positive control was α-hexyl cinnamic aldehyde. Only slight local effects were observed, but no clinical signs or mortality. The stimulation index (SI) values calculated for the test substance concentrations 25%, 50% and 100% were 2.1, 2.4 and 2.8 respectively. Based on these results, 1,4-DMN was not considered a dermal sensitizer.

## 2.2 Short-term studies of toxicity

### (a) Oral administration

***Rat***

In a 14-day oral toxicity dose range finder assay in rats 1,4-DMN, was evaluated to assess its repeat dose toxicity when administered by oral gavage and determine a maximum tolerated dose (MTD) or maximum feasible dose (MFD). 1,4-DMN (purity 99.2%, Lot no. 20131207) was administered undiluted to male Fischer 344 rats (five animals per dose) at dose volumes of 0 (untreated), 0.16, 0.66, and 0.99 mL/kg bw (equal to 0, 160, 670, and 1000 mg/kg bw per day) for up to 14 consecutive days. Surviving animals were sacrificed on day 15 and discarded without further analysis.

At 160 mg/kg bw per day clinical signs were noted: thinness, ruffled fur, and piloerection. At 670 mg/kg bw per day the following clinical signs were noted: thinness, decreased motor activity, ruffled fur, hunched posture, discharge from the eye, hypersalivation, piloerection, and squinty eye. At 1000 mg/kg bw per day clinical signs were: thinness, decreased motor activity, hunched posture, discharge from the eye, hypersalivation, impaired gait, rapid and shallow breathing, and squinty eye, Animals in this highest dose group were either found dead or sacrificed in moribund condition on days 3 or 4.

Under the conditions of this study, a dose of 670 mg/kg bw per day, administered in 0.66 mL/kg bw, was determined to be the MTD for oral administration of 1,4-DMN (Young & Bruce, 2021).

In a 90-day dietary toxicity study, 1,4-DMN (purity 98.8%) was administered to Sprague Dawley rats (CRL:CD(SD)IGSBR) around 6–7 weeks old, weight 167–232 g (males) or 137–177 g (females). Ten rats of each sex per dose received test article for 90 days at concentrations of 0, 500, 2500 or 10 000 ppm (equal to 0, 32, 161 and 677 mg/kg bw per day in males, 0, 38, 186 and 747 mg/kg bw per day in females). Two additional groups (0 and 10 000 ppm) of 10 animals of each sex per dose were observed for an additional 28 days to evaluate the recovery potential from any effects induced by 1,4-DMN.

In addition to standard evaluations (such as body weights, clinical chemistry, haematology, clinical pathology, organ weights and histopathology), each rat was used to evaluate the neurotoxic effects of 1,4-DMN with a functional observational battery (FOB) and automated motor activity assessment performed during week 11 of treatment.

No treatment-related deaths occurred during the study, however one high-dose recovery group male died accidentally during week 14 of the study. In general, no consistent treatment-related clinical signs were noted except for discoloured inguinal fur, which was seen in 60% (12/20) of the high-dose males, 20% (2/10) of the mid-dose males, and one high-dose female. Other clinical signs were sporadic in nature and included rough hair coat, alopecia. and red material around the nose and eyes. These signs were observed in both the treated and control groups and were deemed unrelated to treatment.

Treatment-related effects on food consumption were noted and exhibited a dose–response relationship during the study. On study days 1, 2 and 3, the high-dose groups displayed significantly decreased food consumption compared to controls; the mid-dose group similarly, but only on day 1. Food consumption among the high-dose groups was significantly decreased throughout the study (except during week 1 for females). Also, mid-dose males displayed decreased consumption for seven weeks of the study. Effects on food consumption of the low-dose group were minimal (decreased food consumption during weeks 3 and 7 only). There were no differences in food consumption between the high-dose group and vehicle controls during the recovery period.

As regards the effect on body weight and body weight gain, during the first three days of the study, both high-dose males and females recorded decreased body weights related to their decreased food consumption. In addition, body weights of the high-dose animals were significantly lower than their vehicle control counterparts throughout the study, except for females in week 1. Consequently, body weight gain was significantly impaired in the high-dose group compared to controls, by 66% in males and 68% in females. During the recovery period, body weights of the high-dose animals remained significantly lower than those of controls, but total body weight gain was significantly higher in the high-dose animals than controls in this period.

No treated-related effects due to 1,4-DMN were apparent from ophthalmological observations.

To test neurological parameters FOB and motor activity (MA) assessments were conducted. No significant differences in the FOB or MA parameters were noted during the study, except for decreased forelimb strength in high-dose males, and increased vertical plane time interval (VPTM), at time point 5, in the low-dose males. The decreased forelimb strength was most likely due to the decreased body weight in the high-dose males. The increased VPTM was considered to be sporadic in nature and unrelated to treatment due to the lack of any dose–response relationship.

As regards haematological effects, sporadic, statistically significant differences in mean corpuscular haemoglobin (MCH), haemoglobin (Hb) and mean corpuscular haemoglobin concentration (MCHC) were very minor in nature and deemed to be the result of biological variability rather than related to treatment.

Statistically significant differences between the treated and control groups were noted in various clinical chemistry parameters. Specifically, the albumin to globulin (A : G) ratio, globulin, glucose and triglyceride levels were decreased in high-dose males, while cholesterol levels were increased in high- and mid-dose males. The A : G ratio and blood urea nitrogen (BUN) levels were decreased in high-dose females, while cholesterol levels were increased; BUN levels were also decreased in mid-dose females. Except for the increased cholesterol levels, all other differences noted were considered unrelated to treatment due to their sporadic nature, small size and the values being within the normal range. Sporadic statistically significant differences in various clinical chemistry parameters were noted in the recovery groups but were deemed unrelated to treatment.

*Table 8. Selected haematology and clinical chemistry from the 90-day toxicity study in rats*

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | **0** | **500** | **2500** | **10 000** | **0** | **500** | **2500** | **10 000** |
| Number of animals | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| **Haematology; day 90 (mean ± standard deviation shown)** | | | | | | | | |
| RBC ($\times 10^6$ cells/µL) | 8.46 ± 0.5 | 8.59 ± 0.29 | 8.62 ± 0.26 | 8.33 ± 0.43 | 8.03 ± 0.26 | 7.96 ± 0.26 | 8.07 ± 0.27 | 8.02 ± 0.24 |
| Mean corpuscular volume (fL) | 50.6 ± 2.2 | 48.7 ± 1.7 | 50.2 ± 0.9 | 49.4 ± 1.4 | 52.2 ± 1.3 | 52.1 ± 1.4 | 51.4 ± 1 | 51.3 ± 1.1 |
| Haematocrit (L/L) | 42.7 ± 2.3 | 41.8 ± 1.5 | 43.2 ± 0.9 | 41.1 ± 2.3 | 41.9 ± 1.2 | 41.5 ± 1.4 | 41.4 ± 1.3 | 41.1 ± 1.5 |
| Haemoglobin (g/dL) | 15.3 ± 0.9 | 14.9 ± 0.6 | 15.3 ± 0.4 | 14.5 ± 0.9 | 15 ± 0.4 | 14.8 ± 0.5 | 14.7 ± 0.4 | 14.4* ± 0.4 |
| White blood cells ($\times 10^3$ cells µL) | 6.67 ± 1.67 | 5.55 ± 2.1 | 6.6 ± 2.16 | 6.37 ± 2.35 | 4.5 ± 1.51 | 4.64 ± 1.49 | 4.46 ± 1,72 | 3.84 ± 0.8 |
| Mean corpuscular Hb (pg) | 18.2 ± 0.8 | 17.4 ± 0.7 | 17.8 ± 0.4 | 17.5* ± 0.6 | 18.7 ± 0.5 | 18.6 ± 0.5 | 18.3 ± 0.3 | 18.0* ± 0.4 |
| Mean corpuscular Hb concentration (g/dL) | 35.9 ± 0.6 | 35.6 ± 0.6 | 35.5 ± 0.4 | 35.4 ± 0.4 | 36 ± 0.6 | 36 ± 0.5 | 36 ± 0.4 | 35* ± 0.6 |
| Platelets ($\times 10^3$ cells/µL) | 1143 ± 150 | 1211 ± 113 | 1134 ± 111 | 1068 ± 94 | 1233 ± 91 | 1266 ± 139 | 1230 ± 97 | 1139 ± 61 |
| **Blood chemistry; day 90 (mean ± standard deviation shown)** | | | | | | | | |
| Blood urea nitrogen (mg/dL) | 13 ± 1.4 | 16* ± 3.7 | 14 ± 1.6 | 15 ± 3.3 | 17.4 ± 1.51 | 16.8 ± 1.99 | 15.5* ± 1.9 | 13.8* ± 1.69 |
| Sodium (mmol/L) | 143 ± 1.4 | 142 ± 1.4 | 142 ± 0.7 | 142 ± 1.5 | 142 ± 0.8 | 142 ± 1.3 | 142 ± 1.2 | 141 ± 1 |
| Chloride (mmol/L) | 104 ± 1.1 | 105 ± | 104 ± 0.9 | 104 ± 1.5 | 104.2 ± 0.92 | 104.3 ± 1.83 | 104.3 ± 1.16 | 103.2 ± 1.32 |
| Globulin (g/dL) | 3.2 ± 0.2 | 3.1 ± 0.18 | 3.4 ± 0.16 | 3.0* ± 0.25 | 3.2 ± 0.25 | 3.2 ± 0.15 | 3.3 ± 0.14 | 3.3 ± 0.22 |
| Cholesterol (mg/dL) | 54 ± 8.3 | 63 ± 15.7 | 60* ± 13.2 | 74* ± 10 | 59 ± 10 | 74 ± 21 | 77 ± 9.8 | 116* ± 20.1 |
| Triglycerides (mg/dL) | 59 ± 20.4 | 48 ± 22.3 | 42 ± 15 | 30* ± 8.3 | 23 ± 4.6 | 28 ± 6.7 | 28 ± 7 | 21 ± 4.1 |

Hb: Haemoglobin; Source: Mallett, 2003

* Statistically different from control values with significance $p \leq 0.05$

Various changes noted in the organ to body weight ratios in the mid- and high-dose groups were most likely due to the overall lower rat body weights at the end of the treatment period. Further, in high-dose males, the absolute heart and heart:brain weight ratios were both lower than for controls. Absolute spleen weight was decreased in high-dose males and females, whilst the spleen:brain weight ratio was decreased only in high-dose males. In mid- and high-dose females absolute liver, and liver weight relative to body and to brain weight ratios were all increased compared with controls. After the recovery period, the absolute adrenal and heart weights, and their respective organ:brain weight ratios were all decreased in the high-dose males, and a small increase in relative ovary weight was observed in females. See Table 9 for detail of body and organ weights.

*Table 9. Absolute and relative organ weights from the 90-day toxicity study in rats (All values shown as mean ± standard deviation)*

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Dose rate [ppm]** | **0** | **500** | **2500** | **10 000** | **0** | **500** | **2500** | **10 000** |
| Body weight (g) | 516 ± 45.6 | 480 ± 43.7 | 469 ± 56.0 | 392* ± 37.8 | 262 ± 20.9 | 269 ± 18.3 | 265 ± 15.4 | 228* ± 16.2 |
| Number of Animals | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| **Brain** | | | | | | | | |
| Absolute weight (g) [a] | 2.12 ± 0.08 | 2.12 ± 0.08 | 2.12 ± 0.12 | 2.06 ± 0.07 | 1.91 ± 0.08 | 1.88 ± 0.08 | 1.91 ± 0.07 | 1.91 ± 0.11 |
| Relative to body weight (%) | 0.413 ± 0.039 | 0.444 ± 0.026 | 0.458* ± 0.053 | 0.528* ± 0.04 | 0.729 ± 0.048 | 0.703 ± 0.067 | 0.721 ± 0.038 | 0.843* ± 0.081 |
| **Adrenals** | | | | | | | | |
| Absolute Weight (g) [a] | 0.068 ± 0.014 | 0.059 ± 0.005 | 0.061 ± 0.013 | 0.06 ± 0.006 | 0.069 ± 0.008 | 0.065 ± 0.01 | 0.067 ± 0.01 | 0.06 ± 0.008 |
| Relative to body weight (%) | 0.013 ± 0.003 | 0.012 ± 0.001 | 0.013 ± 0.002 | 0.015 ± 0.002 | 0.026 ± 0.003 | 0.024 ± 0.004 | 0.025 ± 0.004 | 0.026 ± 0.003 |
| Relative to brain weight (%) | 3.235 ± 0.667 | 2.794 ± 0.237 | 2.869 ± 0.651 | 2.9 ± 0.348 | 3.628 ± 0.39 | 3.47 ± 0.545 | 3.528 ± 0.487 | 3.153 ± 0.471 |
| **Liver** | | | | | | | | |
| Absolute Weight (g) [a] | 13.17 ± 1.7 | 12.96 ± 1.88 | 14.38 ± 1.83 | 13.29 ± 1.42 | 6.82 ± 0.55 | 7.11 ± 0.33 | 8.32* ± 1.16 | 9.64* ± 0.92 |
| Relative to body weight (%) | 2.55 ± 0.19 | 2.69 ± 0.2 | 3.07* ± 0.17 | 3.39* ± 0.17 | 2.6 ± 0.1 | 2.65 ± 0.14 | 3.13* ± 0.31 | 4.23* ± 0.21 |
| Relative to brain weight (%) | 621.4 ± 74.9 | 609.7 ± 68 | 679.4 ± 94.5 | 645.7 ± 55.9 | 358.3 ± 27.9 | 379.5 ± 32.4 | 437.1* ± 62.7 | 507.3* ± 62.7 |
| **Heart** | | | | | | | | |
| Absolute Weight (g) [a] | 1.59 ± 0.16 | 1.43 ± 0.16 | 1.53 ± 0.17 | 1.34* ± 0.12 | 0.88 ± 0.076 | 0.914 ± 0.064 | 0.933 ± 0.077 | 0.848 ± 0.06 |
| Relative to body weight (%) | 0.309 ± 0.023 | 0.299 ± 0.028 | 0.327 ± 0.025 | 0.343* ± 0.032 | 0.336 ± 0.019 | 0.341 ± 0.027 | 0.352 ± 0.024 | 0.373* ± 0.017 |
| Relative to brain weight (%) | 75.35 ± 8.38 | 67.48 ± 7.1 | 72.15 ± 8.47 | 65.06* ± 5.68 | 94.63 ± 4.12 | 97.48 ± 8.24 | 102.16 ± 10.09 | 97.63 ± 11.19 |
| **Kidneys** | | | | | | | | |
| Absolute Weight (g) [a] | 3.25 ± 0.37 | 3.03 ± 0.22 | 3.35 ± 0.4 | 3.06 ± 0.29 | 1.803 ± 0.11 | 1.827 ± 0.102 | 1.946 ± 0.193 | 1.858 ± 0.177 |
| Relative to body weight (%) | 0.629 ± 0.038 | 0.633 ± 0.041 | 0.715* ± 0.041 | 0.785* ± 0.09 | 0.69 ± 0.059 | 0.681 ± 0.03 | 0.734 ± 0.047 | 0.815* ± 0.034 |
| Relative to brain weight (%) | 153.5 ± 17.4 | 142.7 ± 8 | 158.2 ± 21.7 | 148.7 ± 12.8 | 358.3 ± 27.9 | 379.5 ± 32.4 | 437.1* ± 62.7 | 507.3* ± 62.7 |
| **Ovaries** | | | | | | | | |
| Absolute Weight (g) [a] | NA | NA | NA | NA | 0.129 ± 0.012 | 0.137 ± 0.019 | 0.131 ± 0.026 | 0.112 ± 0.015 |
| Relative to body weight (%) | NA | NA | NA | NA | 0.049 ± 0.006 | 0.051 ± 0.007 | 0.049 ± 0.009 | 0.049 ± 0.006 |
| Relative to brain weight (%) | NA | NA | NA | NA | 6.752 ± 0.63 | 7.278 ± 1.069 | 6.886 ± 1.351 | 5.883 ± 0.725 |

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Dose rate [ppm]** | **0** | **500** | **2500** | **10 000** | **0** | **500** | **2500** | **10 000** |
| **Spleen** | | | | | | | | |
| Absolute Weight (g)[a] | 0.886 ± 0.143 | 0.794 ± 0.1 12 | 0.776 ± 0.147 | 0.654* ± 04 10 | 0.571 ± 0.065 | 0.519 ± 0.08 | 0.552 ± 0.078 | 0.476* ± 0.086 |
| Relative to body weight (%) | 0.173 ± 0.033 | 0.165 ± 0.018 | 0.165 ± 0.02 | 0.167 ± 0.025 | 0.218 ± 0.022 | 0.193 ± 0.023 | 0.209 ± 0.028 | 0.208 ± 0.029 |
| Relative to brain weight (%) | 41.95 ± 7.62 | 37.36 ± 4.33 | 36.54 ± 6.74 | 31.74* ± 4.66 | 30 ± 3.36 | 27.76 ± 5.17 | 29.01 ± 4.29 | 25.06 ± 4.96 |
| **Testis** | | | | | | | | |
| Absolute Weight (g)[a] | 3.67 ± 0.32 | 26724 ± 0.68 | 3.4 ± 0.95 | 3.66 ± 0.29 | NA | NA | NA | NA |
| Relative to body weight (%) | 0.714 ± 0.075 | 0.775 ± 0.086 | 0.717 ± 0.187 | 0.941* ± 0.12 | NA | NA | NA | NA |
| Relative to brain weight (%) | 173 ± 12.9 | 1 75.4 ± 25.4 | 159.3 ± 44.1 | 178 ± 14.9 | NA | NA | NA | NA |

NA: Not applicable; Source: Mallett, 2003

[a] Group means with standard deviation at the end of terminal necropsy are shown;

* Statistically different from control values with significance $p \leq 0.05$

A range of gross necropsy findings were singular in occurrence and seen across all groups including controls. Therefore, they were deemed unrelated to treatment. No histopathological lesions were noted in any organ except the kidneys. At the top dose of 10 000 ppm there was an increased incidence of chronic progressive nephropathy (not related to increased blood urea nitrogen), a kidney alteration that spontaneously occurs in aging rats, and was also later observed in the male recovery group. Apparently, treatment for 90 days at the limit dose increased the incidence of chronic progressive nephropathy (CPN) significantly above that normally found in aging rats; both incidence and severity were greater in males than females. Chronic progressive nephropathy was not observed in mid- or low-dose females, whilst in the recovery group, high-dose females 3/10 showed CPN, whereas none of the control animals were affected. A higher incidence of basophilic tubules was observed in mid-dose males. Although the study director reported this as CPN, the Meeting decided that this was not the appropriate term, as the rats were not yet old enough, so it should be referred to simply as nephropathy. Moreover, the increased incidence of basophilic tubules seen at mid dose would also progress into nephropathy, and are therefore adverse.

***Table 10. Summary of renal histopathological findings from the 90-day toxicity study in rats***

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Dose rate [ppm]** | **0** | **500** | **2500** | **10 000** | **0** | **500** | **2500** | **10 000** |
| Number of animals examined | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Chronic progressive nephropathy | 1 | 1 | 1 | 5 | 0 | 0 | 0 | 1 |
| Basophilic tubules | 2 | 2 | 6 | 0 | 0 | 0 | 0 | 0 |
| Infiltration, mononuclear cells | 1 | 1 | 3 | 1 | 2 | 3 | 0 | 1 |
| Purulent exudate, renal pelvis | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Hyaline cast(s) | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| Mineralization | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |

Source: Mallett, 2003

The no-observed-adverse-effect level (NOAEL) for 1,4-DMN was 500 ppm (equal to 32 mg/kg bw per day) based on increased relative kidney weight (by 15%) and basophilic tubules in the kidney, and suppressed body weight gain (by 11% in males) at 2500 ppm (equal to 161 mg/kg bw per day) (Mallett, 2003)

**(b) Dermal application**

No data were submitted on this topic.

**(c) Exposure by inhalation**

No data were submitted on this topic.

## 2.3 Long-term studies of toxicity and carcinogenicity

***Rat***

1,4-Dimethylnaphthalene (Purity 98.4%, Lot no. 14D06B01-01) was administered as an admixture in the diet to groups of 85 Sprague Dawley [CD IGS; Crl:CD(SD)] rats of each sex. The first 65 rats of each sex and group were designated for carcinogenicity assessment, and the remaining 20 rats of each sex and group were designated for chronic toxicity evaluation. At the initiation of administration rats were 6–7 weeks old and weighed 199–202 g (males) or 163–165 g (females).

The rats were administered control or test diet ad libitum, seven days per week for a minimum of 52 weeks (chronic toxicity phase) or 104 weeks (carcinogenicity phase), at target concentrations of 0, 150, 500 or 3750 ppm (equal to 0, 8, 27 and 208 mg/kg bw per day for males, 0, 10, 33 and 247 mg/kg bw per day for females). Rats were euthanized at the end of each phase, unless a criterion for early termination was met. The target concentrations were initially based on a 250 g rat consuming 25 g of food per day, and were adjusted throughout the study based on body weight and food consumption to achieve as close as possible to the target doses.

Rats were observed for clinical signs of toxicity weekly throughout the study, with standard evaluations such as body weights, food consumption, clinical pathology and necropsy performed for each rat.

No treatment-related clinical signs or deaths occurred during this chronic and carcinogenicity study. In the chronic toxicity group, four male and two female rats died prior to scheduled termination but their deaths were randomly distributed amongst the groups. Amongst the chronic toxicity rats, the percentage surviving until scheduled termination was 90%–100%. However, with carcinogenicity phase rats, due to the decreased survival rate (28%–35%) of the control, low- and mid-dose females, all female rats were terminated at week 100 of the study, while surviving males were still terminated at week 104. Since sufficient of the high-dose females survived (48%), the study was considered suitable for evaluation of the carcinogenic properties of 1,4-DMN.

***Table 11. Percentage survival in 52/104-week combined toxicity and carcinogenicity study in rats***

| Group | Females (week 100) | Males (week 104) |
|---|---|---|
| Vehicle control | 27.7 | 60.0 |
| Low dose | 35.4 | 38.5 |
| Mid dose | 29.2 | 40.0 |
| High dose | 47.7 | 60.0 |

Source: Mallett, 2011

At ophthalmic examinations, no treatment-related eye abnormalities were observed

*Food consumption*

In high-dose females, food intake was significantly decreased during the entire exposure period compared with controls. In high-dose males, a significant decrease in food intake was observed from week 1–25. In mid-dose females, food intake was only incidentally decreased to a significant extent. The decrease in food consumption observed could have been a palatability effect.

*Body weight and body weight gain - chronic toxicity groups*

A treatment-related and statistically-significant decrease in body weight was noted in male and female rats in the high-dose, chronic toxicity group from week 1 until week 52 in. Body weight gain tended to be lower in males over the first five weeks and for females over the first 10 weeks. Decreases in body weight gain tended to be somewhat sporadic, but statistical significance was achieved several times during this period and these changes were considered treatment-related. In addition, a minimal trend towards reduced body weight gain was noted in high-dose females from week 21 until week 41. Sporadic differences in body weight and body weight gain were noted in the low- and mid-dose groups too, however these changes were not considered treatment-related as there was no consistent trend.

*Body weight and body weight gain - carcinogenicity groups*

In carcinogenicity groups a treatment-related and statistically significant decrease in body weight was noted in male rats from the high-dose group from week 2 until week 104, and in high-dose females from week 1 until termination (week 100). Sporadic changes in body weight gain, which generally attained statistical significance when compared to control values, were noted in male rats from week 1 until week 29. A similar trend was observed in females where a sporadic suppression of body weight gain, which generally attained statistical significance when compared to control values, was noted from week 1 until week 41 of treatment. Sporadic changes in body weight and body weight gain were noted in the low- and mid-dose groups, however, these changes were not considered treatment-related as there was no consistent trend.

*Urinalysis - chronic toxicity groups*

No treatment-related findings were noted in the urinalysis parameters examined. Sporadic, statistically significant decreases in specific gravity were noted in high-dose females at the three- and six-month time points; however as there were no differences at the 12-month time point, this finding was deemed to be the result of biological variation.

*Urinalysis - carcinogenicity groups*

No treatment-related findings were noted in the urinalysis parameters examined. Sporadic differences in pH and volume were noted in high-dose male rats at 18 months and in specific gravity in high-dose males at 24 months, but these findings were deemed the result of biological variation and not considered treatment-related.

*Haematology - chronic toxicity groups*

No treatment-related findings were noted in the haematology parameters examined. Sporadic, statistically significant decreases compared to controls were noted in a range of haematology parameters: white blood cells, MCH, platelets, Hb, mean cell volume (MCV), MCHC, absolute and relative reticulocyte counts, absolute lymphocyte, eosinophils and basophil counts, and relative monocyte counts. However, the changes were very minor in nature, did not exhibit a dose–response relationship or were within historical ranges. Accordingly, these differences were deemed to be the result of biological variability rather than treatment with 1,4-DMN.

*Haematology - carcinogenicity groups*

No treatment-related findings were noted in the haematology parameters examined. Sporadic, statistically significant differences were noted in several haematology parameters (red blood cells, haematocrit, MCH and MCV) when compared to control values, however these changes were very minor in nature or were within historical ranges. Based on this, these differences were deemed to be the result of biological variability rather than treatment with 1,4-DMN.

*Clinical chemistry - chronic toxicity groups*

Statistically significant changes were noted in female rats and included decreases in BUN in high-dose animals at 3, 6 and 12 months, and increases in cholesterol in low- and high-dose animals at 3 and 6 months and in mid- and high-dose animals at 12 months (see Table 12) In addition, increases in cholesterol that were not statistically significant were noted in mid-dose females at 3 and 6 months. A statistically significant increase in cholesterol was seen in mid- and high-dose females at 12 months.

These findings are consistent with the findings in a 90-day study of toxicity. Association with treatment is questionable as these findings were only noted in females and did not display a dose–response relationship. Furthermore, an increase in cholesterol only may be due to dyslipidaemia, hence may not be considered adverse. Nevertheless these results are consistent with the findings in the 90-day study

An additional factor is that similar changes in BUN were not noted at 18 or 23/24 months in animals assigned to the carcinogenicity phase of the study, added to which the direction of the change (that is, a decrease) is not toxicologically relevant.

Sporadic, statistically significant differences in calcium, A:G ratio, glucose, chloride, total bilirubin, γ-glutamyl transpeptidase/transferase (GGTP) and sodium were noted, but were all deemed unrelated to treatment due to their sporadic nature, small size and lack of a dose–response relationship, added to the fact that the values were within normal ranges or were not noted in subsequent blood samplings.

***Table 12. Selective clinical chemistry findings; 12-month chronic toxicity treatment group***

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | **0** | **150** | **500** | **3750** | **0** | **150** | **500** | **3750** |
| Number of animals | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| **Blood chemistry (mean ± standard deviation shown)** | | | | | | | | |
| Sodium (mmol/L) | 149 ± 1.6 | 148 ± 0.9 | 148 ± 1.3 | 150 ± 1.2 | 142 ± 1 | 143 ± 1 | 143 ± 2 | 142 ± 2 |
| Potassium (mmol/L) | 6.4 ± 0.9 | 6.1 ± 0.2 | 5.9 ± 0.3 | 6.4 ± 0.3 | 5.2 ± 0.6 | 5.2 ± 0.5 | 5.2 ± 0.6 | 5.1 ± 0.4 |
| Chloride (mmol/L) | 100 ± 1.9 | 101 ± 1.2 | 101 ± 1.2 | 101 ± 1.4 | 97 ± 1.9 | 97 ± 2.1 | 97 ± 2.3 | 97 ± 1.8 |
| Globulin (g/dL) | 3.5 ± 0.3 | 3.6 ± 0.2 | 3.6 ± 0.1 | 3.6 ± 0.1 | 3.6 ± 0.4 | 3.6 ± 0.2 | 3.7 ± 0.4 | 3.8 ± 0.3 |
| Cholesterol (mg/dL) | 122 ± 20 | 114 ± 32 | 134 ± 27 | 140 ± 22 | 110 ± 21 | 131 ± 25 | 143* ± 34 | 165* ± 29 |
| Triglycerides (mg/dL) | 100 ± 30 | 99 ± 35 | 119 ± 39 | 70 ± 13 | 89 ± 57 | 89 ± 35 | 63 ± 24 | 60 ± 19 |
| Blood urea nitrogen (mg/dL) | 13 ± 2.0 | 12 ± 2 | 12 ± 1 | 12 ± 1 | 13 ± 2 | 14 ± 1 | 13 ± 1 | 10* ± 2 |
| Total bilirubin (mg/dL) | 0.2 ± 0.00 | 0.2 ± 0.03 | 0.2 ± 0.05 | 0.2 ± 0.00 | 0.20 ± 0.00 | 0.23 ± 0.05 | 0.21 ± 0.03 | 0.25* ± 0.05 |

* Statistically different from control values with significance $p \leq 0.05$ Source: Mallett, 2011

*Clinical chemistry - carcinogenicity groups*

Statistically significant increases in cholesterol were noted in high-dose females at 18 and 23 months (see Table 13). A slight, increase in cholesterol (not statistically significant) was also noted in low- and mid-dose females. These were consistent with the findings in the 90-day and chronic toxicity studies. At the high dose there was a relatively large increase in cholesterol in females which remained consistent over time. A connection with treatment cannot be ascertained as these findings were only noted in females.

Sporadic, statistically significant increases in albumin, glucose, total protein and GGTP were also noted, but these were deemed unrelated to treatment due to their occasional nature, small size and lack of a dose–response relationship. More importantly, the values were within normal ranges or were not noted in subsequent blood samplings.

*Table 13. Selected clinical chemistry findings; 24-month carcinogenicity group*

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Male | | | | Female | | | |
| | 0 | 150 | 500 | 3750 | 0 | 150 | 500 | 3750 |
| Number of animals | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 |
| **Blood chemistry (mean ± standard deviation shown)** | | | | | | | | |
| Sodium (mmol/L) | 149 ± 2 | 149 ± 1 | 149 ± 2 | 149 ± 2 | 145 ± 2 | 146 ± 3 | 147 ± 5 | 144 ± 2 |
| Potassium (mmol/L) | 5.8 ± 0.5 | 5.9 ± 0.5 | 5.9 ± 0.6 | 5.8 ± 0.5 | 5.3 ± 0.6 | 5.4 ± 0.6 | 5.0 ± 0.8 | 5.3 ± 0.3 |
| Chloride (mmol/L) | 103 ± 2.1 | 102 ± 1.9 | 102 ± 2.0 | 102 ± 3.2 | 100 ± 3.5 | 101 ± 2.8 | 99 ± 4.2 | 101 ± 2.4 |
| Globulin (g/dL) | 4.1 ± 0.6 | 4.3 ± 0.4 | 4.1 ± 0.5 | 4.2 ± 0.4 | 4.0 ± 0.9 | 3.8 ± 0.4 | 3.9 ± 0.4 | 4.0 ± 0.3 |
| Cholesterol (mg/dL) | 133 ± 37 | 127 ± 28 | 120 ± 46 | 143 ± 37 | 103 ± 22 | 133 ± 34 | 112 ± 28 | 159* ± 29 |
| Triglycerides (mg/dL) | 91 ± 34 | 98 ± 32 | 96 ± 61 | 90 ± 35 | 104 ± 58 | 73 ± 40 | 81 ± 38 | 62 ± 40 |
| γ-Glutamyl transferase (U/L) | 0.6 ± 0.8 | 0.7 ± 0.5 | 0.5 ± 0.7 | 2.0* ± 1.2 | 0.2 ± 0.5 | 0.1 ± 0.4 | 0.5 ± 1.0 | 0.2 ± 0.5 |

* Statistically different from control values with significance $p \leq 0.05$ Source: Mallett, 2011

*Organ weights - chronic toxicity groups*

**S**tatistically significant increases in liver weights were noted in mid-dose males (by 9.8%) and in high-dose males (by 10.1%), and in females (by 20%). Association of these changes with 1,4-DMN administration is equivocal as there were no related histopathology findings in the liver. The changes were therefore considered a result of biological variation and not related to treatment.

Spleen weights were decreased in high-dose females. As there were no histopathology findings for the spleen and no changes in red cell parameters and the decrease was only noted in females, this finding was also considered unrelated to treatment.

*Organ weights - carcinogenicity groups*

Statistically significant differences were found in several organ weight parameters in female rats. Kidney weights were decreased compared to controls in high-dose animals, most likely due to their lower body weights (male lower by 14%, females by 25%). Relative kidney weight were not increased. Adrenal weights in low- and high-dose females were lower than for controls. Though there are decreases in the absolute weight of liver in high-dose females compared to controls, the decreases across the dose levels are not dose-related. Furthermore no histopathological lesions were noted in this organ to support the statistically significant differences, therefore this change was considered incidental in nature.

*Gross pathology - chronic toxicity groups*

Gross necropsy findings were limited, singular in occurrence, and seen across all groups including controls; they were therefore deemed unrelated to treatment. All gross lesions reported at terminal necropsy were considered incidental, background lesions not unexpected in rats.

*Gross pathology - carcinogenicity groups*

All gross lesions observed during necropsy were considered incidental, background lesions not unexpected in rats**.**

*Histopathology - chronic toxicity groups*

The kidney was identified as a target organ of 1,4-DMN. In males there was a minimal to moderate increase in the incidence of proteinosis (deposition of protein) in the tubules of the papilla of the

kidney, and an increase in the incidence of minimal papillary necrosis in males dosed at 3750 ppm when compared to vehicle control males. There was an increase in the incidence of minimal karyomegaly in the tubules of high-dose males compared to the vehicle control males. All three findings were considered treatment-related.

In females there was an increase in the incidence of karyomegaly of the cortical tubules of the kidney. In the females dosed at 500 ppm this finding was minimal in severity, while in the females receiving 3750 ppm karyomegaly was minimal to mild in severity. This finding, which was not seen in the vehicle control group, was considered treatment-related.

Chronic progressive nephropathy, which is a spontaneous change in the kidney most often observed in older male rats, was present in this study at a frequency that was considered consistent with normal background. Chronic progressive nephropathy was not related to the administration of 1,4- DMN. All other histopathological findings were considered unrelated to treatment because of their infrequent occurrence, low severity, and similar distribution among treated and control groups.

*Histopathology- carcinogenicity groups*

No changes related to administration of the test material were noted in the incidence of neoplasms.

Test material-related microscopic findings were limited to renal changes which were present in males and females at 3750 ppm. The findings were more extensive for males than for females. There was also an increase in liver cysts and infiltration of mononuclear cells in high-dose male and females.

Incidence and severity of dilatation of the papillary tubules were greater for males dosed at 3750 ppm than for control males, and the incidence and severity of papillary necrosis, mineralization and pigmentation also greater for both males and females dosed at 3750 ppm than for the controls.

Pelvic proteinosis was present for males and females of all dose groups, however its incidence was increased for males dosed at 3750 ppm (equal to 208 mg/kg bw per day). The frequencies of renal cortical infarction and micro abscesses were also increased for males dosed at 3750 ppm.

***Table 14. Selected pathology findings in kidney, liver and lung at the end of dosing period (12 months for chronic toxicity groups and 24 months for carcinogenicity groups)***

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | **0** | **150** | **500** | **3750** | **0** | **150** | **500** | **3750** |
| | **Kidney** | | | | | | | |
| Number of animals (12 month) | 19 | 18 | 19 | 19 | 20 | 19 | 19 | 20 |
| Chronic progressive nephropathy (all): | 3 | 9* | 6 | 12** | 7 | 0 | 4 | 3 |
| minimal | 3 | 9* | 5 | 9* | 7 | 0 | 3 | 3 |
| mild | 0 | 0 | 1 | 3 | 0 | 0 | 1 | 0 |
| Necrosis, papillary (all): | 0 | 0 | 0 | 5* | 0 | 0 | 0 | 0 |
| minimal | 0 | 0 | 0 | 5* | 0 | 0 | 0 | 0 |
| Proteinosis, tubular; papilla (all) | 0 | 0 | 0 | 13** | 0 | 0 | 0 | 0 |
| minimal | 0 | 0 | 0 | 5* | 0 | 0 | 0 | 0 |
| mild | 0 | 0 | 0 | 5* | 0 | 0 | 0 | 0 |
| moderate | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 |
| Karyomegaly; tubular (all): | 0 | 0 | 0 | 4 | 0 | 0 | 4* | 11** |
| minimal | 0 | 0 | 0 | 4 | 0 | 0 | 4* | 7** |
| mild | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| **Non-neoplastic lesions** | | | | | | | | |
| Number of animals (24 month) | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 |
| Chronic progressive nephropathy | 46 | 48 | 38 | 59** | 26 | 21 | 12 | 33 |

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | **0** | **150** | **500** | **3750** | **0** | **150** | **500** | **3750** |
| Infarction | 5 | 8 | 8 | 30** | 3 | 2 | 1 | 3 |
| Mineralization, papillary | 6 | 8 | 5 | 40** | 13 | 9 | 5 | 27** |
| Cyst, cortex | 3 | 3 | 1 | 10* | 1 | 0 | 1 | 1 |
| Necrosis, papillary | 0 | 2 | 0 | 38** | 0 | 1 | 1 | 6* |
| Micro-abscesses | 0 | 1 | 0 | 12** | 1 | 0 | 0 | 1 |
| Proteinosis, pelvic | 28 | 21 | 16 | 51** | 16 | 9 | 13 | 32** |
| Pigment, papillary | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 1 |
| Dilatation, papillary tubules | 1 | 2 | 0 | 35** | 3 | 1 | 4 | 6 |
| **Neoplastic lesions** | | | | | | | | |
| Number of animals (24 month) | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 |
| Adenoma; benign, primary, incidental | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Sarcoma; malignant, primary, fatal | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Fibrosarcoma; secondary, fatal | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| **Liver** | | | | | | | | |
| Number of animals (12 month) | 19 | - | - | 19 | 20 | - | - | 20 |
| Mononuclear cell infiltration (all): | 11 | - | - | 9 | 10 | - | - | 5 |
| minimal | 11 | - | - | 9 | 10 | - | - | 5 |
| Hyperplasia; biliary (all): | 4 | - | - | 7 | 1 | - | - | 0 |
| minimal | 3 | - | - | 6 | 1 | - | - | 0 |
| mild | 1 | - | - | 1 | 0 | - | - | 0 |
| Cyst | 0 | - | - | 0 | 1 | - | - | 1 |
| Cysts, multiple | 0 | - | - | 0 | 0 | - | - | 1 |
| **Non-neoplastic lesions** | | | | | | | | |
| Number of animals (24 month) | 65 | 40 | 39 | 65 | 65 | 42 | 46 | 65 |
| Basophilic focus | 1 | 0 | 2 | 3 | 0 | 4* | 0 | 0 |
| Clear cell focus | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| Eosinophilic focus | 5 | 2 | 1 | 5 | 1 | 0 | 0 | 3 |
| Cyst | 0 | 0 | 0 | 5* | 2 | 0 | 5 | 9* |
| Degeneration, cystic | 5 | 1 | 1 | 9 | 0 | 0 | 0 | 1 |
| Mononuclear cell infiltration | 44 | 20 | 18 | 48 | 20 | 9 | 16 | 33* |
| Inflammation, chronic active | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Fibrosis | 1 | 0 | 1 | 2 | 1 | 0 | 1 | 0 |
| Haemorrhage | 5 | 0 | 4 | 5 | 1 | 1 | 0 | 3 |
| Pigmentation | 0 | 1 | 1 | 4 | 2 | 0 | 0 | 3 |
| Telangiectasis | 4 | 0 | 0 | 0 | 1 | 0 | 0 | 4 |
| Fibrosis, serosal | 1 | 0 | 1 | 2 | 1 | 0 | 1 | 0 |
| Hyperplasia, bile duct | 20 | 11 | 5 | 28 | 10 | 4 | 6 | 15 |
| Vacuolation, hepatocellular | 14 | 9 | 10 | 9 | 16 | 11 | 3 | 7 |
| Necrosis, hepatocellular | 2 | 1 | 2 | 2 | 3 | 1 | 2 | 6 |
| Tension lipidosis | 1 | 0 | 0 | 3 | 2 | 1 | 0 | 0 |
| **Neoplastic lesions** | | | | | | | | |
| Number of animals (24 month) | 65 | 40 | 39 | 65 | 65 | 42 | 46 | 65 |

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | 0 | 150 | 500 | 3750 | 0 | 150 | 500 | 3750 |
| Hepatocellular adenoma; benign, primary, incidental | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 2 |
| Hepatocellular carcinoma; malignant, primary, fatal | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Hepatocellular carcinoma; malignant with metastasis, primary, fatal | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Hepatocellular carcinoma; malignant with and without metastasis, primary, fatal | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| Cholangioma; benign, primary, incidental | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Hemangiosarcoma; malignant, primary, fatal | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| Hemangiosarcoma; secondary, incidental | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Hemangiosarcoma; secondary, fatal | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cholangiocarcinoma; malignant, primary, incidental | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Malignant lymphoma; fatal | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Lung** | | | | | | | | |
| Number of animals (12 month) | 19 | – | – | 19 | 20 | – | – | 20 |
| Hypertrophy; pneumocyte (all): | 1 | – | – | 1 | 0 | – | – | 0 |
| minimal | 1 | – | – | 1 | 0 | – | – | 0 |
| Histiocytosis; alveolar (all): | 6 | – | – | 10 | 1 | – | – | 6* |
| minimal | 6 | – | – | 10 | 1 | – | – | 5 |
| mild | 0 | – | – | 0 | 0 | – | – | 1 |
| **Non-neoplastic lesions** | | | | | | | | |
| Number of animals (24 month) | 65 | 40 | 39 | 65 | 65 | 42 | 46 | 65 |
| Oedema | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| Haemorrhage | 0 | 1 | 2 | 3 | 2 | 1 | 1 | 1 |
| Bacterial colonies | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cyst, keratin | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Emphysema | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| Inflammation, acute | 1 | 0 | 0 | 1 | 2 | 0 | 1 | 3 |
| Inflammation, chronic | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| Inflammation, granulomatous | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Necrosis | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Pigmentation | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 |
| Fibrosis, pleural | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Foreign body, plant material | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| Hyperplasia, alveolar epithelial cell | 0 | 1 | 1 | 2 | 0 | 0 | 1 | 3 |
| Infiltrate, alveolar macrophage | 8 | 4 | 8 | 14 | 13 | 4 | 4 | 15 |
| Infiltrate, mononuclear | 1 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| Mineralization, alveolar | 2 | 1 | 3 | 0 | 1 | 0 | 0 | 0 |
| Mineralization, vascular | 20 | 16 | 13 | 17 | 15 | 4 | 5 | 6 |
| **Neoplastic lesions** | | | | | | | | |
| Number of animals (24 month) | 65 | 40 | 39 | 65 | 65 | 42 | 46 | 65 |

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | 0 | 150 | 500 | 3750 | 0 | 150 | 500 | 3750 |
| Bronchiolo-alveolar adenoma; benign, primary, incidental | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| Ganglioneuroma; secondary, fatal | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Osteosarcoma; secondary, fatal | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| Mammary carcinoma; secondary, fatal | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 1 |

(Statistical values calculated by the applicant) Source: Mallett, 2011

Statistically different from control values using Fisher's Exact Test with significance: * $p < 0.05$, ** $p < 0.01$

*Conclusion – chronic toxicity*

Daily oral administration of 1,4-DMN via the diet at 3750 ppm per day for one year to male and female Sprague Dawley rats resulted in decreased food consumption with concurrent decreases in body weight and body weight gain. In addition, minimal to moderate histological, treatment-related findings in the kidney (proteinosis, papillary necrosis and karyomegaly) were noted in male rats at 3750 ppm (equal to 208 mg/kg bw per day), while minimal to mild karyomegaly was noted in the kidney of female rats administered 1,4-DMN at doses of 500 or 3750 ppm (equal to 33 and 247 mg/kg bw per day respectively).

*Conclusion – carcinogenicity*

No treatment-related changes were noted in the incidence of neoplasms in animals tested by daily oral administration via diet admixtures of 1,4-dimethylnaphthalene in a carcinogenicity study in rats. The neoplasms observed in the study were typical of those frequently present in aged Sprague Dawley rats. Therefore, the administration of 1,4-DMN to male and female Sprague Dawley rats, under the conditions of this study, did not cause carcinogenicity.

Treatment-related microscopic findings were limited to renal and liver changes present in males and females dosed at 3750 ppm. The findings were more extensive in kidney for males than for females.

The incidence and severity of dilatation of the papillary tubules was higher for males given 3750 ppm (equal to 208 mg/kg bw per day) when compared with control males. Also the incidence and severity of papillary necrosis, mineralization and pigmentation was higher for males and females given 3750 ppm (equal to 208 and 247 mg/kg bw per day for males and females respectively) when compared with relevant controls.

Pelvic proteinosis was present for males and females in all dose groups; however, the incidence was increased in males dosed at 3750 ppm (equal to 208 mg/kg bw per day). Renal cortical infarction and micro-abscesses were increased in males at 3750 ppm.

In view of the above, the NOAEL for systemic toxicity was 500 ppm (equal to the 27 mg/kg bw per day for males, 33 mg/kg bw per day for females) based on reduced body weights, histopathological changes in the kidneys, including dilatation of the papillary tubules, papillary necrosis and mineralization, pelvic proteinosis and renal cortical infarction, all noted in the kidney of rats administered 1,4-DMN at doses of 3750 ppm (equal to 208 mg/kg bw per day for males, 247 mg/kg bw per day for females). The NOAEL for carcinogenicity was 3750 ppm (equal to 247mg/kg bw per day) the highest dose tested. (Mallett, 2011)

## 2.4 Genotoxicity

The data package presented on the genotoxicity of 1,4-DMN consisted of eight studies, five in vitro and three in vivo. Summaries of genotoxicity studies along with the information on doses used, purity and batch number are presented in Tables 15a and b.

### (a) In vitro studies

***Table 15a. Summary of in vitro genotoxicity studies with 1,4-dimethylnaphthalene***

| Type of study | Organisms/cells | Concentration/dose range tested | Purity | Result | Reference |
|---|---|---|---|---|---|
| | | **In vitro** | | | |
| Bacterial mutagenicity (Ames) | *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, and TA1538 | 10, 50, 100, 250, 500 and 1000 µg/plate (± S9)<br>1, 5, 10, 25, 50 and 250 µg/plate (− S9) | 96.4% | Not mutagenic<br>May be considered as support | Lawlor, 1993 |
| Bacterial mutagenicity (Ames) | *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537 | Experiment 1: 0, 0.128, 0.64, 3.2, 16, 80, 400 and 2000 µg/plate (± S9)<br>Experiment 2: 0, 1.6, 4.1, 10.2, 25.6, 64, 160 and 400 µg/plate (± S9) | 98.36% | Not mutagenic | Ballantyne, 2007 |
| Micronucleus test | Human lymphocytes | Experiment I 6.0, 10.5 and 18.3 µg/mL (± S9)<br>Experiment II: 21.3, 37.3 and 65.3 µg/mL (− S9) | 98.91% | Negative | Naumann, 2022 |
| Mammalian gene mutation test | L5178y mouse lymphoma cells | Main test 1: 1, 5, 10, 20, 30, 35, 37.5 and 40 µg/mL (− S9)<br>50, 75, 100, 150, 170, 200, 210 and 220 µg/ mL (+ S9)<br>Main test 2: 50, 75, 100, 150, 170, 200, 210 and 220 µg/ mL (+ S9) | 98.36% | Equivocal [a] | Verspeek-Rip, 2005 |
| Unscheduled DNA synthesis | Rat hepatocytes | 0.25, 0.5, 1, 2.5, 5 and 10 µg/mL | 96.4% | Negative | Ham, 1993 |

### (a) In vivo studies

***Table 15b. Summary of in vivo genotoxicity studies with 1,4-dimethylnaphthalene***

| Type of study | Organisms/cells | Concentration/dose range tested | Purity | Result | Reference |
|---|---|---|---|---|---|
| | | **In vivo** | | | |
| Micronucleus test | Mouse | 225, 450 and 900 mg/kg bw (single dose by gavage) | 96.4% | Negative [b] | Murli, 1993, 2007 |
| Unscheduled DNA synthesis | Rat liver | 500 and 1000 mg/kg bw (single treatment by gavage) | 98.36% | Negative [c] | Beevers, 2007 |
| Transgenic rodent assay | Rat | 70, 210 and 600/670 [d] mg/kg bw (gavage) for 28 consecutive days | 99.2% | Negative | Bruce, 2021 |

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*

[a] Positive outcome at one precipitating dose is likely to have been an artefactual result due to precipitation.

[b] Clinical signs, lethality and the ratio of polychromatic erythrocytes to normochromic erythrocytes (PCE:NCE ratio) indicate systemic exposure. 1000 PCEs (1993) and 2000 PCEs (2007) per animal per sampling time were analysed. In 2007 extra reticulocytes were read from the 1993 slides.

[c] According to EFSA (2017), OECD 486 is no longer valid as a follow-up to positive mutagenicity tests. Therefore, the present study serves as supplementary information only.

[d] High dose (670 mg/kg bw) was adjusted after two deaths were observed on day 3. Two additional animals were added to the high-dose group in place of the two animals that died. From day 3 (day 1 for replacements), the high dose was administered at 600 mg/kg bw per day.

## 2.5 Reproductive and developmental toxicity

### (a) Multigeneration studies

Instead of a two-generation reproduction study, the sponsor provided an extended one-generation reproductive toxicity (EOGRT) study. Though no test guideline existed when performed, it appears to be a reasonable match for the basic phase of OECD 443 (2018).

1,4-Dimethylnaphthalene (purity 98.4%, Batch No. 14D06B01-01) was administered to 24 parental (F0) Sprague Dawley rats of each sex per group. Rats were 8–9 weeks old at first dosing and male rats weighed 219–285 g, females 134–213 g. Test article was provided in admixture with the diet at target concentrations of 0 (basal diet alone), 500, 2000 and 7500 ppm (equal to 0, 40, 160 and 510 mg/kg bw per day). Parental rats were fed the formulated diet for two weeks prior to mating, during the two-week mating period, then for up to 10 weeks for the parental males, and through gestation, lactation and until scheduled necropsy for the parental females.

F1 pups were exposed to 1,4-DMN during the lactation phase of the study, and selected F1 offspring were exposed to the same concentration of the diet admixtures as the parental dams from rearing until scheduled sacrifice.

F1 offspring were culled to 10 pups (five of each sex) when feasible. Approximately 30 pups of each sex per group were selected for additional evaluation. The F1 offspring were subdivided into two cohorts. Rats in cohort 1 were treated until 13 weeks old and then euthanized and subjected to a complete necropsy, while cohort 2 rats were utilized for T-cell-dependant antibody response (TDAR) assays around eight weeks of age. Food consumption, body weight, body weight gain, reproductive performance, organ weights, clinical pathology and histopathology were evaluated during the study, along with offspring body weight (growth), measurement of anogenital distance, survival, and developmental landmarks (vaginal patency and preputial separation for the F1 generation).

***Results – parental animals (F0)***

No treatment-related adverse clinical signs were noted, nor did any treatment-related death occur during the study. Corollary reductions in mean body weight and mean body weight gain were observed throughout the study for the 7500 ppm group animals.

Mean food consumption by both the parental and F1 generations was significantly reduced in the groups receiving 7500 ppm.

Negative food efficiency reflected the pronounced effects seen early in the study (day 3) on food consumption and body weight gain of rats treated at 7500 ppm. By the end of week 1 food efficiency was broadly similar across all groups, although negative food efficiency was seen again in the 500 and 7500 ppm groups over gestation days (GDs) 0–7, and in the 7500 ppm group over postnatal days (PNDs) 0–4.

No treatment-related effects were observed in urinalysis during the study.

With regard to clinical pathology examinations, thyroxine (T4) and thyroid-stimulating hormone (TSH) were not significantly different between the treated and control groups. The most salient changes in clinical pathology parameters were significant increases in GGTP and cholesterol levels in both sexes among the groups receiving 7500 ppm; cholesterol levels were also elevated in the rats treated at 2000 ppm. Another significant finding in serum chemistry profiles was increased triglycerides amongst females receiving 2000 ppm and 7500 ppm. Slight decreases in coagulation parameters (prothrombin time and/or activated partial thromboplastin time) were noted in males and females of all dosed groups but differences were marginal and not considered toxicologically relevant as no other study was available to confirm the results.

*Table 16. Selected haematology and clinical chemistry parental results from the EOGGRT study (mean ± standard deviation shown)*

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Parameter** | **0** | **500** | **2000** | **7500** | **0** | **500** | **2000** | **7500** |
| Number of animals | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| **Haematology (day 90)** | | | | | | | | |
| Prothrombin time (s) | 10.7 ± 0.2 | 10.5* ± 0.2 | 10.4* ± 0.2 | 10.2* ± 0.2 | 10.6 ± 0.3 | 10.6 ± 0.4 | 10.6 ± 0.4 | 10.5 ± 0.6 |
| Activated partial thromboplastin time (s) | 18.6 ± 1.1 | 18.3 ± 0.7 | 17.9 ± 0.6 | 18.3 ± 0.8 | 18.1 ± 0.7 | 17.9 ± 0.7 | 17.3* ± 0.5 | 16.7* ± 0.7 |
| **Blood chemistry (day 90)** | | | | | | | | |
| γ-Glutamyl transferase (U/L) | 0 ± 0 | 0 ± 0 | 0.1 ± 0.1 | 0.3* ± 0.3 | 0.2 ± 0.2 | 0 ± 0 | 0.4 ± 0.4 | 3.1* ± 0.9 |
| Cholesterol (mg/dL) | 53 ± 8 | 62 ± 9 | 77* ± 16 | 84* ± 10 | 42 ± 4 | 51 ± 9 | 68* ± 10 | 94* ± 10 |
| Triglycerides (mg/dL) | 48 ± 14 | 48 ± 13 | 48 ± 16 | 34 ± 6 | 36 ± 10 | 46 ± 11 | 53* ± 14 | 51* ± 12 |
| Thyroxine (µg/dL) | 4.6 ± 1.9 | 4.9 ± 1.8 | 4.6 ± 1.6 | 4.7 ± 1.3 | 3.67 ± 0.62 | 3.21 ± 0.58 | 3.6 ± 0.73 | 3.8 ± 0.56 |
| Thyroid-stimulating hormone (µU/mL) | 2.39 ± 1.41 | 2.69 ± 2.09 | 2.87 ± 1.47 | 3.38 ± 1.74 | 1.87 ± 1.09 | 1.46 ± 0.77 | 2.12 ± 1.84 | 3.17 ± 1.31 |

* Statistically different from control values with significance $p \leq 0.05$; Source: Ryan, 2011a

Treatment with 1,4-DMN resulted in increased liver weights as well as increased relative liver weights in both sexes in either the 2000 ppm and/or 7500 ppm groups. No underlying histological change was associated with the increased liver weights, therefore the change was seen to represent an adaptive response, such as enzyme induction, rather than a direct toxic effect on the liver.

Reductions in a few absolute organ weights were noted in the treated rats and these were considered to be associated with reduced body weight, most likely stemming from food aversion at the time of acclimation to the diet admixtures. No underlying histological change was associated with any of the organ weight changes, even at the highest dose of 7500 ppm, further supporting the conclusion that the reductions in organ weight were secondary to body weight effects.

Gross necropsy findings were few and sporadically distributed across all groups including the vehicle controls.

No histopathological findings were deemed treatment-related and none of the reproductive organs showed evidence of toxicity.

Reproductive performance and litter viability were unaffected by treatment.

***Results – offspring (F1)***

No adverse clinical signs were noted during the study. No statistically significant difference in offspring survival was detected between the treated and control groups. Pup survival was above 90% throughout lactation (PNDs 0–21).

Reductions in body weight parameters were evident in male and female F1 rats receiving 2000 or 7500 ppm 1,4-DMN, and in females receiving 500 ppm prior to and after weaning; these were ascribed, in part, to palatability issues.

Reductions in food consumption were evident in either the 2000 to 7500 ppm treated F1 rats prior to and after weaning and were ascribed, in part, to palatability issues.

Food efficiency was broadly similar across F1 groups throughout their exposure period.

The most salient changes in developmental landmarks were a delay in preputial separation and vaginal patency in the 7500 ppm groups, although this was not unexpected as these developmental landmarks are closely linked to body weight. No other effect attributable to treatment was seen in development or reproductive end-points (anogenital distance, onset of estrus following patency, epididymal/testicular sperm counts, motility and morphology, and vaginal cytology; see Table.17).

***Table 17. F1 litter observations from the EOGGRT study***

| Parameter | | Dose group | | | |
|---|---|---|---|---|---|
| | | Control (0 ppm) | LDT (500 ppm) | MDT (2000 ppm) | HDT (7500 ppm) |
| Anogenital distance/litter; males (cm) | Number | 23 | 22 | 24 | 20 |
| | Mean | 6.6 | 6.4 | 6.3 | 6.1 |
| | SD | 1.1 | 0.56 | 0.52 | 0.62 |
| Anogenital distance/litter; females (cm) | Number | 22 | 22 | 24 | 20 |
| | Mean | 3.2 | 3.1 | 3.2 | 3.1 |
| | SD | 0.4 | 0.3 | 0.2 | 0.3 |
| Age at preputial separation (days) | Number | 20 | 20 | 20 | 20 |
| | Mean | 37 | 38 | 40* | 46* |
| | SD | 2 | 2 | 3 | 3 |
| Body weight at preputial separation (g) | Number | 20 | 20 | 20 | 20 |
| | Mean | 161 | 163 | 168 | 144* |
| | SD | 15 | 19 | 24 | 18 |
| Age at vaginal patency (days) | Number | 20 | 20 | 20 | 19 |
| | Mean | 34 | 36 | 35 | 43* |
| | SD | 2 | 2 | 2 | 4 |
| Body weight at vaginal patency (g) | Number | 20 | 20 | 20 | 19 |
| | Mean | 118 | 122 | 118 | 105* |
| | SD | 12 | 16 | 15 | 13 |
| Onset of first estrus (days post patency) | Number | 20 | 19 | 20 | 19 |
| | Mean | 3 | 2 | 2 | 1 |
| | SD | 3 | 2 | 2 | 2 |
| Onset of first estrus (days post patency, excluding outliers) | Number | 18 | 19 | 20 | 19 |
| | Mean | 2 | 2 | 2 | 1 |
| | SD | 2 | 2 | 2 | 2 |

SD: Standard deviation; Source: Ryan, 2011a

* Statistically different from control values with significance $p \leq 0.05$

No treatment-related urinalysis effects were observed during the study.

As regards clinical pathology, T4 and TSH did differ significantly between the treated and control groups. The most salient changes in clinical pathology parameters were significant increases in GGTP and cholesterol levels in both sexes in the groups receiving 7500 ppm; cholesterol levels were also elevated in the 2000 ppm treatment group. Another significant finding in serum chemistry profiles was increased triglycerides amongst the 2000 ppm and 7500 ppm group females, while triglycerides were reduced in F1 males treated at 2000 ppm and 7500 ppm. Slight decreases in coagulation parameters (prothrombin time and/or activated partial thromboplastin time) were noted in F1 males of all dose groups but not in females, and differences were marginal and not considered toxicologically relevant as no other study could be referred to confirm these results (Table 18).

*Table 18. Selected F1 generation haematology and clinical chemistry data from the EOGGRT study (mean ± standard deviation shown)*

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Doses (ppm)** | **0** | **500** | **2000** | **7500** | **0** | **500** | **2000** | **7500** |
| Number of animals/group | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| **Haematology** | | | | | | | | |
| Prothrombin time (s) | 10.6 ± 0.3 | 10.3* ± 0.1 | 10.3* ± 0.1 | 10.1* ± 0.1 | 10.3 ± 0.3 | 10.3 ± 0.2 | 10.2 ± 0.2 | 10.1 ± 0.1 |
| Activated partial thromboplastin time (s) | 17.6 ± 0.8 | 17.3 ± 0.8 | 16.8 ± 1 | 16.9 ± 0.7 | 17 ± 0.7 | 16.7 ± 0.5 | 16.9 ± 0.6 | 15.5* ± 0.5 |
| **Blood chemistry** | | | | | | | | |
| γ-Glutamyl transferase (U/L) | 0 ± 0.1 | 0 ± 0 | 0 ± 0.1 | 1.9* ± 1.1 | 0.3 ± 0.3 | 0.4 ± 0.4 | 0.7 ± 0.4 | 9.2* ± 9.5 |
| Cholesterol (mg/dL) | 58 ± 11 | 66 ± 8 | 73* ± 15 | 80* ± 10 | 50 ± 11 | 51 ± 10 | 73* ± 14 | 123* ± 23 |
| Triglycerides (mg/dL) | 58 ± 19 | 50 ± 12 | 44* ± 7 | 35* ± 8 | 30 ± 6 | 33 ± 6 | 40* ± 10 | 43* ± 13 |
| Thyroxine (µg/dL) | 3.42 ± 0.47 | 3.76 ± 0.56 | 3.77 ± 0.55 | 3.49 ± 0.39 | 3.55 ± 0.79 | 3.55 ± 0.62 | 3.74 ± 0.48 | 3.6 ± 0.5 |
| Thyroid-stimulating hormone (µU/mL) | 2.7 ± 2.61 | 2.76 ± 1.28 | 3.35 ± 3.23 | 5.64 ± 3.46 | 1.83 ± 1.51 | 0.86 ± 0.68 | 0.95 ± 0.42 | 2.34 ± 1.82 |

* Statistically different from control values with significance $p \leq 0.05$; Source: Ryan, 2011a

Gross necropsy findings were few and sporadically distributed across all groups including vehicle controls. There were no effects at first litter check or during lactation.

As seen in the parental group, treatment with 1,4-DMN resulted in increased liver weights as well as relative liver weights in both sexes, in groups exposed at 2000 ppm (by 18% in males, 13% in females) and/or 7500 ppm (by 24% in males, 36%in females). This change may represent an adaptive response, such as enzyme induction, rather than a direct toxic effect on the liver. Also, reductions in absolute organ weights that were noted in the treated rats and were considered to be associated with reduced body weight.

A single treatment-related finding was seen in the kidney of an adult male rat from the 7500 ppm group (cohort 2). The findings in this animal consisted of moderate karyomegaly (characterized by a band of large cortical tubular cells with very large and prominent nuclei) which was present in the inner region of the renal cortex. No other histopathological findings were reported.

No evidence of immunotoxicity (by TDAR assay or splenic lymphocyte subpopulations) was detected.

In view of the above, it was observed that overall, pronounced reduction in food consumption, likely due to palatability issues, was seen in the parental and F1 offspring receiving 7500 ppm. A similar,but much less pronounced trend was seen in the 2000 ppm group parents and F1 offspring. The reduction in food consumption resulted in decreased body weight, body weight gain, organ weights and alterations in F1 growth and development (delay in preputial separation and vaginal patency at 7500 ppm), while reproductive end-points like mating performance, fertility, fecundity, litter survival, sperm parameters and estrus cyclicity were unaffected by treatment. An additional cohort for developmental neurotoxicity testing was not included in view of the absence of neurotoxicity in any other study, including a 90-day study with extensive neurobehavioural examinations. No evidence of immunotoxicity (TDAR assay or splenic lymphocyte subpopulations) was detected. One observed change was the increased liver weights in the 2000 ppm and 7500 ppm dose groups which were most likely due to an adaptive process (for example enzyme induction) rather than a direct toxic effect, a conclusion supported by the lack of any adverse histological findings.

Accordingly, NOAEL for parental toxicity was 500 ppm in parental males and females (equal to 40 mg/kg bw per day) based on increased liver weights and an increase in plasma cholesterol accompanied by increased triglycerides in females, at a dose of 2000 ppm (equal to 160 mg/kg bw per day). The NOAELs for offspring and for developmental toxicity were both 2000 ppm (equal to 160 mg/kg bw per day), the latter based on delayed vaginal patency and preputial separation, and the former on reduced body weight, both effects seen in the group receiving 7500 ppm (equal to 510 mg/kg bw per day).

As no fertility effects were observed, the NOAEL for reproductive toxicity was 7500 ppm (equal to 510 mg/kg bw/day),the highest dose tested (Ryan, 2011a).

**(b) Developmental toxicity**

***Rat***

The sponsor provided no data for rats. However a conclusion on developmental toxicity in rats could be drawn from extended one-generation reproductive toxicity study of Ryan (2011a), described above.

***Rabbit***

1,4-Dimethylnaphthalene (purity 98.4%, Batch no. 14D06B01-01) was formulated as a solution in a corn oil vehicle and administered by daily oral gavage at dose levels of 0 (vehicle control), 25, 80 or 250 mg/kg bw to 23 time-mated female rabbits per group. Rabbits were approximately five months old and weighed between 2843 and 4785 g on the day of mating. Doses were administered once daily at a constant volume of 1.0 mL/kg bw over GDs 6–28 (day 0 was defined as the observed day of mating), this included the period from implantation through organogenesis and histogenesis.

Clinical observations, maternal body weight, body weight gain and food consumption were monitored throughout gestation. All surviving does were sacrificed on their respective twenty-ninth days of gestation and subjected to caesarean section and gross necropsy. Each uterus was weighed, opened, and inspected for implantation sites. Fetuses were harvested, weighed, and given a gross external examination. In addition, all fetuses were subjected to a visceral examination and processed for skeletal evaluation (vehicle control and high-dose animals were examined), while approximately half of the fetuses from each litter were designated for cephalic examination (vehicle control and high-dose groups were examined).

Mean food consumption was significantly reduced in the does dosed at 250 mg/kg bw per day shortly after treatment initiation (over GDs 6–9 and 9–12); this reduction in food consumption was probably due to palatability issues (Table 19). Corollary reductions in mean body weight gain were observed in the 250 mg/kg bw per day group over GDs 6–9 (Table 20). Body weight gain was lower over GDs 9–12, but statistical significance was not achieved. Absolute body weights tended to be lower in the high-dose group from GDs 9–18, but statistical significance was not achieved. Gravid uterus weights were similar across groups. The changes seen in body weight and/or body weight gain indicated maternal toxicity as gravid uterus weights were unaffected by treatment. In addition, correction of maternal body weight and body weight gain for uterus weight revealed no treatment-related effects.

***Table 19. Mean food consumption during the developmental study in rabbits***

| | | Treatment group and dosage (mg/kg bw per day) | | | |
|---|---|---|---|---|---|
| **Period** | | **Vehicle control 0** | **Low 25** | **Mid 80** | **High 250** |
| Days 0–3 | Number | 21 | 20 | 21 | 18 |
| | Mean | 476 | 461 | 501 | 461 |
| | SD | ±114 | ±82 | ±169 | ±122 |
| Days 3–6 | Number | 21 | 20 | 21 | 18 |
| | Mean | 541 | 519 | 537 | 508 |
| | SD | ±114 | ±102 | ±159 | ±95 |

| | | Treatment group and dosage (mg/kg bw per day) | | | |
|---|---|---|---|---|---|
| | | Vehicle control | Low | Mid | High |
| Period | | 0 | 25 | 80 | 250 |
| Days 6–9 | Number | 21 | 20 | 21 | 18 |
| | Mean | 480 | 403 | 456 | 309* |
| | SD | ±185 | ±103 | ±208 | ±142 |
| Days 9–12 | Number | 21 | 20 | 21 | 18 |
| | Mean | 499 | 379 | 466 | 275* |
| | SD | ±180 | ±100 | ±252 | ±140 |
| Days 12–15 | Number | 21 | 20 | 21 | 18 |
| | Mean | 302 | 237 | 349 | 193 |
| | SD | ±222 | ±164 | ±285 | ±197 |
| Days 15–18 | Number | 21 | 20 | 21 | 18 |
| | Mean | 369 | 238 | 402 | 263 |
| | SD | ±226 | ±184 | ±298 | ±189 |

SD: Standard deviation; Source: Ryan, 2011b

* Statistically different from control values with significance $p \leq 0.05$

***Table 20. Mean maternal body weight gain during the developmental study in rabbits (results shown are weight gain in g over the period)***

| | | Dose (mg/kg bw per day) | | | |
|---|---|---|---|---|---|
| Period | | 0 | 25 | 80 | 250 |
| Days 0–3 | Number | 21 | 20 | 21 | 18 |
| | Mean | 50 | 52 | 41 | 57 |
| | SD | ±55 | ±92 | ±47 | ±82 |
| Days 3–6 | Number | 21 | 20 | 21 | 18 |
| | Mean | 24 | 35 | 9 | 19 |
| | SD | ±37 | ±48 | ±40 | ±42 |
| | N | | | | |
| Days 6–9 | Number | 21 | 20 | 21 | 18 |
| | Mean | 12 | 1 | -9 | −51* |
| | SD | ±53 | ±55 | ±61 | ±82 |
| Days 9–12 | Number | 21 | 20 | 21 | 18 |
| | Mean | 22 | 6 | 30 | −13 |
| | SD | ±72 | ±52 | ±68 | ±104 |
| Days 12–15 | Number | 21 | 20 | 21 | 18 |
| | Mean | -6 | 18 | 11 | −13 |
| | SD | ±107 | ±93 | ±112 | ±110 |
| Days 15–18 | Number | 21 | 20 | 21 | 18 |
| | Mean | 9 | −9 | 3 | 11 |
| | SD | ±92 | ±86 | ±89 | ±122 |
| Days 18–21 | Number | 21 | 20 | 21 | 18 |
| | Mean | −22 | 1 | −29 | −15 |
| | SD | ±76 | ±73 | ±83 | ±70 |

| | | Dose (mg/kg bw per day) | | | |
|---|---|---|---|---|---|
| **Period** | | **0** | **25** | **80** | **250** |
| Days 21–24 | Number | 20 | 19 | 20 | 17 |
| | Mean | 33 | 34 | 14 | −18 |
| | SD | ±63 | ±72 | ±61 | ±160 |
| Days 24–27 | Number | 19 | 19 | 20 | 15 |
| | Mean | 43 | 63 | 35 | 25 |
| | SD | ±83 | ±69 | ±98 | ±76 |
| Days 27–29 | Number | 19 | 18 | 20 | 15 |
| | Mean | 24 | 24 | ~1 | 23 |
| | SD | ±69 | ±47 | ±117 | ±87 |
| Total gain | Number | 19 | 18 | 20 | 15 |
| | Mean | 217 | 299 | 140 | 155 |
| | SD | ±262 | ±157 | ±225 | ±428 |

SD: Standard deviation; Source: Ryan, 2011b

* Statistically different from control values with significance $p \leq 0.05$

One rabbit in the 250 mg/kg bw per day group was found dead on GD 21; evidence of abortion was noted on GD 20 for this rabbit, and the death was probably related to the abortion. No other animals died during the study. Also, seven other rabbits (two controls, two low-, one mid- and two high-dose) underwent abortion over GDs 21 to 27.

No treatment-related clinical signs were noted during the study and gross necropsy findings were limited to those rabbits that underwent abortions. The gross necropsy findings consisted of changes in the gastrointestinal tract (dilatation of stomach and/or intestine) and were probably related to the lack of eating prior to, or during, abortion.

Overall, no treatment-related differences in litter viability were detected. A single statistically significant reduction in total implants was seen for the 80 mg/kg bw per day group. Also, in the 250 mg/kg bw per day group the number of total implants was slightly lower, but not with statistical significance. The lower number of total implants may have been associated with a lower number of corpora lutea, as the groups receiving 80 and 250 mg/kg bw per day also presented on average fewer (but not significantly so) corpora lutea.

Therefore, based on the findings from this study, the maternal NOAEL for 1,4-DMN was 80 mg/kg bw per day based on reduced food consumption and body weight gain at 250 mg/kg bw per day. The embryo/fetal NOAEL was 250 mg/kg bw per day, the highest dose tested (Ryan, 2011b)

## 2.6 Special studies

### (a) Neurotoxicity

The neurotoxic potential of 1,4-DMN was not specifically assessed with tests for this end-point. In a 13-week dietary study in rats (Mallett, 2003) with 1,4-DMN at doses of 0, 500, 2500 and 10 000 ppm (equal to 0, 25, 125 and 500 mg/kg bw per day), the animals were subjected to a FOB and motor activity tests. In that study, the only notable observation was a reduction in forelimb grip strength in males at 500 mg/kg bw per day. Since in these animals, body weight gain was reduced by 34%, it is likely that the reduced forelimb grip strength reflects a poor general condition, rather than being a neurotoxic effect of 1,4-DMN. It was concluded that 1,4-DMN is not neurotoxic.

Since neurotoxicity was not observed in these studies, 1,4-dimethylnaphthalene does not appear to present a neurotoxic hazard. Therefore the requirement for neurotoxicity testing is not triggered.

### (b) Immunotoxicity

Special immunotoxicity studies were not performed and not considered necessary in view of the comprehensive toxicology study database existing for 1,4-DMN. In an extended one generation study, (Ryan, 2011a) a cohort consisting of 10 F1 offspring of each sex per group were used for immunotoxicity assessment and treated until eight weeks old (week 4 post weaning). At least one pup from each litter and sex (male and female from different cohorts) were subjected to investigation of prenatally and postnatally induced immunotoxic effects (lymph nodes and splenic lymphocyte subpopulations analysis).

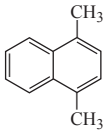
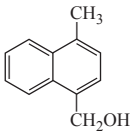
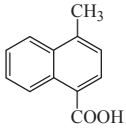
No evidence of immunotoxicity (by TDAR assay or splenic lymphocyte subpopulations) was detected.

### (c) Studies on metabolites

The two metabolites M21 (1-hydroxymethyl-4-methylnaphthalene) and M23 (4-methyl-1-naphthoic acid) are major rat metabolites of 1,4-DMN. It can thus be concluded that they contribute substantially to the toxicological profile of 1,4-DMN and it is highly unlikely that they have greater toxicity than the parent compound.

A quantitative structure–activity relationship (QSAR) assessment report was undertaken for metabolites M21 and M23.

***Table 21. Information on structure of 1,4-DMN, M21 and M23***

| S Number | Molecule | CAS No. | SMILES code | Structure |
|---|---|---|---|---|
| 1 | **1,4-DMN** (1,4-dimethylnaphthalene) | 571-58-4 | Cc1ccc(C)c2ccccc12 |  |
| 2 | **M21** (1-hydroxymethyl-4-methylnaphthalene) | 57322-44-8 | Cc1ccc(CO)c2ccccc12 |  |
| 3 | **M23** (4-methyl-1-naphthoic acid) | 4488-40-8 | Cc1ccc(C(O)=O)c2ccccc12 |  |

SMILES: Simplified molecular input line entry system;

***Table 22. Physical-chemical properties of 1,4-DMN, M23 and M21***

| S Number | Name | Molecular weight (Da)* | Log $K_{ow}$* | Dice similarity coefficient* |
|---|---|---|---|---|
| 1 | 1,4-DMN | 156 | 4.02 | 100% |
| 2 | M21 | 172 (9.4% higher than parent) | 2.8 (1.22 lower than parent) | 80% |
| 3 | M23 | 186 (19.2% higher than parent) | 3.6 (0.42 lower than parent) | 77% |

$K_{ow}$: The *n*-octanol:water partition coefficient;

* Calculated from OECD QSAR Toolbox v. 4.5

Based on Dice similarity coefficients higher than 70%, M21 and M23 (at 77%–80%) are considered similar to the parent molecule.

For metabolite M21, the molecular weight is 172 Da (9.4% higher than the parent), and log $K_{ow}$ found to be 2.8 (1.22 lower than the parent) indicating that urinary excretion is greater than for 1,4-DMN.

For metabolite M23, the molecular weight is 186 Da (19.2% higher than the parent), and log $K_{ow}$ 3.6 (0.42 lower than the parent) indicating that the urinary excretion is greater than for 1,4-DMN. The main structural difference between the parent 1,4-DMN and M21 is the presence of the hydroxyl group

in M21. The presence of the hydroxyl group in M21 promotes its urinary excretion.

M21 and M23 are the downstream metabolites of 1,4-DMN (see Fig. 3). Based on the information provided above and information from the profiling conducted using the OECD QSAR toolbox, it can be concluded that 1,4-DMN and the metabolites M21 and M23 are structurally very similar and their toxicity profile can also be considered similar. Thus, read-across from 1,4-DMN can be applied to M21 and M23.

The genotoxic potential of M21 and M23 was further investigated using a statistical tool (Caesar mutagenicity model) and a rule-based expert model (for example DEREK Nexus). The likelihood of M21 and M23 possessing genotoxic potential was considered to be low because of the reliable negative predictions from DEREK Nexus mutagenicity and chromosome aberration models. Metabolite M21 was positive for mutagenicity by the Caesar mutagenicity model, however the predictions were considered unreliable because the similarity between the target molecule and the source molecules is low according to the Dice similarity coefficient provided by the OECD QSAR toolbox. In addition, the only difference between parent and M21 is a single hydroxyl group, a group that is considered not to confer a high mutagenic potential according to DEREK Nexus and the OECD QSAR toolbox.

In addition. due to the similar physical chemical properties and structural similarity, it is unlikely that M21 has a higher genotoxicity and general toxicity compared to the parent molecule (Khan, 2022).

***Table 23. Prediction of genotoxicity for 1,4-DMN, M21 and M23 by QSAR***

| S. Number | Compound | OECD QSAR alert | Caesar mutagenicity model (applicability domain) | Ames mutagenicity by DEREK Nexus | Chromosome aberration by DEREK Nexus | Overall conclusion on genotoxicity |
|---|---|---|---|---|---|---|
| 1 | 1,4-DMN | None | Negative (out) | Negative | Equivocal | Negative |
| 2 | M21 | None | Positive (in) | Negative | Negative | Negative |
| 3 | M23 | None | Negative (not optimal) | Negative | Negative | Negative |

Apart from M21 and M23 discussed above, there are other metabolites of interest found in various edible commodities of plant and animal origin:

- M01 4-methyl-1-naphthaldehyde,
- M02 the glycine conjugate of M23,
- M03 and M04 (α variant and δ variant) ornithine conjugates of M23,
- M14 1,4-naphthalenedicarboxylic acid,
- M06 dihydroxy-1-methyl-4-naphthoic acid,
- M15 1,4-dimethylnaphthol.

Analysis was made to assess the genotoxic potential of these metabolites. Two state-of-the-art QSAR tools were used as a statistical model (for example, SARAH Nexus) and a rule-based expert model (DEREK Nexus); both are considered suitable for predicting the genotoxicity of pesticides and their metabolites. The assessed end-points were Ames mutagenicity, clastogenicity and aneugenicity.

Metabolites M02, M03 and M04 (glycine conjugate and α/δ ornithine conjugates of M23), were predicted to be negative or inactive by both QSAR models SARAH and DEREK Nexus. the parent molecule and M01 shared the structural alert naphthalene (H20). 1,4-Dimethylnaphthalene was negative in a genotoxicity testing battery and since parent molecule and M01 share the same alert and M01 is also predicted to be negative by DEREK Nexus, the alert was considered to be false positive.

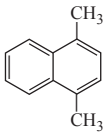
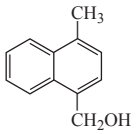
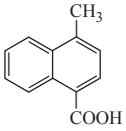
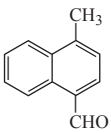
Metabolite M06, was predicted to be positive due to the presence of 2-naphthol and a benzene ring with aliphatic and aromatic arms (H173). The genotoxicity alert due to the presence of 2-naphthol should be considered as false positive. The second genotoxicity alert due to the benzene ring with aliphatic and aromatic arms should also be considered a false positive because of the low reliability of the data in the training set. Also, visually similar compounds from the training set (naphthalene-1,6-diol and naphthalene-1,7-diol) are returned positive for genotoxicity by SARAH Nexus, however they are not classified as genotoxic according to the European Chemicals Agency (ECHA) database.

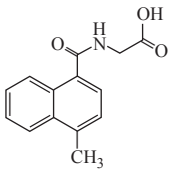
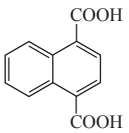
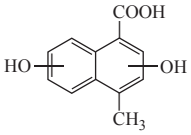
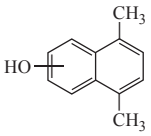
The prediction confidence for the metabolites M06, is very low (9%), which raises concerns over the reliability of the prediction. In addition, metabolite M06, is predicted to be negative by DEREK Nexus. Therefore, based on all the available evidence, the alert was considered a false positive.

Metabolite M14 elicits alerts H-20 and H-173 which were considered false positive, for the reasons described above for other metabolites. Metabolite M15 also contains 2-naphthol which is classified as nonmutagenic according to ECHA, therefore its prediction should be considered a false positive. In addition, both metabolites M14 and M15 were negative according to DEREK Nexus.

In conclusion, the genotoxicity assessment of metabolites of 1,4-DMN by the QSAR tools SARAH Nexus resulted in false positive predictions for M01, M06, M14 and M15. Analysis of the training set reveals that some molecules are listed positive, however they are not classified as mutagens in the ECHA database. Since these metabolites were predicted to be negative by DEREK Nexus, based on a weight of evidence approach, the positive predictions from SARAH Nexus were considered to be false positives and the Meeting concluded that metabolites M01, M06, M14 and M15 were negative for Ames mutagenicity and chromosome aberrations.

***Table 24. Genotoxicity status of 1,4-DMN and its metabolites***

| Code name, Chemical name (IUPAC) | Structure, Molar mass | Source (Percentages shown of total radioactive residue) | Genotoxicity data | SARAH nexus[a] | DEREK Nexus[b] | Overall conclusion |
|---|---|---|---|---|---|---|
| **Parent**<br>1,4-dimethyl-naphthalene<br>1,4-DMN | <br>156.2 g/mol | Plants<br>(potato, 2.9%–98%)<br>Ruminants<br>(muscle, 4.7%)<br>Poultry<br>(egg yolk, 39%; whole egg, 29%; muscle, 7.9%–35%; fat, 89%–94%; liver, 1.5%) | Genotoxicity<br>Data submitted.<br>No genotoxicity alert | Negative (100% conf.)<br>Hypothesis H-20 | Equivocal for chromosome aberration | Negative |
| **M21**<br>1-Hydroxy-methyl-4-methyl-naphthalene | <br>172.2 g/mol | Plants<br>(potato, 1.0–65%) | Based on the high structural similarity of around 80%, it was proposed to group this metabolite with the parent[c] | | | Negative. Based on the high structural similarity of approx. 80%, it is proposed to group this metabolite with the parent[e] |
| **M23**<br>4-methyl-1-napthaoic acid | <br>186.2 g/mol | Plants<br>(potato, 1.5%–2.3%)<br>Ruminants<br>(kidney, 1.3%)<br>Poultry<br>(egg white, 71%; egg yolk, 13%; whole egg, 26%; muscle, 34%–47%; liver, 54%) | Based on the high structural similarity of around 80%, it was proposed to group this metabolite with the parent[d] | Negative (29% conf.)<br>Hypothesis H-20 (12% conf.) | Inactive (No mis-classified or unclassified features) | Negative |
| **M01**<br>4-methyl-1-naphthal-dehyde |  | Processed commodity after hydrolysis<br>(potato, 9.2%) | Downstream of M21 – goes to M23 (Between M21 and M23) | Positive (10% conf.)<br>Hypothesis H-20 (10% conf.) | Inactive (No mis-classified or unclassified features) | Negative |

| Code name, Chemical name (IUPAC) | Structure, Molar mass | Source (Percentages shown of total radioactive residue) | Genotoxicity data | SARAH nexus[a] | DEREK Nexus[b] | Overall conclusion |
|---|---|---|---|---|---|---|
| **M02**<br>Glycine conjugate of 4-methyl-1-naphthaoic acid |  | Ruminants (milk, 18%; kidney, 17%) | Glycine conjugate of M23 | Negative (29% conf.) Hypothesis H-20 (12% conf.) | Inactive (No mis-classified or unclassified features) | Negative |
| **M03**<br>(δ variant)<br>**M04**<br>(α variant)<br>Ornithine conjugates of 4-methyl-1-naphthaoic acid | | Poultry (egg yolk, 16%; whole egg, 12%; liver, 6.4%) | | Negative (28% conf.) Hypothesis H-20 (21% conf.) | Inactive (No mis-classified or unclassified features) | Negative |
| **M14**<br>1,4-Naphtha-lene-dicarboxylic acid |  | Poultry (muscle, 4.2%–5.3% liver, 5.4%) | | Negative (26% conf.) Hypothesis H-20 (20% conf.) | Inactive (No mis-classified or unclassified features) | Negative |
| **M06**<br>Dihydroxy-1-methyl-4-naphthoic acid |  | Ruminants (kidney, 1.3%; muscle, 4%) | | Positive (11% conf.) Hypothesis H-25 (9% conf.) Hypothesis H-173 (31% conf.) | Inactive (No mis-classified or unclassified features) | Negative |
| **M15**<br>1,4-Dimethyl-naphthol |  | Processed commodity (potato, identified as minor metabolite, but not quantified | Ring hydroxylation yields 1,4-DMN | Positive (7% conf.) Hypothesis H-25 (7% conf.) | Inactive (No mis-classified or unclassified features) | Negative |

[a] DEREK Nexus: Ames mutagenicity; conf.: Confidence; Source- Khan, 2023

[b] SARAH Nexus: Ames mutagenicity and chromosome aberration;

[c] Greater than 10% after i.p. administration in urine, but only traces after oral administration. However, two direct downstream metabolites (M23 and 4-methyl-1-naphthoic acid glucuronide); these are taken into consideration here as it is assumed that downstream metabolites can only be found if the respective metabolic precursors were present before. Therefore, M21 was present in rat metabolism after oral administration at more than 10%. In addition, QSAR and read-across assessment were conducted. M21 was found negative regarding genotoxicity according to the QSAR models. According to Dice score, similarity was 80%. Profiling with OECD QSAR Toolbox showed no additional alerts compared to the parent. Based on the high structural similarity it was proposed this metabolite be grouped with the parent.

[d] M23 is covered by toxicity studies conducted with the parent. M23 was found in urine at greater than 10% after i.p. administration, but only at 6% after oral administration. However, one direct downstream metabolite (4-methyl-1-naphthoic acid glucuronide) was also found after oral administration at 6.1%. Downstream metabolites are taken into consideration as it is assumed that they can only be found if the respective metabolic precursors were present before. Therefore, M23 was present in rat metabolism after oral administration at more than 10%. In addition, QSAR and read-across assessment was conducted and M23 was found to be negative regarding genotoxicity according to the QSAR models. According to Dice score, similarity to its parent was 80%. Profiling with OECD QSAR Toolbox showed no additional alerts compared with the parent. Based on the high degree of structural similarity it was proposed this metabolite be grouped with the parent

[e] M21 was found to be negative regarding genotoxicity according to the QSAR models. According to its Dice score, similarity to its parent was 80%. Profiling with OECD QSAR Toolbox showed no additional alerts compared to the parent. Based on the high structural similarity it was proposed this metabolite be grouped with the parent

### (d) Expert statement on comparison of 1,4-dimethylnaphthalene and other alkylated naphthalenes in terms of metabolism and toxicity

The sponsor provided an expert statement (Weterings & Freudenthal, 2004) in which metabolism and toxicity of 1,4-DMN was compared with other alkylated naphthalenes. The authors reviewed and discussed information on ADME studies of various alkylated naphthalenes (listed in Table 25), conducted with mice, rats and Guinea pigs and administered either orally or intraperitoneally. Single-dose administration and repeat-dose administration were also compared.

A summary of this statement is provided below.

***Table 25. Alkylated naphthalenes and their abbreviations evaluated in the statement of Weterings & Freudenthal***

| Abbreviation | Name |
|---|---|
| 2-MN | 2-methylnaphthalene |
| 1-IPN | 1-isopropylnaphthalene |
| 2-IPN | 2-isopropylnaphthalene |
| 1,2-DMN | 1,2-dimethylnaphthalene |
| 1,4-DMN | 1,4-dimethylnaphthalene |
| 1,6-DMN | 1,6-dimethylnaphthalene |
| 2,6-DMN | 2,6-dimethylnaphthalene |
| 2,6-DIPN | 2,6-diisopropylnaphthalene |

Weterings, & Freudenthal, 2004

Alkylated naphthalenes are rapidly absorbed, presumably by passive diffusion through the lipophilic matrix of the intestinal membrane, and widely distributed through the body with a preference for adipose tissue and skin. After a single oral or intraperitoneal dose to mammals, the alkylnaphthalenes studied demonstrate very similar rates of absorption and excretion. Excretion is essentially complete within 72 hours.

Distribution and disappearance after repeated administration was investigated with 2,6-DIPN. After daily oral administration to rats for 17 or 31 days, adipose tissue and skin contained higher concentrations of 2,6-DIPN than those of rats subject to a single dose. 2,6-Diisopropylnaphthalene concentrations in blood, liver and kidneys corresponded to those in these tissues observed at 6–8 hours after a single oral administration. After administration, all alkylnaphthalenes disappeared rapidly from most tissues except adipose tissue and skin. Except for 2-MN, the disappearance curves were biphasic. No accumulation was observed.

All alkylnaphthalenes were extensively metabolized in rat, mouse and Guinea pig. Unchanged parent compound is a minor component in urine or bile. Apart from the unchanged substance, rat urine contained metabolites formed by three different pathways.

The number of sulfur-containing metabolites reflects the amount of highly reactive epoxide intermediates formed, and consequently the toxicity of the parent compounds. The amount of sulfur-containing metabolites excreted in urine differed significantly between naphthalene (5.5%), 1,2-DMN (35%), 1,4-DMN (1.5%) and 1-6-DMN (22%). Since there is very little of the glutathione conjugate formed from the metabolism of 1,4-DMN, it was concluded that very little of the reactive epoxide metabolites are formed. This is supported by the observation that glutathione depletion, observed at high doses of administered naphthalenes, was very low for 1,4-DMN compared to other alkylated naphthalenes.

Of the many alkylated naphthalenes, 1,4-DMN and 2,6-DIPN revealed equally low cytotoxicity and equally low rates of formation of reactive metabolite.

The statement also compares toxicological end-points of 1,4-DMN with end-points of other alkylnaphthalenes. Two 90-day feeding studies conducted with 1,4-DMN and 2,6-DIPN showed that the target organ and the toxic effects are the same, and occur at similar dose levels, providing convincing

evidence that the toxicity of 1,4-DMN and 2,6-DIPN are equivalent.

Chronic toxicity and carcinogenicity studies conducted with 1-MN and 2-MN in mice showed glutathione depletion and related pulmonary alveolar proteinosis which shall be typical for alkylnaphthalenes. However, the authors concluded that due to the low ring-oxidation profile of 1,4-DMN, chronic toxicity and carcinogenicity effects from 1,4-DMN are unlikely.

Chronic dietary studies with 1-MN or 2-MN provided evidence that there are unlikely to be any effects on reproduction from alkylnaphthalenes at doses up to 140 mg/kg bw per day.

Developmental toxicity was evaluated based on the three studies: a prenatal developmental toxicity study of 2,6-DIPN in rats, a limited two-generation study with 2,6-DIPN in pregnant mice and an oral study with pregnant rats. For reproductive toxicity assessment, the authors considered three studies: the subchronic 90-day study with 1,4-DMN in rats and two mouse studies with 1-MN and 2-MN. Developmental and reproductive toxicity was not affected by any of alkylnaphthalenes investigated. Therefore, it was concluded that there is no cause for concern over developmental toxicity due to alkylnaphthalenes, including 1,4-DMN (Weterings & Freudenthal, 2004).

## 4. Microbiological aspects

The possible impact of 1,4-dimethylnaphthalene residues on the human intestinal microbiome was considered for evaluation.

A search of literature available in the public domain did not identify information describing any direct or indirect experimental evidence that addressed the possible impact of 1,4-DMN residues on the human intestinal microbiome. No experimental data were submitted by the sponsor in this regard.

## 5. Observations in humans

**Report on medical surveillance on manufacturing plant personnel**

No exposure-related health effects during manufacture of the active substance are known.

The product is intended for application by trained personnel only, using specialised equipment. In the last 27 years during which the product has been applied to stored potatoes in the USA, Canada, Europe and other regions, no clinical cases or poisoning incidents have been reported.

Neither data on exposure of the general public nor epidemiological studies are available.

No published literature regarding health effects after exposure of the general public are available. (Jeanette ,2022).

# Comments

## Biochemical aspects

After oral administration, radiolabelled 1,4-DMN is rapidly and almost completely absorbed, widely distributed, metabolized, and essentially completely excreted within 48 hours, with urine being the primary route of excretion (72% of administered radioactivity) (Dohn, Ostermann & Woodbury, 2012). After intraperitoneal administration of [$^{14}$C]1,4-DMN, radioactivity was found in faeces (40.8% of administered dose, AD, within 72 hours) which confirms biliary excretion. After intraperitoneal administration of [$^{3}$H]1,4-DMN the maximum level of radioactivity in plasma ($C_{max}$ = 0.53 kBq/mL) was reached during the fourth hour after administration of the compound ($T_{max, plasma}$ = ca 4 hours).

Limited information is available on the distribution of 1,4-DMN after oral administration. However, after intraperitoneal administration, 1,4-DMN and its metabolites were widely and rapidly distributed in rats. The highest concentrations were observed in adipose tissue, followed by liver, kidneys, spleen and adrenals. After 72 hours tissue levels, including in adipose tissue, were low (less than 2 % of AD), indicating that neither 1,4-DMN nor its metabolites accumulate (Kilanowicz, Sapota, & Daragó, 2000).

After a single oral dose of 28.6 mg/kg body bw 1,4-DMN, it was detected only in trace amounts in urine (Dohn, Ostermann & Woodbury, 2012). After intraperitoneal administration at 28 mg/kg bw, 1,4-DMN and its metabolites were excreted rapidly (plasma half-life of eight hours) and mainly via urine (56.5% of AD within 72 hours), but also via faeces (40.8% of AD within 72 hours) (Kilanowicz, Sapota, & Daragó, 2000).

After intraperitoneal administration, apart from the unchanged substance (1,4-DMN), the rat urine contained metabolites obtained via three different pathways:

- side chain hydroxylation, forming 1-hydroxymethyl-4-methylnaphtalene (M21; trace amounts in urine) followed by oxygenation to form 4-methyl-1-naphthoic acid (M23; 6% in urine) and further metabolism to, for example, 4-methyl-1-naphthoic acid glucuronide (6.1 % in urine);
- ring hydroxylation;
- the formation of reactive epoxide intermediates, followed by their conjugation with glutathione or other organic xenobiotics that can be cleaved by enzymatic hydrolysis to naphthothiophenols, resulting in cysteine-*S*-conjugates, which in turn are enzymatically converted to the corresponding thionaphthol by the release of pyruvate and ammonia. In a further step, metabolites with accessible sulfhydryl groups can be subjected to methylation, yielding 1,4-dimethyl-methylthionaphthalene.

From in vitro studies of the metabolism of 1,4-DMN with microsomes, no unique metabolite was identified with human microsomes compared to mice, rats and dogs. It was observed that rat metabolism was slower than metabolism in humans, dogs or mice (Erk, 2022).

## Toxicological data

The acute oral median lethal dose ($LD_{50}$) of 1,4-DMN was greater than 2000 mg/kg bw and the dermal $LD_{50}$ was greater than 2000 mg/kg bw. The inhalation median lethal concentration ($LC_{50}$) of 1,4-DMN was greater than 4.15 mg/L (Rajendran, 1993). 1,4-Dimethylnaphthalene was slightly to moderately irritating to skin (Johnson, 1993c) and irritating, but reversibly so, to the eyes in rabbits (Johnson, 1993c). It was not skin sensitizing in a local lymph node assay (LLNA) (Van Huygevoort, 2011).

In repeat-dose toxicity studies on rats the main effects were on the kidney.

In a 90-day dietary toxicity study in rats, 1,4-DMN was administered at concentrations of 0, 500, 2500 or 10 000 ppm (equal to 0, 32, 161 and 677 mg/kg bw per day for males, 0, 38, 186 and 747 mg/kg bw per day for females). The no-observed- adverse-effect level (NOAEL) for 1,4-DMN was 500 ppm (equal to 32 mg/kg bw per day) based on increases in relative kidney weight (by 15%), basophilic tubules in the kidney and a suppression of body weight gain (by 11% in males) at 2500 ppm (equal to 161 mg/kg bw per day) (Mallett, 2003).

In a 104-week combined chronic toxicity and carcinogenicity study in rats, 1,4-DMN was administered in the diet at concentrations of 0, 150, 500 or 3750 ppm (equal to 0, 8, 27 and 208 mg/kg bw per day for males, 0, 10, 33 and 247 mg/kg bw per day for females). The NOAEL for systemic toxicity was 500 ppm (equal to the 27 mg/kg bw per day) based on reduced body weight and histopathological changes in the kidneys of both sexes at 3750 ppm (equal to 208 mg/kg bw per day). The NOAEL for carcinogenicity was 3750 ppm (equal to 208 mg/kg bw per day), the highest dose tested (Mallett, 2011).

The Meeting concluded that 1,4-dimethylnaphthalene is not carcinogenic in rats.

1,4-Dimethylnaphthalene was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found.

The Meeting concluded that 1,4-dimethylnaphthalene is unlikely to be genotoxic.

In view of the lack of genotoxicity and the absence of carcinogenicity in rats, the Meeting concluded that 1,4-DMN is unlikely to pose a carcinogenic risk to humans.

In an extended one-generation reproductive toxicity study in rats, 1,4-DMN was administered at dietary concentrations of 0, 500, 2000 or 7500 ppm (equal to 0, 40, 160 and 510 mg/kg bw per day).

The NOAEL for parental toxicity was 500 ppm (equal to 40 mg/kg bw per day), based on increased liver weights, increased cholesterol accompanied by increased triglycerides in females at 2000 ppm (equal to 160 mg/kg bw per day). The reproductive NOAEL was 7500 ppm (equal to 510 mg/kg bw per day), the highest dose tested. The offspring/developmental NOAEL was 2000 ppm (equal to 160 mg/kg bw per day), based on delayed vaginal patency and preputial separation, and reduced body weight at 7500 ppm (equal to 510 mg/kg bw per day) (Ryan, 2011a).

In a developmental toxicity study in rabbits, 1,4-DMN was administered by gavage at dose levels of 0 , 25, 80 or 250 mg/kg bw per day. The maternal NOAEL was 80 mg/kg bw per day based on reduced food consumption and body weight gain at the 250 mg/kg bw per day. The embryo/fetal NOAEL was 250 mg/kg bw per day, the highest dose tested (Ryan, 2011b).

The Meeting concluded that 1,4-dimethylnaphthalene is not teratogenic.

No specific data were submitted regarding neurotoxicity, but no evidence of neurotoxicity was reported in routine toxicological studies with 1,4-DMN.

The Meeting concluded that 1,4-dimethylnaphthalene is unlikely to be neurotoxic

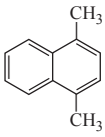
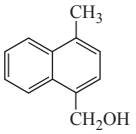
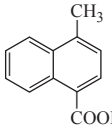
No specific data were submitted regarding immunotoxicity, but no evidence of immunotoxicity was reported in routine toxicological studies with 1,4-DMN.

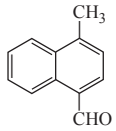
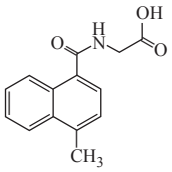
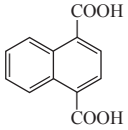
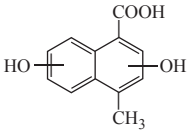
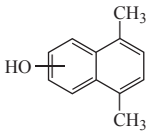
The Meeting concluded that 1,4-dimethylnaphthalene is unlikely to be immunotoxic.

## Toxicological data on metabolites and/or degradates

There were in total nine metabolites identified. These were: M21 (1-hydroxymethyl-4-methylnaphthalene), M23 (4-methyl-1-naphthoic acid), M01 (4-methyl-1-naphthaldehyde), M02 (glycine conjugate of M23), M03 (α variant) and M04 (δ variant) both of which are ornithine conjugates of M23), M14 (1,4-naphthalenedicarboxylic acid, M06 (dihydroxy-1-methyl-4-naphthoic acid), and M15 (1,4-dimethylnaphthol). Information on these metabolites is summarized in Table 26.

***Table 26. Summary overview of toxicological characterization of plant/livestock metabolites***

| **Compound, codes and structure** | **Major rat metabolite** (> 10% AD) | **Genotoxicity assessment** (data, QSAR, read-across) | **General toxicity** | **Toxicological reference values** |
|---|---|---|---|---|
| **1,4-Dimethylnaphthalene** (1,4-DMN)  | Parent | Not genotoxic (data) | Dataset | ADI 0–0.08 mg/kg bw<br>ARfD: Unnecessary |
| **M21** (1-Hydroxymethyl-4-methylnaphthalene)  | Traces in urine, but > 12.1% of AD based on downstream metabolites | Not genotoxic (QSAR) | Covered by parent | Parent ADI |
| **M23** (4-Methyl-1-naphthaoic acid)  | 6.0% in urine, but > 12.1% including downstream metabolites | Not genotoxic (QSAR) | Covered by parent | Parent ADI |

| Compound, codes and structure | Major rat metabolite (> 10% AD) | Genotoxicity assessment (data, QSAR, read-across) | General toxicity | Toxicological reference values |
|---|---|---|---|---|
| **M01**<br>(4-Methyl-1-naphthaldehyde)<br> | M01 is a metabolite of M21 and is metabolized into M23 | Not genotoxic (QSAR) | No data | Parent ADI |
| **M02**<br>(Glycine conjugate of 4-methyl-1-napthaoic acid)<br> | Glycine conjugate of M23 | Not genotoxic (QSAR) | No data | Parent ADI |
| **M03** (δ variant)<br>**M04** (α variant)<br>Ornithine conjugates of M23,4-methyl-1-napthaoic acid (two stereoisomers) | Ornithine conjugates of M23 | Not genotoxic (QSAR) | No data | Parent ADI |
| **M14**<br>(1,4-naphthalenedicarboxylic acid)<br> | Not found in rats | Not genotoxic (QSAR) | No data | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **M06**<br>(Dihydroxy-1-methyl-4-naphthoic acid)<br> | Not found in rats | Not genotoxic (QSAR) | No data | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **M15**<br>(1,4-dimethylnaphthol)<br> | Not found in rats | Not genotoxic (QSAR) | No data | TTC Cramer class III value: 1.5 µg/kg bw per day |

ADI Acceptable daily intake; RA read across; AD Administered dose; TTC Threshold of toxicological; concern
QSAR Quantitative structure–activity relationship

## Microbiological aspects

There was no information available in the public domain, and no experimental data was submitted that addressed the possible impact of 1,4-DMN residues on the human intestinal microbiome.

## Human data

No exposure-related health effects during manufacture or use of 1,4-DMN are known.

No information on accidental or intentional poisoning in humans was available.

The Meeting concluded that the existing database on 1,4-dimethylnaphthalene was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

## Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) for 1,4-dimethylnaphthalene of 0–0.3 mg/kg bw based on the NOAEL of 27 mg/kg bw per day in the 104-week combined chronic toxicity and carcinogenicity study in rats, and using a safety factor of 100. This was supported by the NOAEL of 32 mg/kg bw per day in the 90-day rat dietary study.

The Meeting concluded that it was not necessary to establish an acute reference dose (ARfD) for 1,4-dimethylnaphthalene in view of its low acute oral toxicity and the absence of developmental toxicity or any other toxicological effects likely to be elicited by a single dose.

***Levels relevant to risk assessment of 1,4-dimethylnaphthalene***

| Species | Study | Effect | NOAEL | LOAEL |
|---|---|---|---|---|
| Rat | 90-day dietary toxicity study | Toxicity | 500 ppm, equal to 32 mg/kg bw per day | 2500 ppm, equal to 161 mg/kg bw per day |
| | 104-week combined chronic toxicity and carcinogenicity study [a] | Toxicity | 500 ppm, equal to 27 mg/kg bw per day | 3750 ppm, equal to 208 mg/kg bw per day |
| | | Carcinogenicity | 3750 ppm, equal to 208 mg/kg bw per day [c] | - |
| | Extended one-generation reproductive toxicity study [a] | Reproductive toxicity | 7500 ppm equal, to 510 mg/kg bw per day [c] | - |
| | | Parental toxicity | 500 ppm equal, to 40 mg/kg bw per day | 2000 ppm, equal to 160 mg/kg bw per day |
| | | Offspring toxicity | 2000 ppm, equal to 160 mg/kg bw per day | 7500 ppm, equal to 510 mg/kg bw per day |
| Rabbit | Developmental toxicity study [b] | Maternal toxicity | 80 mg/kg bw per day | 250 mg/kg bw per day |
| | | Embryo/fetal toxicity | 250 mg/kg bw per day [c] | - |

[a] Dietary administration [b] Gavage administration [c] Highest dose tested

*Acceptable daily intake (ADI) applies to 1,4-dimethylnaphthalene, M01, M02, M03, M04, M21 and M23, expressed as 1,4-dimethylnaphthalene*

0–0.3 mg/kg bw

*Acute reference dose (ARfD)*

Unnecessary

### Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure.

***Critical end-points for setting guidance values for exposure to 1,4-dimethylnaphthalene***

| ***Absorption, distribution, excretion and metabolism in mammals*** | |
|---|---|
| Rate and extent of oral absorption | Rapid; extensive, $T_{max}$ = 4 hours |
| Dermal absorption | No data |
| Distribution | Wide; highest concentrations in the adipose tissue and liver |
| Potential for accumulation | No evidence of accumulation |

| | |
|---|---|
| Rate and extent of excretion | Rapid and nearly complete via urine and faeces; 99% within 48 hours |
| Metabolism in animals | Extensively metabolized mainly through hydroxylation on a methyl group, to form M21 and M23, and ring hydroxylation |
| Toxicologically significant compounds in animals and plants | 1,4-Dimethylnaphthalene, metabolites M01, M02, M03, M04, M21, M23 |
| ***Acute toxicity*** | |
| Rat, $LD_{50}$, oral | > 2000 mg/kg bw |
| Rat, $LD_{50}$, dermal | > 2000 mg/kg bw |
| Rat, $LC_{50}$, inhalation | > 4.15 mg/L |
| Rabbit, dermal irritation | Moderately irritating |
| Rabbit, ocular irritation | Slightly irritating, but reversible |
| Mouse, dermal sensitization | Not sensitizing (LLNA) |
| Guinea pig, dermal sensitization | No data |
| ***Short-term studies of toxicity*** | |
| Target/critical effect | Body weight gain, liver weight, kidney effects |
| Lowest relevant oral NOAEL | 32 mg/kg bw per day (rat) |
| Lowest relevant dermal NOAEL | No data |
| Lowest relevant inhalation NOAEC | No data |
| ***Long-term studies of toxicity and carcinogenicity*** | |
| Target/critical effect | Body weight gain, kidney effects |
| Lowest relevant NOAEL | 27 mg/kg bw per day (rat) |
| Carcinogenicity | Not carcinogenic |
| ***Genotoxicity*** | Unlikely to be genotoxic |
| ***Reproductive toxicity*** | |
| Target/critical effect | Liver weights, cholesterol and triglycerides |
| Lowest relevant parental NOAEL | 40 mg/kg bw per day (rat) |
| Lowest relevant offspring NOAEL | 160 mg/kg bw per day (rat) |
| Lowest relevant reproductive NOAEL | 510 mg/kg bw per day, highest dose tested (rat) |
| ***Developmental toxicity*** | |
| Target/critical effect | Reduced body weight and food consumption |
| Lowest relevant maternal NOAEL | 80 mg/kg bw per day (rabbit) |
| Lowest relevant embryo/fetal NOAEL | 250 mg/kg bw per day, highest dose tested (rabbit) |
| ***Neurotoxicity*** | No data |
| **Studies on toxicologically relevant metabolites** | |
| ***M01, M02, M03, M04, M21, M23*** | QSAR data only; all negative for genotoxicity |
| ***Microbiological data*** | No data submitted |
| ***Human data*** | No clinical cases or poisoning incidents have been recorded |

## Summary

| | Value | Study | Safety factor |
|---|---|---|---|
| ADI | 0–0.3 mg/kg bw [a] | Two-year study of toxicity and carcinogenicity (rat) | 100 |
| ARfD | Unnecessary | | |

[a] Applies to 1,4-dimethylnaphthalene,. M01, M02, M03, M04, M21 and M23, expressed as 1,4-dimethylnaphthalene

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# Carbofuran

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## Explanation

Carbofuran is the International Organisation for Standardisation (ISO)-approved common name for 2,3-dihydro-2,2-dimethylbenzofuran-7-yl *N*-methylcarbamate which has the Chemical Abstracts Service (CAS) number 1563-66-2. Carbofuran is a carbamate insecticide that acts by reversibly inhibiting the activity of cholinesterases thus inhibiting the breakdown of the neurotransmitter acetylcholine. This results in an increase of both the level and duration of the neurotransmitter action at acetylcholine receptors.

Carbofuran can be manufactured from two different starting chemicals: catechol or 2-nitrophenol (*o*-nitrophenol, ONP). The starting material affects the impurity profile of technical grade carbofuran.

Carbofuran has been previously evaluated by the Joint FAO and WHO Meeting of Pesticide Residue (JMPR) in 1976, 1979, 1980, 1982, 1996, 2002 and 2008. It was evaluated at the present Meeting as part of the periodic review program of the Codex Committee on Pesticide Residues (CCPR). New studies submitted for this evaluation include new genotoxicity studies on carbofuran and its metabolites, pharmacodynamic studies, a 21-day dermal exposure toxicity study (and associated preliminary dose-ranging studies) and a suite of endocrine disruption studies. Several older studies, not previously evaluated by JMPR, were also considered. Where appropriate, summaries of studies previously evaluated by JMPR have been included in the monograph. Many older studies were not conducted in accordance with current national or international test guidelines. All these studies were evaluated, however only those studies that contributed additional, useful information and were considered to be of acceptable quality and regulatory value have been summarized in this monograph.

A literature search was conducted using US National Institutes of Health, National Library of Medicine PubMed, and the search term "carbofuran". A total of 1465 references were retrieved. While all these publications have been reviewed, only those of value is assessing the compound and of appropriate quality have been summarized. Unless otherwise stated, all critical studies were conducted in compliance with good laboratory practice (GLP) and statements of quality assurance were provided.

The JMPR Meeting in 1996 established an acceptable daily intake (ADI) of 0–0.002 mg/kg body weight (bw) based on the no-observed-adverse-effect level (NOAEL) for inhibition of erythrocyte acetylcholinesterase (AChE) at 0.22 mg/kg bw per day in a four-week dog dietary study and using a safety factor of 100. The Meeting in 2002 established an acute reference dose (ARfD) of 0.009 mg/kg bw based on the NOAEL of 0.22 mg/kg bw per day from a four-week study in dogs, and using a safety factor of 25, the latter because the relevant toxic effects of carbofuran are "peak effects", that is, they are dependent on the maximum concentration $C_{max}$.

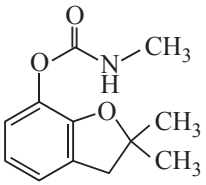
At the 2008 Meeting newly submitted AChE inhibition studies in rats (adults and pups) and a newly submitted human volunteer study (conducted in 1976) were evaluated; relevant data from short-term studies of toxicity in dogs, which had been considered by previous Meetings, were also re-examined. Based on the new and re-evaluated studies the ARfD was withdrawn and a new ARfD established at 0.001 mg/kg bw, based on the overall NOAEL of 0.03 mg/kg bw per day for inhibition of brain AChE activity in rat pups aged 11 days and a safety factor of 25. This NOAEL was supported by the lower confidence limit on the benchmark dose for a 10% response ($BMDL_{10}$) of 0.03 mg/kg bw, extrapolated from data on inhibition of brain AChE activity from three studies with 11-day-old rat pups. A safety factor of 25 was considered appropriate because the acute toxic effects of carbofuran are dependent on $C_{max}$ rather than area under the concentration–time curve (AUC), and data indicated that the sensitivities of humans and laboratory animals (rats, dogs) to inhibition of AChE by carbofuran were similar. Given the apparent higher sensitivity of younger animals, the ARfD was considered as adequately protective of infants and children since it was based on the NOAEL from a study in rat pups. At the same time the Meeting noted that this ARfD was lower than the current ADI of 0–0.002 mg/kg bw. The 2008 Meeting therefore concluded that the ADI and ARfD for carbofuran should be based on the same NOAEL, and revised the ADI to 0–0.001 mg/kg bw based on the overall NOAEL of 0.03 mg/kg bw from studies of acute toxicity in rats, and using a safety factor of 25.

# Evaluation for acceptable daily intake

## 1. Biochemical aspects

### 1.1 Absorption, distribution and excretion

*Figure 1. Chemical structure of carbofuran*



**(a) Oral route**

No new data was provided. Based on the 1996 JMPR evaluation (JMPR, 1997):

In a study first evaluated by the 1976 JMPR male Swiss white mice given an oral dose of 2 mg/kg bw of $^{3}$H-labelled carbofuran eliminated 37–67% of the dose in the urine within the first 24 h. The main metabolic pathway starts with hydroxylation, to give 3-hydroxycarbofuran, and continues with oxidation, resulting in the formation of 3-keto-carbofuran. Breakage of the carbamate ester linkage results in liberation of phenolic derivatives and their corresponding conjugates, principally glycosides.

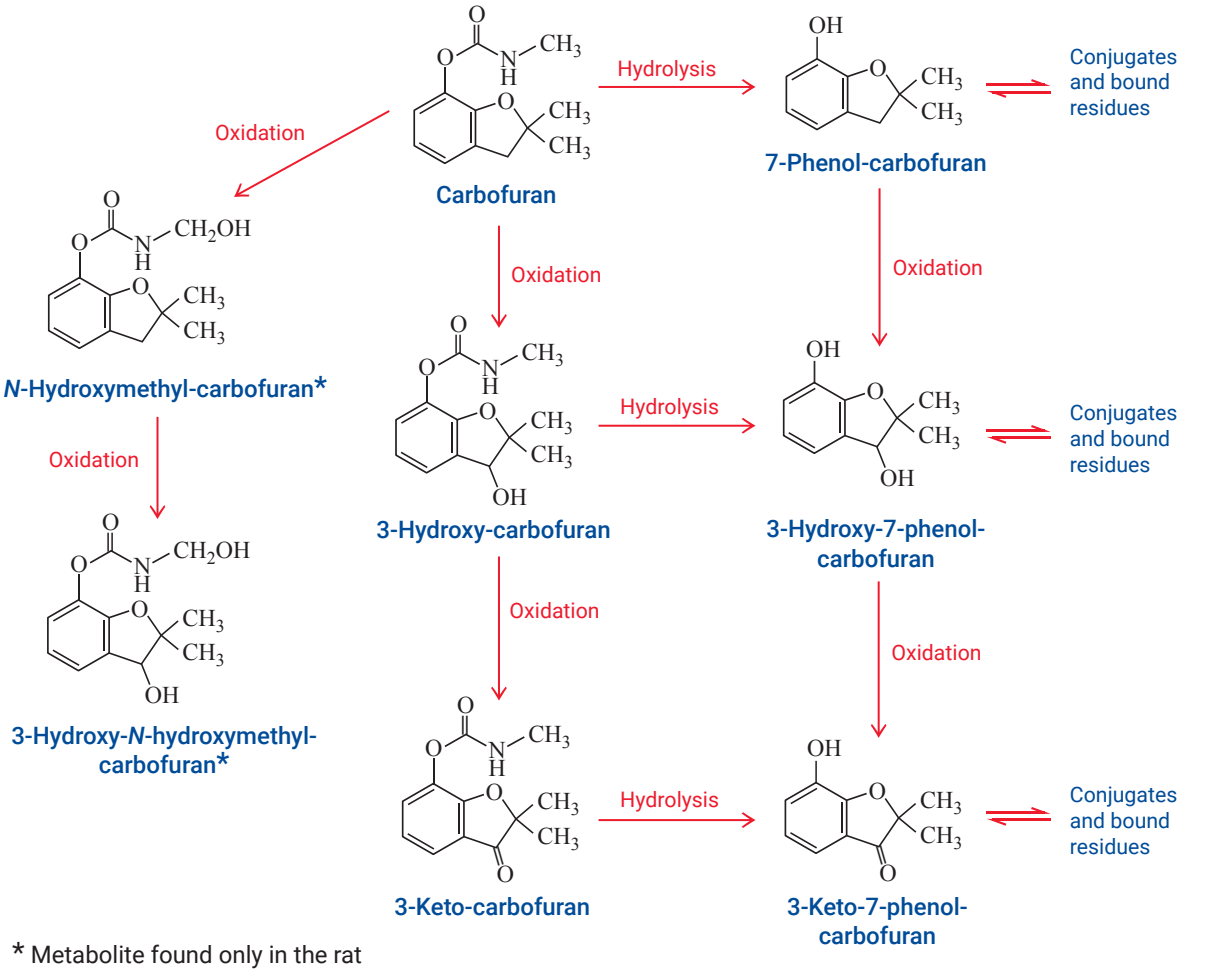
In a study first evaluated by the 1976 JMPR, rats were given either 0.4 mg/kg bw [$^{14}$C-carbonyl]carbofuran or 4 mg/kg bw [$^{14}$C-phenyl ring]carbofuran. Measurement of $^{14}$C-carbon dioxide expired by the rats treated with [$^{14}$C-carbonyl]carbofuran indicated that about 45% of the administered dose was hydrolyzed within 32 h; total urinary excretion amounted to 38% and faecal excretion to about 4% of the dose. After administration of the ring-labelled compound, total urinary excretion was about 92% and faecal excretion was 3% of the dose. Most of the radiolabel was eliminated within the first 24 h after treatment, indicating rapid oxidative cleavage of the ester linkage and rapid elimination. Analysis of metabolites in urine showed that 3-hydroxy-carbofuran, 3-keto-carbofuran phenol, 3-hydroxy-*N*-hydroxymethyl-carbofuran, 3-hydroxymethyl-carbofuran, and carbofuran phenol were the major metabolites. In vitro in rat liver homogenates, the main metabolites were 3-hydroxycarbofuran and carbofuran phenol derivatives, indicating that the metabolism in vivo follows the same general pattern observed in vitro. In a further group of rats given a single oral dose of 1 mg/kg bw, analysis of various tissues showed residues ranging from 0.06 ppm in bone to < 1.4 ppm in liver. By eight hours after treatment, all of the residue levels were < 0.8 ppm.

In plants, carbofuran is metabolized by hydroxylation, hydrolysis, and conjugation resulting in glycosides. After oral administration of alfalfa residues of carbofuran to rats, the urinary metabolites were glucuronides and sulfates, indicating cleavage of the conjugates and reconjugation.

Carbofuran is thus rapidly absorbed, metabolized, and eliminated, primarily via the urine, in the species investigated. Hydroxylation, hydrolysis, and conjugation reactions are the major metabolic steps. The resulting metabolites are either esters or cleavage products of the ester linkage.

A scheme of the proposed metabolic pathway is given in Fig. 1.

*Figure 2. Proposed metabolic pathway of carbofuran (JMPR 1996 meeting)*




(Redrawn from JMPR, 1997)

### (b) Dermal route

No data was submitted.

## 1.2 Biotransformation

### (a) In vitro

No in vivo studies were submitted. Carbofuran is a major in vivo and in vitro metabolite of carbosulfan. Extensive data on the biotransformation of carbosulfan and its downstream metabolites, including carbofuran, have been evaluated in the JMPR 2023 carbosulfan monograph (see previous monograph).

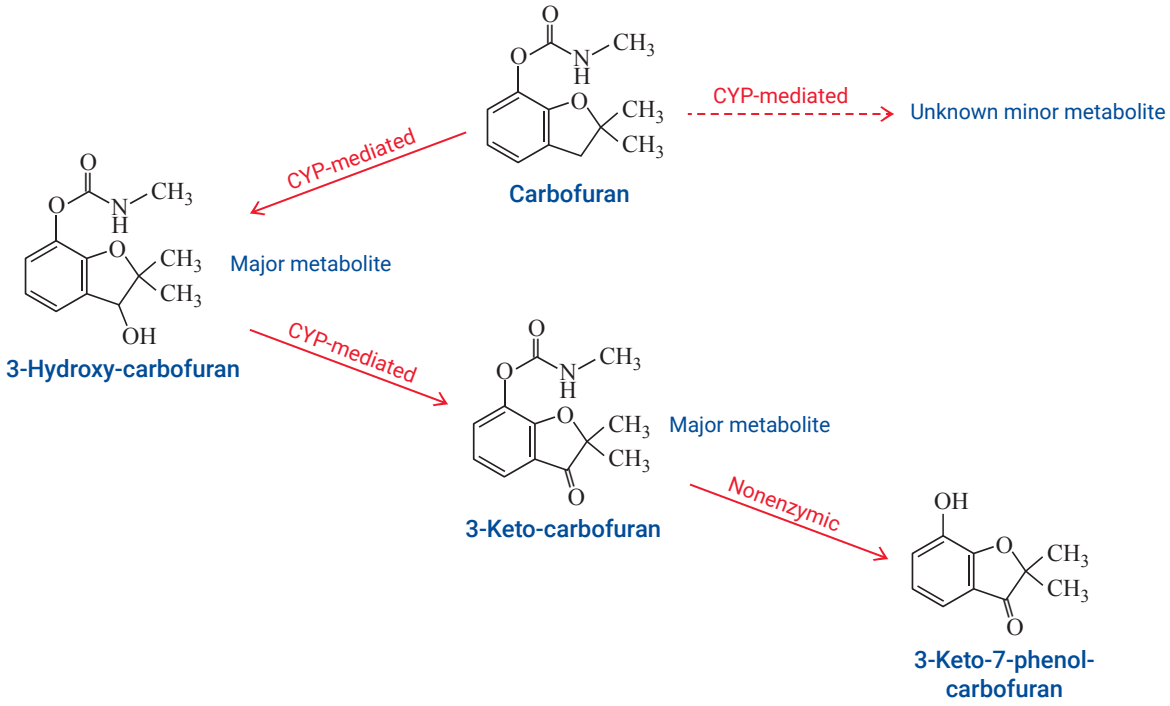
In a non-GLP, peer-reviewed published paper that has not previously evaluated by JMPR, the in vitro metabolism of carbofuran (purity not stated) was investigated in the following preparations:

- fresh human hepatocytes,
- pooled rat liver microsomes (prepared from adult male Long–Evans rats, number and sex not stated),
- pooled mouse liver microsomes (prepared from adult male CD-1 mice, number and sex not stated),
- human liver microsomes (17 donors, age and sex not stated)
- recombinant 1 human P450 (CYP) enzymes (CYP1A1, 1A2, 1B1, 2A6, 3A4, 3A5, 3A7, 4A11, 2B6, 2C8, 2C9*1, 2C9*2, 2C9*3, 2C18, 2C19, 2D6*1, 2D6*10, and 2E1),
- recombinant rat CYP enzymes (CYP3A1, 3A2, 2C6, 2C11, and 2C13).

Enzyme kinetic assays on the pooled liver microsomes were performed by incubating serial concentrations of carbofuran (final concentration 12.5–3200 µM) with microsomes. The initial combination of substrate and the NADPH-regenerating system was pre-incubated at 37°C for five minutes. The reactions were initiated by the addition of ice-cold microsomes (protein concentration 1 mg/mL) followed by incubation for 10 minutes at 37°C. To determine metabolic activity of human liver microsomes from different individuals, incubations with carbofuran (final concentration 1600 µM) were conducted for 10 minutes at 37°C using a protein concentration of 1 mg/mL. Reactions were terminated by the addition of an equal volume of acetonitrile. Control reactions were performed under identical conditions using microsomes prepared from Sf9 cells. Enzyme kinetic assays using fresh human hepatocytes were performed by incubating serial concentrations of carbofuran (final concentration 200–3200 µM) with the hepatocytes. The initial combinations of substrate and hepatocyte culture media were pre-incubated at 37°C for five minutes. The reactions were initiated by the addition of ice-cold human hepatocytes (0.1 million cells/0.25 mL) followed by incubation for 30 minutes at 37°C. After 29 minutes the mixtures were centrifuged for one minute to sediment the cells, and the supernatants were transferred to 250 µL of acetonitrile. The suspended cells were pooled to prepare S9 fractions. Protein concentration was determined from S9 fractions to express the maximum reaction velocity ($V_{max}$) per mg of protein. Metabolic activity assays designed to screen human CYP enzymes for metabolic activity towards carbofuran were performed by incubation with the appropriate substrate (final concentration 1600 µM) with an NADPH-regenerating system. After pre-incubation at 37°C for five minutes the reactions were initiated by the addition of ice-cold CYP enzymes (final concentration 50 pmol/mL) with gentle mixing, then incubated for 30 minutes at 37°C. The control reactions were performed under identical conditions with a Sf9 insect cell control. After the screening assays were completed, enzymes shown to have the greatest impact on metabolism were selected for further characterization. Enzyme kinetic assays for human CYP3A4 and 2C19 were performed by incubating serial dilutions of carbofuran (final concentration 25–1600 µM) with 50 pmol/mL of CYP enzyme for 10 minutes as described above. The control reactions were performed under identical conditions using Sf9 cells. To demonstrate the role of CYP3A4 in carbofuran metabolism in HLM (protein concentration 1 mg/mL), a CYP3A4 specific inhibitor, ketoconazole (final concentration 0–10 µM), was added to the reaction mixture simultaneously with 1600 µM carbofuran. Carbofuran metabolites were analyzed using high-performance liquid chromatography (HPLC), metabolites being identified from their retention times in comparison with the retention times of known purified carbofuran metabolites.

The proposed in vitro metabolic pathways with human, rat, and mouse liver microsomes are shown in Figure 3. The major microsomal metabolite of carbofuran was 3-hydroxy-carbofuran. This 3-hydroxy-carbofuran is then metabolized by CYPs to 3-keto-carbofuran, which is then non-enzymatically converted to 3-keto-7-phenol-carbofuran. There were no qualitative differences in in vitro metabolism of carbofuran across species. However, rodent-derived biomaterials generated quantitatively more metabolites than human biomaterials. This difference correlated with the kinetic parameters for ring hydroxylation of carbofuran, where human liver microsomes had a lower affinity (higher Michaelis–Menten constant, $K_M$) and a lower intrinsic clearance rate than rodent liver microsomes. In the CYP enzyme screening study arm, only CYP2A6, 4A11, 2C18, 2D6*10, and 2E1 had no detectable activity toward carbofuran. All other evaluated CYP enzymes were involved in ring hydroxylation of carbofuran. With regards to this reaction CYP3A4, 1A2, and 2C19 were the most active. Normalization of the data with respect to average CYP enzyme levels in native human liver microsomes demonstrated that in this species metabolism is primarily mediated by CYP3A4 (with a percentage total normalized rate of 77.5), although CYP1A2 and 2C19 play ancillary roles (with percentage total normalized rates of 9.0 and 6.0, respectively). In human biomaterials, CYP3A4 had about three-fold lower $K_M$ than CYP1A2 and 2C19. Rat CYP3A1 and 3A2 and human CYP3A4 had similar $K_M$ values. Studies with single-donor human liver microsomes demonstrated up to five-fold interindividual differences in carbofuran ring hydroxylation rates. This correlated ($r^2$ = 0.96) with interindividual differences in CYP3A4 content as determined by immunoassay. The role of CYP3A4 in the metabolism of carbofuran was verified by co-incubations of ketoconazole, a specific CYP3A4 inhibitor, with carbofuran and human liver microsomes. Ketoconazole, a competitive/mixed inhibitor of CYP3A4, inhibited carbofuran ring hydroxylation with a half-maximal inhibitory concentration value ($IC_{50}$) of 0.31 M. Chlorpyrifos, an irreversible non-competitive inhibitor of CYP3A4, inhibited carbofuran hydroxylation with an $IC_{50}$ of 39 µM. Neither CYP3A4 inhibitor completely inhibited carbofuran metabolism, even at the highest concentrations used (Usmani, Hodgson & Rose, 2004).

*Figure 3. Proposed metabolic pathway of carbofuran in human, rat, and mouse liver microsomes*




(Redrawn from Usmani, Hodgson & Rose, 2004)

*Table 1. Kinetic parameters for ring hydroxylation of carbofuran by pooled human, rat, and mouse hepatic liver microsomes, HLM, RLM, MLM, human and rat cytochrome P450 enzymes*

| | Kinetic parameters (mean of three determinations shown) | | |
|---|---|---|---|
| | $K_M$[a] (mM) | $V_{max}$[a] | $K_M / V_{max}$[b] |
| **Biomaterial** | | **Liver microsomes** | |
| Human | 1.974 | 3.30 | 1.70 |
| Rat | 0.207 | 5.50 | 26.6 |
| Mouse | 0.551 | 13.3 | 24.1 |
| | | **Primary hepatocytes** | |
| Human | 2.066 | 0.64 | 0.31 |
| | | **Cytochrome P450s** | |
| Human CYP3A4 | 0.742 | 40.3 | 54.3 |
| Human CYP1A2 | 0.238 | 22.0 | 92.3 |
| Human CYP2C19 | 0.199 | 18.1 | 90.8 |
| Rat CYP3A1 | 0.514 | 53.8 | 105 |
| Rat CYP3A2 | 0.751 | 106 | 141 |
| Rat CYP2C6 | 0.091 | 13.3 | 146 |
| Rat CYP2C11 | 0.224 | 31.7 | 142 |
| Rat CYP2C13 | 0 | 0 | 0 |

Source: Usmani, Hodgson & Rose, 2004

[a] $V_{max}$ is expressed as nmol/mg protein/minute for liver microsomes and human hepatocytes; $V_{max}$ is expressed as nmol/nmol isoforms/minute for human and rat CYP450s;

[b] $V_{max}/K_M$ is expressed as µL/mg protein/minute for liver microsomes; $V_{max}/K_M$ is expressed as µL/nmol isoforms/minute for human and rat CYP450s

In a second non-GLP, peer-reviewed published paper that has not previously evaluated by JMPR, in vitro human liver homogenate and hepatic microsomal metabolism were evaluated in seven species of mammal. Recombinant CYP enzyme metabolism of carbofuran was also assessed. Pooled hepatic microsomes were prepared from 10 humans (four females and six males, aged between 21 and 62 years), male DBA/2 mice (number and sex not stated), SD rats (number and sex not stated), beagle dogs (number and sex not stated), cynomolgus monkeys (number and sex not stated), Göttingen minipigs (number and sex not stated) and New Zealand White rabbits (number and sex not stated). The production of potential metabolites of carbofuran was investigated using human liver homogenate and liver microsomal preparations. The standard incubation mixture for the microsomal preparations consisted of 0.15 mg of pooled liver microsomal protein ($n = 10$), 50 or 100 µM carbofuran, 1 mM NADPH, and 200 µL of 0.1 M phosphate buffer at pH 7.4, all in a final volume of 200 µL. The carbofuran was prepared once a week in dimethylsulfoxide (DMSO) with a final concentration of 1% in the reaction mixture. After a two minute incubation at 37°C in a shaking incubator block, the reaction was initiated by adding NADPH and was allowed to proceed for 30 minutes before being stopped with ice-cold acetonitrile containing an internal standard. Incubations were performed in triplicate. The production of main carbofuran metabolites from recombinantly expressed CYP enzymes (rCYPs) was also measured. The standard incubation mixture consisted of 100 µM carbofuran, 0.1 M phosphate buffer at pH 7.4, 1 mM NADPH, and 50 pmol CYP per 1 mL of rCYPs, all in a final volume of 200 µL. The reaction was initiated by adding the recombinant enzymes to the pre-incubated mixture and was allowed to proceed for 30 minutes at 37°C in an incubator block without agitation.

Identification of metabolites was by liquid chromatography with tandem mass spectrometry (LC-MS/MS) using relevant authentic chemical standards. The measurement of enzyme kinetic parameters in microsomal samples was performed by utilizing a standard incubation mixture containing carbofuran final concentrations ranging from 2.5 to 300 µM. The same incubation mixture and methods, except, for the 20-minute incubation time, were applied as previously described. Nonlinear regression was applied to calculate the kinetic parameters $V_{max}$ and $K_M$. The intrinsic clearance value was determined by dividing $V_{max}$ by $K_M$. The results are presented in Tables 2 and 3 as the mean (± standard error), obtained from three replicates. In the established experimental conditions for carbofuran metabolites, the reaction rate was observed to be linear for at least 60 minutes of incubation and up to a concentration of 0.15 mg/mL of microsomal protein. In vitro to in vivo extrapolated (IVIVE) liver clearances were calculated using in vitro data, liver microsomal protein quantity per gram.

The biotransformation of carbofuran in hepatic preparations of these species was qualitatively the same and yielded five identified metabolites: 3-hydroxy-carbofuran, 3-hydroxy-7-phenol-carbofuran, 3-keto-carbofuran, 3-keto-7-phenol-carbofuran, and 3-phenol-carbofuran. Consistent with other data the results of this study demonstrated that carbofuran is primarily metabolized (detoxified) via the 3-hydroxy-carbofuran pathway. A secondary (minor) pathway from this study involved the formation of 3-phenol-carbofuran.

Based on in vitro microsomal elimination studies (Figure 3), mammals could be divided into two groups: slower metabolizers (mouse, human and rabbit) and faster metabolizers (rat, monkey, dog and minipig). The disappearance curve for rabbit and mouse liver hepatic microsomes closely resembled that for human liver, and these had the slowest disappearance rates (64%, 74% and 67% of carbofuran remaining after 60 minutes incubation, respectively). The dog and minipig liver hepatic microsomes showed the fastest disappearance rates (29% and 21% of carbofuran remaining, respectively). A species-dependent variation in the proportion of different metabolites formed was also observed. The highest levels of 3-hydroxy-carbofuran (47.8, 73.1 and 89.5 µM at 20, 40 and 60 minutes of incubation, respectively) and 3-keto-carbofuran (1.9, 5.1, and 7.3 µM at 20, 40 and 60 minutes of incubation, respectively) were observed with dog liver microsomes. Conversely, the lowest levels of 3-hydroxy-carbofruan (11.4, 13.5, and 17.1 µM at 20, 40 and 60 minutes of incubation, respectively) were seen with the human liver microsomes. The maximum concentration of 7-phenol-carbofuran was detected with the human liver microsomes after 20 minutes of incubation (7.6 µM), with rat and mouse liver microsomes following closely behind (6.3 and 6.1 µM, respectively).

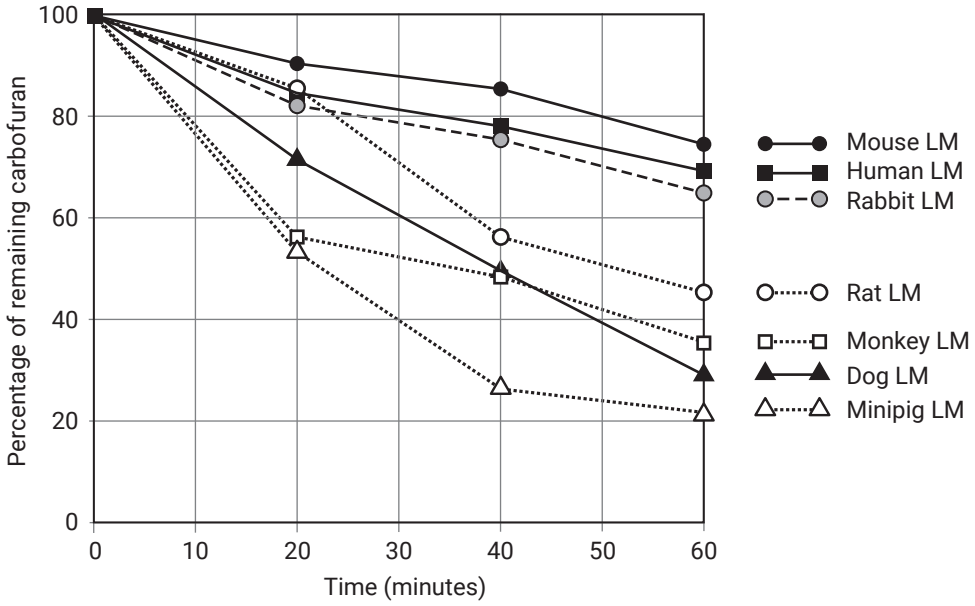
Interspecies differences in the intrinsic clearance of 3-hydroxy-carbofuran was apparent (Table 2). Human and rat liver microsomes displayed comparable affinity with $K_M$ values of 174.2 and 180.3 µM respectively. However, human liver microsomes had the lowest $V_{max}$ value of

8.1 nmol/(mg protein/minute), reflecting its greatest affinity and least capacity. On the other hand, minipig liver microsomes displayed the highest affinity with the lowest $K_M$ value of 74.3 µM and the highest capacity of 32.4 nmol/(mg protein/minute). The catalytic efficacy of rat liver microsomes was found to be the lowest among the mammalian microsomes with a value of 26.7 µL/(mg protein/minute), while minipigs displayed the highest efficiency at 430.1 µL/(mg protein/minute). The formation of 3-phenol-carbofuran also revealed interspecies differences in kinetic parameters. Minipig and monkey liver microsomes demonstrated the greatest affinity with the smallest $K_M$ values of 56.3 and 28.6 µM respectively, and the least capacity with the lowest possible $V_{max}$ values of 1.9 and 0.78 nmol 3-phenol-carbofuran/(mg protein/minute). Rabbit liver microsomes displayed the lowest affinity with a $K_M$ value of 980 µM. Dog liver microsomes demonstrated the highest capacity with a value of 12.8 nmol 3-phenol-carbofuran/(mg protein/minute) and the highest catalytical efficiency of 76.1 µL/(mg protein/minute). Mouse hepatic microsomes were found to have the lowest efficiency with a value of 9.8 µL/(mg protein/minute).

Based on IVIVE, biologically relevant differences in hepatic clearance of carbofuran (that is, differences of two-fold or more compared with humans) were apparent in the study (Table 2). Rodent liver microsomes had a 2.3- to 2.4-fold higher estimated hepatic clearance (detoxification) of carbofuran than comparable human liver microsomes. Dog liver microsomes had a 5.6-fold higher estimated hepatic clearance of carbofuran compared to human preparations. The estimated hepatic clearance of carbofuran by rabbit and human liver microsomes was approximately the same.

Based on in vitro studies using recombinant human CYPs, CYP3A4 is the predominant enzyme catalyzing the conversion of carbofuran to 3-hydroxy-carbofuran (Table 3). Carbofuran conversion to 3-hydroxy-carbofuran was substantially (by 70%) inhibited by the CYP3A4 inhibitor ketoconazole. These findings are consistent with those of Usmani, Hodgson & Rose (2004). The major CYP isoform involved in the formation of 7-phenol-carbofuran was CYP1A2 (Abass et al., 2023).

***Figure 4. Time-dependent degradation of carbofuran over incubation time***




(Redrawn from Abass et al., 2023)

*Table 2 Mean in vitro kinetic parameters of carbofuran metabolic pathways in liver microsomes and extrapolated in vivo hepatic clearance values for 3-hydroxy-carbofuran and 7-phenol-carbofuran*

| | $V_{max}$ (nmol/(mg prot. × min.) | $K_M$ (µM) | $CL_{int}$ (µL/(mg prot. × min.)) | In vivo hepatic clearance; CLH (mL/min.) | Animal to human differences (-fold difference) |
|---|---|---|---|---|---|
| **Species** | | | **3-Hydroxycarbofuran** | | |
| Human | 8.1 | 174 | 46.4 | 9.6 | - |
| Rat | 11.3 | 180 | 26.7 | 19.1 | 2.0 |
| Mouse | 11.2 | 154 | 72.3 | 40.2 | 4.2 |
| Dog | 19.8 | 108 | 183.3 | 30.3 | 3.1 |
| Rabbit | 10.1 | 75.5 | 132.4 | 31.5 | 3.3 |
| Minipig | 32.4 | 74.3 | 430.1 | 35.4 | 3.7 |
| Cynomolgus monkey | 21.8 | 99.4 | 211.1 | 37.4 | 3.9 |
| **Species** | | | **7-Phenol-carbofuran** | | |
| Human | 8.4 | 640.4 | 12.9 | 4.0 | - |
| Rat | 4.3 | 379.7 | 11.3 | 9.6 | 2.4 |
| Mouse | 8.6 | 870.0 | 9.8 | 9.1 | 2.3 |
| Dog | 12.8 | 168.2 | 76.1 | 22.6 | 5.6 |
| Rabbit | 6.1 | 980.4 | 6.2 | 4.9 | 1.2 |
| Minipig | 1.9 | 56.3 | 33.7 | 11.0 | 2.7 |
| Cynomolgus monkey | 0.78 | 28.6 | 27.2 | 10.4 | 2.6 |

min.: Minute; $V_{max}$: Maximum reaction velocity; $K_M$: Michaelis–Menten constant; $CL_{int}$: Internal clearance;

Source: Abass et al., 2023

*Table 3. Mean metabolic kinetic values of main carbofuran pathways in human recombinant CYP enzymes*

| | $V_{max}$ (nmol/(nmol P450 × min.)) | $K_M$ (µM) | $CL_{int}$ (µL/(nmol P450 × min.)) | Relative contribution |
|---|---|---|---|---|
| **CYP450 isoform** | | **3-Hydroxycarbofuran** | | |
| CYP1A1 | 152.7 | 176.6 | 864.7 | 0.0 |
| CYP1A2 | 98.6 | 248.0 | 397.5 | 6.2 |
| CYP1B1 | 66.3 | 304.0 | 218.1 | 0.0 |
| CYP2B6 | 15.2 | 341.2 | 44.5 | 0.3 |
| CYP2C19 | 101.9 | 331.7 | 307.2 | 1.3 |
| CYP3A4 | 185.8 | 74.4 | 2497.3 | 92.2 |
| CYP4A11 | 66.0 | 570.4 | 115.8 | Not determined |
| | | **7-Phenol-carbofuran** | | |
| CYP1A2 | 103.2 | 117.7 | 876.8 | 90.1 |
| CYP2C19 | 55.4 | 162.2 | 341.5 | 9.9 |

Source: Abass et al., 2023

min.: Minute; $V_{max}$: Maximum reaction velocity; $K_M$: Michaelis–Menten constant; $CL_{int}$: Internal clearance;

**(b) In vivo**

No data was submitted on this topic, but such studies not routinely expected.

### 1.3 Effects on enzymes and other biochemical parameters

Abass et al. (2023) evaluated the effect of carbofuran on human liver P450 enzymes using a cocktail assay in pooled human liver microsomes (derived from prepared from four females and six males, between 21 and 62 years old). Based on the $IC_{50}$ values for CYP3A4-catalyzed metabolism of omeprazole and midazolam, carbofuran was an inhibitor of human CYP3A4 in vitro and CYB2B6 (Abass et al., 2023).

## 2. Toxicological studies

### 2.1 Acute toxicity

#### (a) Lethal doses

This assessment considered the available data on carbofuran as an active constituent. Studies using formulated products that have been previously considered by JMPR have not been included.

***Table 4. Summary of lethal dose studies with carbofuran***

| Route | Purity | Source | Species | Sex | Result | Comments | References |
|---|---|---|---|---|---|---|---|
| **Oral** | Not stated | Not stated | Rat neonatal | M + F | 8.11 | $LD_1$ = 0.56 mg/kg bw<br>$LD_{99}$ = 117.37 mg/kg bw<br>Slope = 33.2; R = 0.83 | Rucci, Becci & Parent, 1980 |
| **Oral** | Not stated | Not stated | Rat weanling | M + F | 7.30 | $LD_1$ = 1.80 mg/kg bw<br>$LD_{99}$ = 29.72 mg/kg bw<br>Slope = 64.4; R = 0.96<br>Notably weanling rats have lower $LD_1$, $LD_{99}$ and dose response slope than neonatal rats | Rucci, Becci & Parent, 1980 |
| **Oral** | 97% | Catechol | Rat | M + F | Male $LD_{50}$: 13.3 mg/kg bw<br>Female $LD_{50}$: 5.6 mg/kg bw | Clinical signs consistent with cholinergic toxicity (tremor, decreased locomotion, chromorhinorrhea, chromodacryorrhea,oral discharge) were present at 0.5 hour following dosing and persisted until study day 2 | Norvell, 1983a |
| **Oral** | 96% | ONP | Rat | M + F | Male $LD_{50}$: 13.2 mg/kg bw<br>Female $LD_{50}$: 5.3 mg/kg bw | Clinical signs consistent with cholinergic toxicity (tremors, decreased locomotion, abdominogenital staining, and oral, ocular, and nasal discharge) occurred | Norvell, 1983b |
| **Oral** | Not stated | Not stated | Rat | M + F | Male $LD_{50}$: 8.0 mg/kg bw:<br>Female $LD_{50}$: 10.5 mg/kg bw | All deaths occurred within 3 hours of dosing; Clinical signs observed during the 14-day observation period included gasping, head tilting, tearing, loss of co-ordination, hyperactive to external stimuli, erect hair, moist rales, staggering, blood-like tears, nasal discharge, anogenital staining, sporadic shaking, tremors, and convulsions<br>All surviving rats recovered within 24 hours of dosing and gained weight by end of study | Elleman, 1979 |
| **Oral** | 96.6% | Not stated | Rat | M + F | Male $LD_{50}$: 14.9 mg/kg bw<br>Female $LD_{50}$: 13.9 mg/kg bw | Clinical signs observed during the 14-day observation period included tremors, exophthalmos, lacrimation, salivation, and piloerection. All clinical signs subsided within 24 hours of dosing in the surviving rats except for piloerection which lasted up to 14 days in some rats. All surviving rats gained weight. | Sabol, 1979a |
| **Oral** | 96.6% | Not stated | Rat | M + F | Male $LD_{50}$: 5.2 mg/kg bw<br>Female $LD_{50}$: 5.8 mg/kg bw | Clinical signs observed during the 14-day observation period included tremors, exophthalmos, constricted pupils, lacrimation, salivation, piloerection. All clinical signs subsided within 24 hours of dosing in survivors except piloerection which lasted up to 14 days in some rats.<br>All survivors gained weight. | Sabol, 1979b |

| Route | Purity | Source | Species | Sex | Result | Comments | References |
|---|---|---|---|---|---|---|---|
| Dermal (occlusive) | 96.1% | Not stated | Rabbit | M + F | **Abraded:** Male $LD_{50}$: 2703 mg/kg bw Female $LD_{50}$: > 2010 mg/kg bw **Intact:** Male $LD_{50}$: 4405 mg/kg bw | Clinical signs observed during the 14-day observation period were consistent with cholinesterase inhibition. Included tremors, decreased activity, ataxia, nasal discharge, constricted pupils and salivation; Rabbits recovered from above signs within 5 days of dosing All but two survivors gained weight by end of study | Mehta, 1998 |
| **Inhalation** | No studies supplied on carbofuran technical | | | | | | |

$LD_{50}$: Median lethal dose; $LD_1$: 1% lethal dose $LD_{99}$: 99% lethal dose ONP: *o*-Nitrophenol; R: Goodness of fit for regression analysis

### (b) Dermal irritation

No studies supplied on carbofuran technical.

### (c) Ocular irritation

Since deaths would be expected at the normal dose of 100 mg per eye in a primary eye irritation study, a study was conducted using 5% of the normal dose. Carbofuran technical was instilled into the conjunctival sac of two New Zealand White rabbits at a dose of 5 mg. Eyes were scored using the Draize method at 1, 24, 48, 72 and 96 hours post dosing, then again at seven days post dosing. Iritis and minimal conjunctival redness were observed in both rabbits at the one-hour scoring. All irritation resolved within 24 hours. Within minutes of dosing both rabbits displayed miosis (both eyes), hypernea and hyperactivity. These reactions persisted for approximately two hours, after which the rabbits again appeared normal. Carbofuran technical was minimally irritating to the eye (Palazzolo, 1963a).

### (d) Dermal sensitization

A skin sensitization study was conducted on carbofuran technical according to the Draize method. The test material was administered as a 0.1% (w/v) solution in propylene glycol intracutaneously to the test sites of five male Guinea pigs every other day for a total of ten injections. The first injection consisted of 0.05 mL while the remaining nine injections were 0.10 mL each. The test animals were challenged with 0.05 mL two weeks after the tenth injection. A concurrent positive control group, which received 0.1% (w/v) aqueous solution of dinitrochlorobenzene, followed the same dosing regimen as the test animals. The irritation scores of test animals following the challenge injection were like their irritation scores following the initial injection. Animals in the positive control group displayed definite sensitization responses following the challenge application. Carbofuran technical was considered not to be a sensitizer (Schoenig, 1967a).

## 2.2 Short-term studies of toxicity

### (a) Oral administration

***Dog***

In a preliminary, non-GLP dose range finding study previously evaluated by JMPR in 1996, carbofuran technical (purity 96.1%) was administered in the diet to one male and one female beagle dog at dietary concentrations of 0, 18, 32, 56, 100, 316 or 1000 ppm (equivalent to 0, 0.45, 0.8, 1.4, 2.5, and 8 mg/kg bw per day). The treatment period was 14 days for all groups except for the 18 ppm group which was changed to doses of 1000 ppm on day 4, and this continued for another 10 days. No deaths occurred. Clinical signs consistent with a cholinergic toxidrome (tremors, emesis, and salivation, the latter only in males) occurred in the 1000 ppm group. Body weights were depressed in males receiving 316 ppm, and in females at 1000 ppm. Mean food consumption on day 14 was reduced with increasing dose at 32 ppm and above in males (reduced by 28%–58%) and at 56 ppm and above in females (reduced by 19%–56%).

Plasma acetylcholinesterase (AChE) activity was depressed in a dose-related manner already on day 3 starting from 32 ppm and at all dose levels starting from day 5, except for the first three days of the 18 ppm dosing, where values remained within the limits of normal variation. Inhibition ranged from 16% (within the historical control range) in animals at 18 ppm, to 92% and above at 1000 ppm. No change in erythrocyte cholinesterase activity was detected at any of the levels tested. The recommended dosage levels for the one-year feeding study were 0, 10, 20 and 500 ppm (Burtner, 1982).

In four-week repeat dietary exposure toxicology study was designed to identify a NOAEL with respect to clinical signs and inhibition of AChE activity. Groups of four male beagle dogs were fed diets containing carbofuran (purity 99.6%) at concentrations of 0 or 5 ppm for four weeks. This study was previously evaluated by JMPR in 1996 but updated by the sponsor in 2005. The dogs were examined daily for deaths and clinical signs. Blood samples for measurement of plasma and erythrocyte AChE activity were collected within the 30 minutes following food withdrawal. Cholinesterase activity at 37°C was estimated using the method of Ellman et al. (1961). No further details of the method were given, and it was not clear whether the experimental conditions were appropriate to minimize spontaneous reactivation of the enzyme. Haematology and clinical chemistry investigations were not performed. Anatomic pathology examinations and measurements of brain AChE activity were not conducted since the dogs were not killed at the end of the study. Treatment with carbofuran had no effects on clinical signs, mortality, body weight, food consumption, or plasma AChE activity. At the pretest investigation, erythrocyte AChE activity in dogs to be treated with carbofuran was significantly lower (by 21%; $p < 0.05$) than for the control group and continued to be lower (by 25.1%–37% compared to controls) for the rest of the study. In carbofuran-treated animals a reduction of less than 20% in erythrocyte AChE compared with pretest levels occurred on days 1, 7, 14 and 28. On day 3, erythrocyte AChE levels in carbofuran-treated animals were 72.6% of their pretest value. However, this was regarded as an isolated finding of no toxicological significance. Confirming the findings of previous JMPR evaluations, the NOEL for inhibition of erythrocyte AChE was 5 ppm (equal to 0.22 mg/kg bw per day) the highest dose tested (Bloch et al., 2005a).

***Table 5. Erythrocyte activity in dogs given diets containing 0 or 5 ppm of carbofuran for four weeks***

| | | Dietary concentration (ppm) | | | | |
|---|---|---|---|---|---|---|
| **Incubation time** | | **0** | | **5** | | |
| | | Erythrocyte AChE activity (µmol-SH/mL) | | | | |
| | | Mean | % of pretest | Mean | % of pretest | % of control |
| Pretest | | 3.14 | 100.0% | 2.48* | 100.0% | 79.0% |
| Day 1 | | 2.99 | 95.2% | 2.24* | 90.3% | 74.9% |
| Day 3 | | 2.86 | 91.1% | 1.80* | 72.6% | 62.9% |
| Day 7 | | 3.23 | 102.9% | 2.37* | 95.6% | 73.4% |
| Day 14 | | 3.27 | 104.1% | 2.44* | 98.4% | 74.6% |
| Day 28 | | 3.14 | 100% | 2.18* | 87.9% | 69.4% |
| Day 1–28: | mean | 3.01 | | 2.21 | | |
| | standard deviation | 0.15 | | 0.22 | | |
| | variance | 0.02 | | 0.05 | | |

* Significantly different from control group at $p < 0.05$; Source: Bloch et al., 2005a

In a study previously evaluated by JMPR in 1996 and 2002 and updated by the sponsor in 2005, groups of four male and four female beagle dogs were given diets for 13 weeks containing carbofuran (purity 99.6%) at concentrations of 0, 10, 70 or 500 ppm, the highest concentration being reduced to 250 ppm after six days owing to toxicity. These dietary concentrations resulted in mean carbofuran doses equal to 0, 0.43, 3.1 and 11 mg/kg bw per day for males and females combined. Additional groups consisting each of two males and two females, were given the control diet or the diet containing carbofuran at the highest concentration, and these were kept for a further four weeks after dosing ceased to study the reversibility of any effects. The dogs were examined daily for deaths and clinical signs. Food consumption was recorded daily. Hearing tests were performed before the start of the study, at the end of dosing and at the end of the recovery period. Ophthalmoscopic examinations and electrocardiography

were also carried out, with an additional examination after six weeks of dosing. Blood samples (for haematology and clinical chemistry) and urine were taken seven days before the start of treatment, at 6 and 13 weeks, and after the recovery period. For estimation of erythrocyte AChE activity, blood samples were taken 30 minutes after the daily two hour feeding period, at seven days before the start of the treatment, on test days 1 and 3, and at the end of weeks 1, 2, 6 and 13 of the study (also 11 days later for males). A further sample was taken after the recovery period. The cerebellum was isolated at autopsy and immediately assayed for AChE activity. Cholinesterase activity at 37°C was estimated based on the method of Ellman et al. (1961). However, no further details of the method were given, and it is not clear whether the experimental conditions were appropriate to minimize spontaneous reactivation of the enzyme. Selected organs were processed for histopathological examination. There were no test article-associated adverse effects on findings from ophthalmology, electrocardiography, hearing tests, haematology, urinalysis, clinical chemistry (other than AChE activity), organ weights or anatomical pathology, this last, excepting a single instance of jejunal intussusception (invagination).

One dog receiving 500 ppm died after five days, due to a jejunal intussusception. Gastrointestinal intussusception is a known consequence of carbamate-induced anticholinesterase poisoning in dogs (Corfield et al., 2008; Menchetti et al., 2022). Dogs at all doses showed hyperaemia (of the ear pinnae, abdominal skin, and oral mucous membranes) and salivation, these signs being most severe early in the study. Skin and mucosal hyperaemia is consistent with the effects of anticholinesterases on neuronal and non-neuronal ACh-mediated regulation of cutaneous blood flow (Cracowski & Roustit, 2020; Kuirzen et al., 2007). The clinical signs consistent with cholinergic toxicity, occurred at the highest dietary concentration, and included ataxia, vomiting and tachypnoea. At this concentration there was decreased food consumption by up to 50%–56% and loss in body weight of up to 17% (predominantly in males). These observations were mostly made during the first weeks, and reversed when the concentration of carbofuran in the diet was reduced.

Inhibition of erythrocyte AChE activity was also seen at all three doses, representing 73%, 35% and 13% of that of concurrent controls at 10, 70 and 500 ppm, respectively, on day 1 in males, when inhibition was generally maximal (Table 2). In females at 10, 70 and 500 ppm, the activities on day 1 were 83%, 28% and 14% of that of concurrent controls, respectively. Notably, on pretest day 7, females of the two highest dose groups already showed unexplained decreased levels of erythrocyte AChE activity (76% and 77% of concurrent control). No significant inhibition ($p < 0.05$) of brain AChE activity was observed. However, since administration of the test substance was terminated on the day before necropsy, the data on brain AChE activity were considered unreliable, as spontaneous reactivation of the enzyme could have occurred. Confirming the findings of previous JMPR evaluations, the study LOAEL was 10 ppm (equal to 0.43 mg/kg bw per day, average intake in males and females) the lowest dose tested, due to a greater than 20% inhibition of erythrocyte AChE activity and the occurrence of clinical signs consistent with cholinergic toxicity (salivation, hyperaemia) at this dietary concentration (Bloch et al., 2005b).

***Table 6. Erythrocyte and brain Che activity in dogs given diets containing carbofuran for 13 weeks***

| | Dietary concentration (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | 0 | 10 | 70 | 500/250 | 0 | 10 | 70 | 500/250 |
| | Erythrocyte acetylcholinesterase (µmol-SH/mL) | | | | | | | |
| Pretest, day –7 | 2.49 | 2.50 | 2.78 | 2.95 | 2.85 | 3.06 | 2.18 | 2.19 |
| % of control | 100 | 100 | 112 | 118 | 100 | 107 | 76 | 77 |
| Day 1 | 2.34 | 1.71 | 0.81* | 0.31* | 2.64 | 2.18 | 0.73* | 0.37* |
| % of control | 100 | 73 | 35 | 13 | 100 | 83 | 28 | 14 |
| Day 3 | 2.61 | 2.07 | 1.15* | 1.20* | 3.11 | 2.52 | 1.05* | 0.80* |
| % of control | 100 | 79 | 44 | 46 | 100 | 81 | 34 | 26 |
| Day 7 | 2.66 | 1.95 | 1.07* | 0.57* | 2,96 | 2.46 | 1.03* | 0.53* |
| % of control | 100 | 73 | 40 | 21 | 100 | 83 | 35 | 18 |

| | Dietary concentration (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | 0 | 10 | 70 | 500/250 | 0 | 10 | 70 | 500/250 |
| Day 14 | 2.68 | 2.05 | 1.15* | 0.68* | 3.12 | 2.54 | 1.00* | 0.63* |
| % of control | 100 | 76 | 43 | 25 | 100 | 81 | 32 | 20 |
| Week 6 | 3.15 | 2.35 | 1.33* | 0.67* | 3.53 | 3.12 | 1.30* | 0.62* |
| % of control | 100 | 75 | 42 | 21 | 100 | 88 | 37 | 18 |
| Week 13 | 2.37 | 1.54 | 0.81* | 0.50* | 2.22 | 2.09 | 0.92* | 0.46* |
| % of control | 100 | 65 | 34 | 21 | 100 | 94 | 41 | 21 |

* Significantly different from control group at $p < 0.05$; Source: Bloch et al., 2005b

In a non-GLP study previously evaluated by JMPR in 1996 and 2002, groups of six male and six female beagle dogs were fed diets for one year containing technical grade carbofuran (purity 96.1%) at concentrations of 0, 10, 20 or 500 ppm (equivalent to 0, 0.25, 0.5 and 12 mg/kg bw per day). Dogs at 500 ppm showed emesis and weight loss and were given control diet with the test diet from five months onwards, in unstated proportions of test to control diet. As a result the equivalence of the high dose to 12 mg/kg bw per day is likely to be an overestimate of carbofuran consumption. The animals were observed daily for clinical signs and deaths. Ophthalmic examinations were carried out before treatment, at six months, then just before termination of the study. Blood was taken from all animals for haematology and clinical chemistry before the start of the study and thenceforth monthly. Dogs were weighed before the start of the study and weekly thereafter. Food consumption was measured daily for the first five weeks of the study and weekly thereafter. Blood samples for estimation of AChE activity were taken three times before the start of the study, at study days 3, 7 and 14, then monthly. The samples were taken approximately one hour after the end of the daily two hour feeding period. Urine was analyzed before treatment and then at 60-day intervals. At termination the animals were necropsied. A gross pathological examination was undertaken, and selected organs were weighed and processed for histopathological examination. A sample of brain (cerebellum) was taken for estimation of brain AChE activity.

One male in the high dose cohort died. Decreased body weight relative to controls was observed throughout the study in the highest dose group in both sexes, with a 34% loss in males and 17% in females at the end of the study. A net body weight loss (negative total body weight change) was evident at the end of the study. Food consumption was significantly decreased in the highest dose groups starting at week 44 in males (by 33% compared to controls) and week 32 in females, peaking at week 44 (by 42% compared to controls) and achieving a 20% reduction compared to controls by the end of the study. In males however, this was accompanied by high variability over time throughout the remaining weeks, as well as between individuals within the group, with the final mean difference from controls being 11% at the end of the study. Emesis and loose stools were seen, mostly in animals at the highest concentration. Effects on ophthalmic parameters were described and considered not to be related to treatment. Decreased erythrocyte volume fraction, haemoglobin (Hb) concentration and erythrocyte count were seen in males at 500 ppm. Changes in electrolytes, as well as decreased total serum protein in both sexes and decreased serum albumin in males, were also seen at this high concentration.

Plasma AChE activity was inhibited in males of all dosed groups. In the group at 10 ppm the reduction was around 20% especially in males during the first five months, however this returned to control levels afterwards. Erythrocyte AChE activity was inhibited only at the highest dietary concentration in males and then only sporadically. In females at the lowest concentration, plasma AChE activity was always greater than 80% that of concurrent controls. Erythrocyte AChE activity was not significantly inhibited in females. At termination, biologically significant ($p < 0.05$) depression of brain AChE activity was seen in males at the highest concentration (76% that of concurrent controls). No depression in brain AChE activity was seen in females.

At the highest dietary concentrations, the absolute weights of the liver and heart were decreased (by 38% and 20% in males and females respectively), which, however, was not reflected in the organ weights when expressed relative to either body weight or to brain weight. Absolute brain weight was

also decreased (by as much as 15%) in males of the same dose group, while in females this decrease was only marginal (2%). Compared with controls an absolute weight loss of testes (reduced by 35%), ovaries (reduced by 11%), thyroid (reduced by 30% and 20% in males and females respectively.) and pituitary in females (reduced by 25%) was also recorded, along with an increase in the absolute weights of adrenals (by 11% and 19% in males and females respectively.) and an increase in the weights of adrenals relative to body weight by 70% and 43% in males and females respectively. Gross pathological examination revealed decreased body fat and alopecia at this, the highest dose. Degeneration of the seminiferous tubules and/or the presence of giant tubules and/or aspermia were seen in four of the five surviving males receiving the highest dietary concentration, and in one of six given 20 ppm. Atrophy of the seminiferous tubules is a common finding in laboratory dogs, occurring in 25%–40% of dogs under twelve months old, decreasing with age to 14%–17% in dogs between 12 and 36 months old (Goedken et al., 2008). Based on this data, and in contrast to the previous JMPR evaluations, the current evaluation considered the effects in the seminiferous tubules of dogs to be consistent with the known spontaneous incidence of this phenomenon in this species. Accordingly, the effect was not regarded as being test article-related or adverse. Inflammatory changes in the lung (minimal to moderate) were observed at a higher incidence (7/11 animals) in the dogs exposed at 500 ppm. Confirming the findings of previous JMPR evaluations, the NOAEL was 20 ppm (equivalent to 0.5 mg/kg bw per day) based on a greater than 20% inhibition of erythrocyte AChE activity in males at the next highest dose of 500 ppm (equivalent to 12 mg/kg bw per day) (Taylor, 1983).

***Table 7. Erythrocyte cholinesterase activity in dogs given diets containing carbofuran for one year***

| | Mean erythrocyte cholinesterase values | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Day 3 | | Day 7 | | Day 14 | | 1 Month | | 6 Months | | 12 Months | |
| Dose (ppm) | M | F | M | F | M | F | M | F | M | F | M | F |
| 0 | 1.49 | 1.43 | 1.46 | 1.45 | 1.53 | 1.48 | 1.46 | 1.45 | 1.54 | 1.49 | 1.66 | 1.65 |
| 10 | 1.30 | 1.54 | 1.38 | 1.51 | 1.32 | 1.55 | 1.40 | 1.63 | 1.39 | 1.48 | 1.56 | 1.68 |
| % change [a] | –13 | 0 | –5 | 0 | –14 | 0 | –4 | 0 | –10 | 1 | –6 | 0 |
| 20 | 1.42 | 1.54 | 1.45 | 1.52 | 1.48 | 1.55 | 1.54 | 1.51 | 1.50 | 1.38 | 1.71 | 1.58 |
| % change [a] | –5 | 0 | 1 | 0 | –3 | 0 | 0 | 0 | –3 | –7 | 0 | –4 |
| 500 | 1.49 | 1.54 | 1.51 | 1.61 | 1.39 | 1.58 | 1.33 | 1.56 | 1.13* | 1.30 | 1.35 | 1.55 |
| % change [a] | 0 | 0 | 0 | 0 | –9 | 0 | 9 | 0 | –27 | –13 | –19 | –6 |

[a] Percentage change compared with control (0 ppm); M: Male; F: Female; Source: Taylor, 1983

* Significantly different from control group at $p < 0.05$

### (b) Dermal application

In a non-GLP, dose range finding study previously evaluated by JMPR in 1996, carbofuran technical (purity 96.9%, batch M-607210) was applied to the shaved skin of four New Zealand White rabbits of each sex per group for approximately six hours per day at rates of 0, 100, 300 or 1000 mg/kg bw for a total of seven consecutive days. No clinical signs of toxicity and no local irritation were observed. The treatment did not affect body weight or food consumption. A 30% depression in plasma AChE activity was measured in males at 100 and 300 mg/kg bw per day, and a 47% reduction at 1000 mg/kg bw per day. Brain AChE activity was reduced by 26% in males at 100 mg/kg bw per day, 49% in those receiving 300 mg/kg bw per day, and 54% in males at 1000 mg/kg bw per day. These reductions were not statistically significant. No lesions related to treatment were observed at gross necropsy. Based on the information obtained in this study, the recommended dosage levels for the 21-day dermal study were 0, 10.0, 100, 1000 mg/kg bw per day (Kedderis, 1985a).

In the main study (previously evaluated by JMPR in 1996), carbofuran technical (purity 96.9%) was applied to the shaved skin of six New Zealand White rabbits of each sex per group for approximately six hours per day at dosage levels of 0, 10.0, 100 or 1000 mg/kg bw per day for a total of 21 consecutive days. All animals remained healthy throughout the study, except for one control female which died on study day 10 with clinical signs indicative of enteritis. A slight decrease in mean food consumption was noted at week 2 (by 16%) and week 3 (by 17%) in females receiving the highest dose, which led to

slightly decreased mean body weights (depressed by 7% and 5%, respectively compared to controls). Increased food consumption observed during week 1 for females in the 10.0 and 100 mg/kg bw per day groups was not considered treatment-related. There were no statistically significant differences between control and treated animals as regards body weight, haematology, clinical chemistry, AChE activities (erythrocyte, plasma, or brain), or organ weights. No lesions related to test material administration were observed from gross necropsy or histopathologic examination of tissues. Confirming the findings of previous JMPR evaluations, the NOAEL was 1000 mg/kg bw per day, the highest dose tested (Kedderis, 1985b).

**(c) Exposure by inhalation**

No studies were submitted on this topic.

## 2.3 Long-term studies of toxicity and carcinogenicity

*Mouse*

In a non-GLP carcinogenicity study previously evaluated by JMPR in 1980 and 1996, carbofuran technical (purity 95.6 %) was administered in the diet for up to two years to 100 CD-1 mice of each sex per group at levels of 0, 20, 125 or 500 ppm (equal to 0, 2.8, 18 and 70 mg/kg bw per day). The mice were observed daily for signs of overt toxicity, moribundity and mortality. Detailed clinical observations, body weights and food consumption values were recorded weekly. Haematological, biochemical (including brain AChE activity) and urinalysis studies were conducted on 10 animals of each sex per group at 6, 12 and 18 months and on 20 animals of each sex per group at 24 months of dosing. Anatomical pathology evaluations were conducted on 10 mice of each sex per group at 6, 12 and 18 months, and on all survivors at the end of the study. There were no test article-associated adverse effects on mortality, behaviour, haematology findings, clinical chemistry findings (except for brain AChE activity) or anatomical pathology findings.

Brain AChE values were significantly different to controls at all sampling time points at 500 ppm (27%–52% inhibition in males; 41%–55% inhibition in females) and most of the time points at 125 ppm (16%–31% inhibition in males; 20%–31% inhibition in females). Based on these results, it was concluded that carbofuran technical was not carcinogenic in the mouse at dietary levels up to 500 ppm. The NOAEL for carcinogenicity was 500 ppm (equal to 70 mg/kg bw per day), the highest dose tested. The NOAEL for toxicity was 20 ppm (equal to 2.8 mg/kg per day) based on a greater than 20% inhibition of brain plasma AChE activity (seen particularly in females) at 125 ppm (equal 18 mg/kg bw per day). These NOAELs are consistent with previous JMPR evaluations of this study (Goldenthal, 1983).

***Table 8. Brain cholinesterase activity in mice given diets containing carbofuran for two years***

| Study month | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | 0 | 20 | 125 | 500 | 0 | 20 | 125 | 500 |
| | Mean brain cholinesterase activity, [% of control value] (U/g tissue) | | | | | | | |
| 6 | 55.4 | 53.5 | 46.4 | 40.5* [73%] | 58.2 | 56.1 | 46.3* [80%] | 34.6* [59%] |
| 12 | 47.5 | 58.4 | 39.8 | 26.1* [55%] | 54.7 | 52.8 | 39.4* [72%] | 28.4* [52%] |
| 18 | 49.0 | 49.5 | 39.9* [81%] | 31.2* [64%] | 50.4 | 46.5 | 36.7* [73%] | 22.8* [45%] |
| 24 | 54.7 | 52.9 | 37.8* [69%] | 26.3* [48%] | 57.0 | 53.9 | 39.4* [69%] | 26.7* [47%] |

* Significantly different from control group at $p < 0.05$; Source: Goldenthal, 1983

*Rat*

In a non-GLP carcinogenicity study previously evaluated by JMPR in 1979, 1980 and 1996, carbofuran technical grade (purity 95.6%) was administered in the diet for two years to groups of 90 male and 90 female CD rats to provide concentrations of 0, 10, 20 or 100 ppm (equivalent to 0, 0.5, 1 and 5 mg/kg bw per day). Groups of 10 animals of each sex from each dose group were killed at 6, 12, and 18 months, and blood and brain samples collected for determination of AChE activity. Statistical analyses, when performed, used t-test comparisons, which the Meeting considered inappropriate. The treatment did not adversely affect general appearance, mortality, food consumption, body weight parameters, ophthalmoscopic findings, haematological parameters, urinary parameters, organ weights in males or anatomical (including neoplastic) pathology findings. Reduced body weight gain (at ca 90% of the control group value) occurred over weeks 0 to 104 in the high-dose female group. Clinical chemical examinations revealed statistically significant inhibition of AChE activity in plasma, erythrocytes, and the brain in rats at 100 ppm. The maximal reduction of AChE activity in males was by 37% in plasma, 24% in erythrocytes, and 25% in brain; the corresponding reduction values for females at 100 ppm were by 26% in plasma, 19% in erythrocytes, and 43% in brain. No relevant inhibition of AChE activity was seen in erythrocytes or the brain of animals at 10 or 20 ppm (Case & Wilson, 1979). Confirming the findings of previous JMPR evaluations, the NOAEL for toxicity was 20 ppm (equivalent to 1 mg/kg bw per day) based on a greater than 20% inhibition of brain AChE activity at 100 ppm (equivalent to 5 mg/kg bw per day). The NOAEL for carcinogenicity was 100 ppm (equivalent to 5 mg/kg bw per day), the highest dose tested (Goldenthal 1982a).

***Table 9. Body weight gain and cholinesterase activities in rats given diets containing carbofuran for two years***

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Effect/time point** | **0** | **10** | **20** | **100** | **0** | **10** | **20** | **100** |
| | **Mean body weight gain, weeks 0–104 (g)** | | | | | | | |
| Mean bw gain | 615 | 652 | 638 | 577 | 393 | 409 | 404 | 352 |
| percentage of control | 100 | 106 | 104 | 94 | 100 | 104 | 103 | 90 |
| | **Mean erythrocyte cholinesterase activity units of activity** | | | | | | | |
| Study month 6 | 11.9 | 12.1 | 11.7 | 11.8 | 13.9 | 13.1 | 12.4 | 12.1* |
| percentage of control | 100 | 102 | 98 | 99 | 100 | 94 | 89 | 87 |
| Study month 12 | 14.2 | 14.5 | 13.4 | 11.7* | 14.6 | 14.0 | 13.7 | 13.0 |
| percentage of control | 100 | 102 | 94 | 82 | 100 | 96 | 94 | 89 |
| Study month 18 | 22.5 | 21.3 | 20.0 | 17.1* | 20.6 | 20.0 | 20.6 | 18.1* |
| percentage of control | 100 | 95 | 89 | 76 | 100 | 97 | 100 | 88 |
| Study month 24 | 16.1 | 15.7 | 15.1 | 13.1* | 17.7 | 17.7 | 17.0 | 14.3* |
| percentage of control | 100 | 98 | 94 | 81 | 100 | 100 | 96 | 81 |
| | **Mean brain cholinesterase activity units of activity** | | | | | | | |
| Study month 6 | 10.6 | 12.2 | 11.8 | 10.6 | 17.1 | 16.1 | 15.1 | 13.3 |
| percentage of control | 100 | 115 | 111 | 100 | 100 | 94 | 88 | 78 |
| Study month 12 | 10.5 | 10.4 | 10.4 | 9.3 | 11.4 | 10.6 | 11.2 | 9.4 |
| percentage of control | 100 | 99 | 99 | 89 | 100 | 93 | 98 | 82 |
| Study month 18 | 13.9 | 14.4 | 13.9 | 10.4* | 15.4 | 15.7 | 14.9 | 11.4* |
| percentage of control | 100 | 104 | 100 | 75 | 100 | 102 | 97 | 74 |
| Study month 24 | 10.5 | 10.8 | 10.4 | 8.3* | 15.1 | 13.3 | 13.3 | 8.6* |
| percentage of control | 100 | 103 | 99 | 79 | 100 | 88 | 88 | 57 |

* Significantly different from control group at $p < 0.05$; Source: Goldenthal, 1982a

## 2.4 Genotoxicity

Based on the in vitro genotoxicity studies, high purity carbofuran (99.8% purity) was consistently shown not to be genotoxic: it was negative in the bacterial reverse mutation assay, mammalian forward mutation assay and in vitro micronucleus test. However, irrespective of the starting material for synthesis there is evidence of genotoxicity in vitro with some lower purity (99% or lower) carbofuran batches (E2700-114A, E2700-112D, E2700-112E, E2700-154A, E2915-100A, RHB-09/10-0112, RHB-09/10, RHB-11). In bacterial reverse mutation assays, evidence was based on positive findings with *Salmonella typhimurium* strain TA 1535 in the absence of metabolic activation. This implies the capacity of relevant batches (E2700-114A, E2700-112D, E2700-112E, RHB-09/10, RHB-09/10-0112, RHB-11) to produce base pair substitutions at histidine mutation G46 (target DNA sequence GGG). Batches E2915-100A and E2700-154A produced forward mutations in the mouse lymphoma L5178Y $tk^{+/-}$ assay (MLA). No in vivo gene mutation assay was submitted for evaluation. Carbofuran did not induce chromosomal aberrations or micronuclei in vivo.

Overall, these results imply that more recent (2023), highly purified carbofuran (purity 99.8%) is unlikely to be genotoxic. According to EFSA (2009) a more efficient method of producing high purity carbofuran (greater than 98%) was developed in the 1980s. This is consistent with the current findings that the higher purity batches of carbofuran produced recently have not proved genotoxic. The in vitro studies available, however, suggest that some older, lower purity (99% and below, evaluated in 1983) carbofuran batches may be genotoxic based on their ability to cause base pair substitutions in bacterial reverse mutation assays and forward mutations in the MLA assay. The United States Environmental Protection Agency (US EPA) has reported that studies conducted with impurities found in *o*-nitrophenol-derived carbofuran indicated that two impurities were mutagenic in *S. typhimurium* strains TA98 and TA100 in the presence of S9 activation. This implies that these two impurities can produce both frameshift (TA 98) and base pair substitution (TA 100) mutations (Gahli, 1997). No impurity data on carbofuran was supplied and no information on the impurities identified by US EPA (Gahli, 1997) were supplied.

### (a) In vitro studies

***Table 10. Summary of in vitro genotoxicity studies on carbofuran***

| End-point | Test system | Concentrations | Lot number, Starting material, Purity % | Result and comments | Reference |
|---|---|---|---|---|---|
| Bacterial reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537<br>*E. coli* WP2 uvrA (pKM101) | 1st test: plate incorporation assay, 0 to 5000.0 µg/plate (± S9)<br>2nd test: pre-incubation, 0 to 5000.0 µg/plate (± S9) | G0101:118<br>Not stated<br>99.8% | Negative<br>Precipitate at 5000 µg/plate | Woods, 2023a |
| Bacterial reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538 | Plate incorporation method<br>0–10 000 µg/plate (± S9) | E2915-100A<br>ONP1<br>96% | Negative<br>1.6-fold and 1.5-fold dose-related increases in revertants compared with solvent control in TA1535 and TA100 (–S9); increases of this magnitude were not considered a positive response<br>Precipitate at 10 000 µL/plate | Haworth et al., 1983a |

| End-point | Test system | Concentrations | Lot number, Starting material, Purity % | Result and comments | Reference |
|---|---|---|---|---|---|
| Bacterial reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538 | Plate incorporation method 0–10 000 µg/plate (± S9) | E2700-154A<br>Catechol<br>97.6% | Negative<br>1.8-fold and 1.4-fold increases in revertants compared with solvent control in TA1535 and TA100 (–S9); this size of increase did not satisfy criteria for a positive response<br>Precipitate at 10 000 µL/plate | Haworth et al., 1983b |
| Bacterial reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538 | Plate incorporation method 0–10 000 µg/plate (± S9) | E2700-116A<br>Catechol<br>98% | Negative<br>1.8-fold and 1.4-fold increase in revertants over the solvent control in strains TA1535 and TA100 -S9. The magnitude of this increase did not satisfy the criteria for a positive response.<br>Precipitate at 10 000 µL/plate | Haworth et al., 1983c |
| Bacterial reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538 | Plate incorporation method 0–10 000 µg/plate (± S9) | E2700-114A<br>Catechol<br>98% | Positive in TA 1535 (–S9)<br>2.0-fold increase over the solvent control with a dose-response in TA1535 (–S9)<br>Precipitate at 6667 µL/plate and above | Haworth et al., 1983d |
| Bacterial reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538 | Plate incorporation method 0–10 000 µg/plate (± S9) | E2700-112E<br>Catechol<br>99% | Negative<br>1.7-fold increase in revertants over the solvent control with a dose-response in TA1535 (–S9); the size of this increase did not satisfy the criteria for a positive response<br>Precipitate at 10 000 µL/plate | Haworth et al., 1983e |
| Bacterial reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538 | Plate incorporation method 0–10 000 µg/plate (± S9) | E2700-112D<br>Catechol<br>ca 80% | Positive in TA1535 (–S9)<br>2.2-fold increase in revertants over the solvent control with TA1535 (–S9)<br>Precipitate at 10 000 µL/plate | Haworth et al., 1983f |
| Bacterial reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538 | Plate incorporation method 0–10 000 µg/plate (± S9) | RHB-11-0202<br>Catechol<br>98% | Negative<br>1.9-fold dose increase in revertants over the solvent control in TA1535 (–S9); the size of this increase did not satisfy the criteria for a positive response.<br>Precipitate at 10 000 µL/plate | Haworth et al., 1983g |

| End-point | Test system | Concentrations | Lot number, Starting material, Purity % | Result and comments | Reference |
|---|---|---|---|---|---|
| Bacterial reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538 | Plate incorporation method<br>0–10 000 µg/plate (± S9) | RHB-09/10-0112<br>Catechol<br>98.3% | Positive in TA1535 (–S9)<br>2.2-fold increase in revertants over solvent control in TA1535 (–S9)<br>Precipitate at 10 000 µL/plate | Haworth et al., 1983h |
| Bacterial reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538 | Plate incorporation method<br>0–10 000 µg/plate (± S9) | E2700-154A<br>Catechol<br>97.6% | Negative | DeGraff, 1983a |
| Bacterial reverse mutation | *S. typhimurium* TA98, TA100, TA1537, TA1538 | Experiments 1 & 2: 0–5000 µg/plate<br>Experiment 3: 0–9436 µg/plate | RHB-09/10<br>Catechol<br>98.3% | Positive in TA1535 (–S9)<br>3-fold increase in revertants over the solvent control in TA1535 (–S9) | Farrow, 1983a |
| Bacterial reverse mutation | *Salmonella typhimurium*. TA98, TA100, TA1537, TA1538 | Experiments 1 & 2: 0–5000 µg/plate<br>Experiment 3: 0–9436 µg/plate | RHB-11<br>Catechol<br>98% | Positive with TA1535 (–S9)<br>Showed a dose-related mutagenic response in TA1535 at all dosage levels (–S9) | Farrow, 1983b |
| Bacterial reverse mutation | *Salmonella typhimurium*. TA98, TA100, TA1537, TA1538 | Experiments 1 & 2: 0–5000 µg/plate<br>Experiment 3: 0–9436 µg/plate | E2700-112E<br>Catechol<br>99% | Positive in TA1535 (–S9)<br>> Two-fold increase in revertants over solvent control in TA1535 (–S9) | Farrow, 1983c |
| Mammalian gene mutation | Mouse lymphoma L5178Y $tk^{+/-}$ | Preliminary toxicity study: 0–1000 µg/mL (± S9)<br>Main study: 0–316 µg/mL (± S9) | E2915-100A<br>ONP<br>96% | Positive (±S9)<br>Positive findings at adequate cytotoxicity levels were observed at 100 and 75 ug/mL in the absence of S9 and 1335 and 1001 ug/mL in the presence of S9. | Kirby, 1983a |
| Mammalian gene mutation | Mouse lymphoma L5178Y $tk^{+/-}$ | Preliminary toxicity study: 0 to 10 000 µg/mL (± S9)<br>Main study: 0–211 µg/mL (–S9) 90–1780 µg/mL (+S9) | E2700-154A<br>Catechol<br>97.6% | Positive (–S9)<br>Positive findings at adequate cytotoxicity levels were observed at 119 and 89 ug/mL (–S9) | Kirby, 1983b |
| Mammalian gene mutation | Chinese Hamster ovary cells, HPRT | Preliminary toxicity study: 0–2000 µg/mL (± S9)<br>Main study: 0–1250 µg/mL (± S9) | G0101:118<br>Starting material not stated<br>99.8% | Negative<br>Precipitation at ≥ 1000 µg/mL | Woods, 2023b |

| End-point | Test system | Concentrations | Lot number, Starting material, Purity % | Result and comments | Reference |
|---|---|---|---|---|---|
| Chromosomal aberration | Chinese Hamster ovary cells | First study:<br>Two-hour treatment<br>0–2500 µg/mL<br>(+S9)<br>Second study:<br>12-hour treatment<br>0–2000 µg/mL<br>(–S9) | E2915-100A<br>ONP<br>96% | Negative | Thilagar, 1983a |
| In vitro micronucleus test | Human peripheral blood lymphocytes | Preliminary toxicity tests:<br>3-hour exposure<br>0 to 2000 µg/mL<br>(±S9)<br>20-hour exposure<br>0–2000 µg/mL<br>(–S9)<br>Main tests:<br>3-hour exposure<br>0–1025 µg/mL<br>(+S9)<br>3-hour exposure<br>0–1250 µg/mL<br>(–S9)<br>20-hour exposure<br>0 –350 µg/mL<br>(–S9) | G0101:118<br>Starting material not stated<br>99.8% | Negative<br>Precipitation at 2000 µg/mL<br>Negative | Woods, 2023c |
| Unscheduled DNA synthesis | Rat primary hepatocytes | 18-hour treatment<br>0–100 µg/mL<br>(–S9) | E2700-154A<br>Catechol<br>97.6% | Negative | Thilagar, 1983b |

ONP: *o*-Nitrophenol; i.p.: Intraperitoneal; HPRT: Hypoxanthine–guanine phosphoribosyl transferase

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*;
+S9: With S9 activation; –S9: Without S9 activation; ±S9: With and without S9 activation

### (b) In vivo studies

*Table 11. Summary of in vivo genotoxicity studies*

| End-point | Test system | Dose and route | Starting material, Purity % | Result and comments | Reference |
|---|---|---|---|---|---|
| Chromosomal aberration | Rat bone marrow cells | 0, 0.6, 2, and 6 mg/kg bw per day by oral gavage for 5 consecutive days with fixation at 6 h following last dose in the oral dosing cohort | Catechol 96% | Negative, but with experimental limitations; fixation before time needed for 1.5 normal cell cycles (12–18 hours)<br>Toxicity manifesting as >10% reduction in body weight at the high dose<br>Bone marrow exposure not experimentally confirmed but highly probable given known disposition of carbofuran following oral dosing | Putman, 1983a |
| Chromosomal aberration | Rat bone marrow cells | 0, 1, 6, and 10 mg/kg bw per day by oral gavage for 5 consecutive days with fixation at 6 h following the last dose in the oral dosing cohort | ONP 96% | Negative, but with experimental limitations; fixation before time needed for 1.5 normal cell cycles (12–18 hours)<br>Clinical signs consistent with cholinergic toxicity at the high dose<br>Bone marrow exposure not experimentally confirmed but highly probable given known disposition of carbofuran following oral dosing | Putman, 1983b |
| Chromosomal aberration | Mouse bone marrow cells | Dose ranging study: 0, 5, 10 and 20 mg/kg bw<br>Main study: 0, 1.25, 2.5 and 5 mg/kg bw | Unknown 99% | Negative<br>Dose ranging study: all animals dosed at ≥ 10 mg/kg bw died. Animals at 10 mg/kg bw had clinical signs including tremors and ataxia prior to death. At 5 mg/kg bw clinical signs included hypoactivity, and laboured respiration<br>Bone marrow exposure not experimentally confirmed but highly probable given known disposition of carbofuran following oral dosing | Erexson, 2005a |
| Chromosomal aberration | Mouse bone marrow cells | Main study: 0, 1.25, 2.5, 5.0 mg/kg bw with fixation times of 18- and 42-hours following dosing | Unknown 99% | Negative<br>Three of 18 animals dosed at 5.0 mg/kg bw died. Clinical signs observed at 5.0 mg/kg bw included hypoactivity, irregular respiration and tremor. At 1.25 and 2.5 mg/kg bw hypoactivity and irregular respiration were seen in all animals 15 minutes post dose.<br>Bone marrow exposure not experimentally confirmed but highly probable given known disposition of carbofuran following oral dosing | Erexson, 2005b |

ONP: *o*-Nitrophenol;

## 2.5 Reproductive and developmental toxicity

### (a) Multigeneration studies

In a non-GLP, three generation reproduction study evaluated by JMPR in 1980 and 1996, groups of Charles River CD rats (10 males and 20 females per group for the first and second generations; 12 males and 24 females per group for the third generation) were maintained on a diet providing concentrations of carbofuran (purity 95.6%) at 0, 20 or 100 ppm (equal to 0, 1.2 and 6 mg/kg bw per day for males, 0, 1.9 and 9.7 mg/kg bw per day for females). Each generation was mated twice to produce two litters.

There were no carbofuran-associated adverse effects on general behaviour, appearance, fertility index or survival of the parental rats. No carbofuran-associated adverse anatomical pathology findings or effects on organ weights were seen at necropsy in the F0, F1, or F2 parental animals or the F2b or F3b litters. Parental body weight gain was lower in the 100 ppm groups throughout the course of treatment (non-adverse, ca 93% of control weight gains over weeks 0–28). This effect was associated with lower food consumption, particularly in males. The survival of pups from the F1a, F2a, and F3a generations at 100 ppm was reduced at lactation day (LD) 4 in comparison with controls (by 4%, 7% and 10% in the F1a, F2a, and F3a generations respectively). Dehydration was seen in pups from some F3a and F3b litters at the 100 ppm dose. The mean weights of pups at 100 ppm were significantly ($p < 0.05$) and adversely lower (greater than 10% reduction) than those of the controls on post partum day (PPD) 21 in all generations. Various organ weights of F2 parental animals and F3b pups at 20 and 100 ppm differed from controls to a statistically significant extent. The reproductive NOAEL was 100 ppm (equal to 6 mg/kg bw per day), the highest dose tested and consistent with previous JMPR evaluations. The parental NOAEL was 100 ppm (equal to 6 mg/kg bw per day), the highest dose tested, but differing from the previous JMPR evaluation due to a different interpretation of the adversity attached to the effects of carbofuran on body weight. The developmental NOAEL was 20 ppm (equal to 1.2 mg/kg bw per day), based on reductions in the growth and survival of pups at 100 ppm (equal to 6 mg/kg bw per day), and consistent with previous JMPR evaluations (Goldenthal, 1982b).

### (b) Developmental toxicity

***Rat***

The following study of Barron, Giesler & Ras (1978) was evaluated by JMPR in 1980 and 1996 (see below) but was not submitted for the current assessment:

> In a study first evaluated by the 1980 JMPR (Annex 1, reference 34), carbofuran (technical-grade; purity, 95.6%) was administered to groups of 24 pregnant Charles River rats by intubation in 0.25% carboxymethylcellulose at doses of 0, 0.1, 0.3, or 1 mg/kg bw per day on days 6–15 of gestation. Transient, dose-dependent clinical signs (chewing motions) were observed at doses > 0.1 mg/kg bw per day for a short period after treatment. Overt signs of toxicity in animals at 0.3 mg/kg bw per day included rough coats and lethargy; at the high dose, lacrimation, increased salivation, trembling, and convulsions were also seen. One female at the high dose died. The treatment had no effect on the body weights of dams or pups. Reproductive parameters (e.g. numbers of corpora lutea, implantations, and resorptions and litter size) were not adversely affected by treatment. Skeletal examination revealed a higher incidence of some variations in ossification of sternebrae in treated animals but with no clear dose–response relationship. Similar alterations were prevalent in the control group at an incidence of 47% of fetuses. There was no evidence of teratogenicity. A NOAEL was not determined because transient clinical signs were observed at the lowest dose (Barron, Giesler & Ras, 1978).

In a non-GLP prenatal developmental toxicity study in rats, carbofuran technical (purity 95.6%) was administered by gavage to groups of 24 presumed-pregnant Sprague Dawley CD rats per group during gestation days (GDs) 6–15 at dosages of 0, 0.1, 0.3 or 1.0 mg/kg bw per day. There were no carbofuran-associated adverse effects on maternal mortality, maternal body weight, the number of viable litters, the number of dead litters, sex ratio, average litter size, number of corpora lutea, fetal variations or fetal malformations. An increased incidence of adverse clinical signs (lethargy, rough coat, chewing motions) occurred at 0.3 mg/kg bw per day. An increased incidence of clinical signs consistent with a cholinergic crisis (lethargy, excessive lacrimation, pale eyes, excessive salivation, trembling, chewing motions and convulsions) occurred in the 1.0 mg/kg bw per day group. The maternal NOAEL was 0.1 mg/kg bw per day based on an increased incidence of adverse clinical signs at 0.3 mg/kg bw per

day, with an increased incidence of clinical signs consistent with a cholinergic crisis at 1.0 mg/kg bw per day. The developmental NOAEL was 1 mg/kg bw per day, the highest dose tested (Rao, 1978a).

In another non-GLP prenatal developmental toxicity study previously evaluated by JMPR 1996, pregnant Charles River CD rats (25 per dose level) were dosed by oral gavage with carbofuran (purity 95.6%) at 0, 0.25, 0.50 or 1.20 mg/kg bw per day over GDs 6–19. There were no carbofuran-associated effects on appearance, behaviour, maternal body weight gain, number of corpora lutea, total implantations, early and late resorptions, post-implantation loss, number of viable fetuses, sex distribution, mean fetal body weight, mean fetal body length, the number of litters with malformations or the number of litters with developmental variations. The maternal and developmental NOAEL was 1.20 mg/kg bw per day, the highest dose tested, and consistent with JMPR 1996 (Rodwell, 1980).

In a preliminary non-GLP prenatal developmental toxicity study previously evaluated by JMPR in 1996, pregnant Charles River COBS® CD rats (10 per dietary concentration level) were exposed to carbofuran (purity 95.6%) in the diet at 0, 20, 60, 120, 160 or 200 ppm (equal to 1.30, 4.07, 8.44, 12.3 and 15.1 mg/kg bw per day) over GDs 6–19. There were no test article-associated adverse effects on mortality, mean numbers of viable or nonviable fetuses, early or late resorptions, total implantations, corpora lutea or mean post implantation losses. No teratogenic effect was observed. Clinical signs noted during the study included dried red matter in the nasal region, soft stool, and matting of the haircoat in the treated groups. Dose-related mean maternal body weight losses and decreases in mean food consumption (not statistically significant) were noted during the first two days of treatment in the 60, 120, 160 and 200 ppm groups. Over the entire treatment, mean body weight gain in the 60 ppm group was comparable to controls, but in the 120 ppm group weight gain was depressed by ca 10% compared with controls, and in the 160 and 200 ppm groups it was severely depressed (by approximately 21% and 20% respectively) compared to controls. Based on the results obtained in this range-finding study, dietary dosage levels for the main developmental toxicity study in rats were set at 0, 20, 60 and 160 ppm. (Rodwell, 1985).

For Rodwell's main study, a study amendment, but not the full study report, was supplied. The main, non-GLP, study was evaluated by JMPR in 1996:

> The main study was conducted with groups of 40 pregnant Charles River COBS rats fed diets giving concentrations of 0, 20, 60, or 160 ppm carbofuran (purity, 95.6%), equal to 0, 1.5, 4.4, and 11 mg/kg bw per day, on gestation days 6–19. Treatment had no effect on appearance or behaviour, except for a slight increase in hair loss, matting of the ventral haircoat, and soft stools, particularly in females at 60 and 160 ppm. A dose-related reduction in body-weight gain was seen at doses ≥60 ppm during the treatment period, with single instances of body-weight loss in animals at 60 and 160 ppm at the beginning of treatment. Increases in body-weight gain of treated animals during the subsequent lactation period resulted in very similar body weights in all groups at the end of lactation. Food consumption was reduced during the first few days of treatment in animals at 60 and 160 ppm. No differences between groups were observed in reproductive parameters (such as the numbers of corpora lutea, implantations, and resorptions and litter size). The mean weights of pups at 160 ppm were decreased throughout lactation. The incidence of malformations was not increased. The NOAEL for maternal toxicity was 20 ppm, equal 1.5 mg/kg bw per day, on the basis of reduced body-weight gain of the dams at doses ≥60 ppm. The NOAEL for pup toxicity was 60 ppm, equal to 4.4 mg/kg bw per day, on the basis of reduced weights of pups at 160 ppm (Rodwell, 1981).

The submitted amendment to Rodwell (1981) provided corrected and/or updated data regarding group mean maternal food consumption during gestation, additional individual maternal observation data and statistical analysis on the mean maternal body weight change during gestation and lactation. The amended data had no material effect of the interpretation of Rodwell's original study.

***Rabbit***

In a study first evaluated by the JMPR in 1980, and re-evaluated by JMPR in 1996, groups of 17 pregnant New Zealand White rabbits were orally (gavage) dosed with carbofuran (purity 95.6%) at doses of 0, 0.2, 0.6 or 2 mg/kg bw per day over GDs 6–18. Notably, the animals used in the study had active coccidiosis infections (confirmed by microscopic anatomical pathology examinations). No carbofuran-associated effects were apparent on maternal body weight, maternal macroscopic pathology findings, uterine examination and reproductive findings, fetal parameters, fetal variations, or fetal malformations. The deaths of three females at 2 mg/kg bw per day were potentially related to treatment. The cause of death

of two females at 0.6 mg/kg bw per day was not determined. An increased incidence of trembling occurred at 0.6 mg/kg bw per day. An increased incidence of clinical signs consistent with a cholinergic crisis (trembling, loss of muscle control, salivation, sneezing, chewing motions, and reduced food and water consumption) occurred in rabbits receiving 2.0 mg/kg bw per day. The maternal NOAEL was 0.6 mg/kg bw per day, based on the occurrence of clinical signs consistent with cholinergic crisis seen at 2 mg/kg bw per day; this was consistent with JMPR's 1980 and 1996 evaluations. The developmental NOAEL was 2 mg/kg bw per day, the highest dose tested (Rao, 1978b).

The sponsor supplied an incomplete copy of Laveglia (1981), which lacked the key table on fetal morphological observations (Table 7). Several other deficiencies (an incomplete table of contents, missing Table 6) were also noted. The 1996 JMPR evaluation of this non-GLP study included the following:

> Carbofuran (purity, 95.6%) was administered by gavage at doses of 0, 0.12, 0.5, or 2 mg/kg bw per day on gestation days 6–18 to groups of 20 pregnant New Zealand White rabbits. One dam at 2 mg/kg bw per day died of an unknown cause. Matting and/or staining of the haircoat was seen at a somewhat higher frequency in the treated animals. Although the mean maternal body weight was reduced during treatment, no clear dose–response relationship was seen. A marked reduction in body-weight gain was observed at 2 mg/kg bw per day early in the treatment; no data were available on food consumption. Reproductive parameters (numbers of corpora lutea, implantations, and resorption, litter size, fetal body weight) were not affected by treatment. A slightly increased incidence of misaligned sternebrae was seen in fetuses at 2 mg/kg bw per day. The NOAEL was 0.5 mg/kg bw per day, on the basis of reduced body weight in dams at 2 mg/kg bw and the increase in skeletal variations (Laveglia, 1981).

## 2.6 Special studies

### (a) Neurotoxicity

In a 28-day neurotoxicity range-finding study previously evaluated by JMPR in 1996, carbofuran (purity 98.6%) was administered in the diet for 28 consecutive days to five Sprague Dawley CD female rats per group at dietary concentrations of 0, 50, 200, 500, 1000, 3000 or 6000 ppm (equivalent to 0, 2.5, 10, 25, 50, 150, and 300 mg/kg bw per day). Rats were observed once daily for clinical signs and twice daily for mortality. Body weights were recorded weekly. Gross necropsy of surviving animals were performed on day 28. Two males receiving 6000 ppm died by day 7. No other deaths occurred during the study. Clinical signs generally occurred with a frequency that increased with dosage. The most significant clinical signs included ataxia, exophthalmos, hypersensitivity to touch, loss of muscle control, splayed hindlimbs, staggered gait and tremors. Other treatment-related clinical signs included abdominogenital staining, abdominal gripping, chromodacryorrhea, chromorhinorrhea, decreased faeces, decreased locomotion, dehydration, lacrimation, oral discharge, unkempt appearance and unthriftiness. No treatment-related clinical signs were noted among animals receiving 50 ppm or less. At 500 ppm and higher doses body weights and body weight gains were reduced significantly ($p < 0.05$) and adversely, by at least 10% compared to controls. No gross lesions were noted during necropsy. The suggested dosage levels for the subchronic neurotoxicity screen were 0, 8, 50, 500 and 1000 ppm (Freeman, 1993).

In a subchronic neurotoxicity study, carbofuran technical (purity 98.6%) was administered in the diet for 13 weeks to ten Sprague Dawley CD rats of each sex per group at dose levels of 0, 50, 500 or 1000 ppm (equal to 0, 2.4–4.7, 27.3–46.1 and 55.3–92.2 mg/kg bw per day for males, 0, 3.1–4.8, 35.3–50.7 and 64.4–100 mg/kg bw per day for females). Rats were observed once daily for clinical signs and twice daily for mortality. Body weights and food consumption were recorded weekly. Functional observational battery (FOB) and motor activity testing occurred during the pretest interval and following week 4, 8 and 13 of treatment. At termination survivors were perfused and eviscerated; the nervous systems of five rats of each sex from the 1000 ppm and control groups were examined for neuropathological lesions. There were no carbofuran-associated effects on mortality.

Clinical signs (oral discharge, exophthalmos, loss of muscle control, splayed hindlimbs, staggered gait and tremor) consistent with cholinergic toxicity occurred in rats receiving 500 ppm and above. Other clinical signs noted at 500 ppm and above included abdominogenital staining, chromorhinorrhea, decreased faeces, dehydration, haematuria, unthriftiness, and pale eyes, ears, feet, and tail. All carbofuran-exposed rats exhibited significant ($p < 0.05$) reductions in body weight gain by at least 13% compared to controls. Males receiving 1000 ppm exhibited sporadic incidences of decreased

food consumption when compared to controls. Food consumption was not significantly different among males receiving 500 ppm or less to that of controls. Females in the high-dose group exhibited decreased food consumption during the first week of the study. Females in all treated groups exhibited sporadic increases in food consumption during the remainder of the study. In the FOB evaluations at study week 4, gait impairment (characterized by one or more of the following: ataxia, staggered gait, splayed hindlimbs, exaggerated hindlimb flexion) occurred in rats dosed at 500 ppm and above. Reduced hindlimb grip strength and tail flick latency occurred at 1000 ppm in males. Exophthalmos occurred in females at doses of 500 ppm and above and increased tail flick latency occurred at 1000 ppm. No adverse FOB findings occurred in males at week 8 or 13 assessments. In study week 8 gait impairment and exophthalmos occurred in females at 500 ppm and above. In the high-dose females decreased hindlimb grip strength and an increased number of urine pools occurred at 1000 ppm. In study week 13, increased landing foot splay was seen in treated females at all dietary concentrations. Exophthalmos and gait impairment occurred in females at doses of 500 ppm and above, and reduced forelimb grip strength occurred at 1000 ppm. Whole body tremors occurred in males at 500 ppm and above during the open field test at study weeks 4 and 8. Whole body tremors and abnormal posture was recorded in females at 500 ppm and above during the open field test during study weeks 4, 8 and 13. There were no significant differences in motor activity levels of treated males when compared to controls at any test period during the study. Females receiving 1000 ppm exhibited decreased motor activity when compared to controls following the fourth and eighth week of treatment. Females receiving 500 ppm or less displayed no significant differences in motor activity when compared to controls during any testing interval during the study. There were no carbofuran-associated adverse anatomical pathology findings. The LOAEL for neurotoxicity was 50 ppm (equal to 3.1 mg/kg bw per day), the lowest dose tested, based on increased landing foot splay in females at all carbofuran dietary concentrations. The LOAEL for systemic toxicity was as 50 ppm (equal to 2.4 mg/kg bw per day) the lowest dose tested, based on adversely reduced body weight gain (by more than 10% compared to controls) seen in all carbofuran-treated animals (Freeman, 1994).

***Table 12. Mean body weight gain in the subchronic neurotoxicity study with carbofuran in rats***

| | Dietary concentration (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Parameter** | **0** | **50** | **500** | **1000** | **0** | **50** | **500** | **1000** |
| Mean body weight gain (g) | 350 | 281 | 242 | 215 | 140 | 122 | 118 | 101 |
| Percentage of control | 100 | 80 | 69 | 61 | 100 | 87 | 84 | 72 |

Source: Freeman, 1994

In a developmental neurotoxicity study previously evaluated by JMPR in 1996, groups of 24 mated female Sprague Dawley (CD) rats were fed diets containing carbofuran (purity 99.1%) at 0, 20, 75 or 300 ppm (equal to 0, 1.7–1.73, 4.95–6.915 and 8.57–31.38 mg/kg bw per day) from GD 6 until LD 10. The wider ranges in carbofuran intake at the 75 and 300 ppm concentrations were consistent with fluctuations in food consumption during gestation. Physical observations were made, and body weight and food consumption measured for all maternal animals at selected intervals throughout the gestation and lactation periods. The numbers of live pups was recorded, and litter parameters such as pinna detachment, incisor eruption, eye opening, motor activity, auditory startle response, swimming development, learning and memory, and brain weights were evaluated. Neuropathological examinations were performed on six pups of each sex per group that were killed on postnatal days (PNDs) 11 and 60. Maternal animals were killed after weaning of the last litter. There were no carbofuran-associated effects on maternal mortality, maternal physical observations, pregnancy status, gestation length, delivery, maternal anatomical pathology findings, pup sex distribution, pup external malformations, pup auditory startle response, pup motor activity, pup brain weight or pup brain microscopic anatomical pathology.

Significantly reduced ($p < 0.05$) maternal body weight gain occurred at 75 ppm and above on GDs 6–10. This was followed by significant compensatory body weight gain ($p < 0.05$) over GDs 10–15 in the high-dose dams. However, the high-dose maternal body weight gain was lower by 11% compared to controls (significant at $p < 0.05$) over GDs 15–20. Mean maternal body weights were significantly

lower ($p < 0.05$) on GD 4 at 75 ppm and on GDs 4 and 11 at 300 ppm. The litter survival index was significantly reduced ($p < 0.05$) at 300 ppm.

Significant ($p < 0.05$) and adverse reductions (by less than 10% compared to controls) in pup weights occurred during lactation at dietary concentrations 75 ppm and above. Pup viability and weaning indices were significantly reduced ($p < 0.05$) at doses of 75 ppm and above. Most of the pups died before LD 4. Due to the large number of missing and autolyzed pups the cause of many deaths remained unknown. The development of vaginal patency and preputial separation were significantly delayed ($p < 0.05$) at dietary concentrations of 75 ppm and above, as was lower incisor eruption. Pinna detachment was significantly delayed ($p < 0.05$) at 300 ppm. The effects on incisor eruption and pinna detachment were however within the relevant historical control range. Group mean head angle when swimming was significantly reduced ($p < 0.05$) compared to controls at 75 ppm and above (angle evaluations assess position of the head in the water; as development proceeds, pups begin to hold more of their head out of the water). Although pups in the dose groups receiving 75 ppm and above demonstrated improved angle scores over time, the scores were consistently lower than for the controls. Overall, pups receiving 75 ppm or above exhibited delays in swimming head angle development when compared to controls. Pups derived from the high-dose maternal cohort showed reduced acquisition and memory in the water Y-maze test on PND 24. Mean trial times for male and female pups in the 300 ppm dose group were increased for all six trials when compared to controls. The differences were statistically significant ($p < 0.05$) in two out of the six trials for both sexes. The lack of any pattern in the females' trial times indicated that the female pups did not learn the task. Males in the high-dose group did learn the task, although both short- and long-term memory were compromised compared to controls. There were no effects on learning and memory in the water Y-maze test conducted on PND 60.

The maternal NOAEL was 20 ppm (equal to 1.7 mg/kg bw per day) based on an adverse reduction in maternal body weight parameters at 75 ppm (equal to 4.95 mg/kg bw per day). The developmental NOAEL was 20 ppm (equal to 1.7 mg/kg bw per day) based on adverse effects on pup body weight parameters, pup viability and weaning indices, pup deaths and evidence of developmental delay (delayed vaginal patency, delayed preputial separation, delay in swimming head angle development) at 75 ppm (equal to 4.95 mg/kg bw per day); these findings were consistent with the JMPR 1996 evaluation (Ponnock, 1994).

***Table 13. Findings in the dietary exposure developmental neurotoxicity study in rats***

| | Mean maternal body weight change during gestation (g) | | | |
|---|---|---|---|---|
| **Dietary dose** | **Days 0-6** | **Days 6-10** | **Days 10-15** | **Days 15-20** |
| 0 ppm | 28 | 14 | 30 | 73 |
| 20 ppm | 25 | 12 | 27 | 69 |
| 75 ppm | 25 | 1* | 31 | 70 |
| 300 ppm | 23 | –27* | 46* | 65* |

| | Mean maternal body weights during lactation (g) | | | | | | |
|---|---|---|---|---|---|---|---|
| **Dietary dose** | **Day 0** | **Day 4** | **Day 11** | **Day 21** | **Day 0-4** | **Day 4-11** | **Day11-21** |
| 0 ppm | 289 | 305 | 318 | 316 | 17 | 13 | –2 |
| 20 ppm | 282 | 295 | 310 | 308 | 14 | 15 | –3 |
| 75 ppm | 276 | 285* | 303 | 310 | 9 | 18* | 6 |
| 300 ppm | 259* | 263* | 285* | 317 | 12 | 18 | 33* |

| | Mean pup weights during lactation (g) | | | | | |
|---|---|---|---|---|---|---|
| **Dietary dose** | **Day 0** | **Day 4, pre cull** | **Day 4, post cull** | **Day 11** | **Day 17** | **Day 21** |
| 0 ppm | 6.2 | 9.6 | 9.6 | 24.7 | 38.9 | 51.1 |
| 20 ppm | 6.0 | 9.0 | 9.0 | 23.2 | 38.4 | 48.7 |
| 75 ppm | 5.8* | 7.4* | 7.4* | 18.6* | 31.8* | 40.9* |
| 300 ppm | 5.2* | 7.0* | 7.0* | 15.2* | 26.2* | 35.6* |

| | Litter survival index, pup live birth viability and pup weaning index | | | |
|---|---|---|---|---|
| **Dietary dose** | **Survival index** | **Pup live birth index** | **Pup viability index** | **Pup weaning index** |
| 0 ppm | 100 | 97.7 | 98.5 | 87.0 |
| 20 ppm | 100 | 99.4 | 94.7 | 84.8 |
| 75 ppm | 87.5 | 97.7 | 83.4* | 72.9* |
| 300 ppm | 39.1* | 91.9 | 33.8* | 69.6 |

| | Mean developmental landmark data (days to criteria) | | | | | |
|---|---|---|---|---|---|---|
| **Dietary dose** | **Pinna detachment** | **Upper incisor eruption** | **Lower incisor eruption** | **Eye opening** | **Vaginal patency** | **Preputial separation** |
| 0 ppm | 2.52 | 10.89 | 11.85 | 13.93 | 31.92 | 44.96 |
| 20 ppm | 3.00 | 10.92 | 12.25* | 14.15* | 32.28 | 45.51 |
| 75 ppm | 2.93 | 10.97 | 12.50* | 14.29* | 32.85* | 47.94* |
| 300 ppm | 3.19* | 11.00 | 12.33* | 14.33* | 34.93* | 48.88* |
| **Historical control data** | | | | | | |
| Mean | 2.5 – 2.7 | 9.5 – 9.7 | 10.8 – 11.0 | 13.7 – 13.8 | - | - |
| Standard deviation | (0.5 – 0.6) | (0.5 – 0.7) | (10.8 – 11.0) | (0.3-0.6) | - | - |
| Number of studies | 42 | 42 | 42 | 42 | - | - |

* Significantly different from control group at $p < 0.05$; Source: Ponnock, 1994

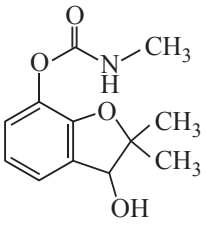
### (b) Immunotoxicity

No studies were submitted on this topic.

### (c) Studies on metabolites

***3-Hydroxy-carbofuran***

***Figure 5. 3-Hydroxy-carbofuran***




The metabolite 3-hydroxy-carbofuran is an AChE inhibitor that has, based on its acute toxicity, similar potency to its parent. The acute oral $LD_{50}$ of 3-hydroxy-carbofuran (purity 96%) was 21.9 mg/kg bw in males and 8.3 mg/kg bw in females (Freeman, 1984a). In a second study (not GLP) the acute oral $LD_{50}$ of 3-hydroxy-carbofuran (purity not stated) in SD rats was determined as 17.9 mg/kg bw (Schoenig, 1967b). In both studies the predominant clinical signs (tremors, hyphemia, fibrillary action, exophthalmus, salivation, oral discharge, lacrimation, chewing motions, haemorrhagic nasal discharge, hypernea and cyanosis, chromodacryorrhea, chromorhinorrhea and abdominogenital staining) were all consistent with a cholinergic crisis.

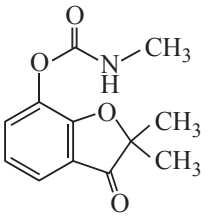
In a repeat oral (dietary) exposure toxicity study (not GLP), 15 SD rats of each sex per dose were fed diets for 90 days containing 3-hydroxy-carbofuran (purity not stated) at 0, 10, 30 or 100 ppm (equivalent to 0, 0.5, 1.5 or 5 mg/kg bw per day). There were no test article-associated adverse effects on behaviour, body weight, food consumption, haematology, clinical chemistry and urinalysis findings, organ weights or anatomical pathology findings. A female in the 10 ppm group was found dead during week 2. This death was attributed to an acute respiratory infection. Males exposed at 10 ppm had significantly higher liver weights, liver to body and brain weight ratios, spleen to brain weight ratios, kidney to brain weight ratios and brain to body weight ratios. These findings occurred in the absence of anatomical pathology correlates. Male rats at 30 ppm had increased liver to body weight ratios, which

however was not observed at the highest dose and lacked anatomical pathology correlates. Given the lack of any consistent dose or sex-related response and the lack of anatomical pathology correlates these findings were not considered attributable to the effects of 3-hydroxy-carbofuran. Despite evidence of the possible anticholinesterase activity of 3-hydroxy-carbofuran in the acute oral toxicity studies, AChE activity levels were not measured. Because of the key limitations of this study (lack of AChE measurement), a NOAEL was not identified (Plank, 1969).

Metabolite 3-hydroxy-carbofuran (purity 99.1%) was not genotoxic in a bacterial reverse mutation assay, did not induce forward mutations in a Chinese hamster ovary HPRT test, and did not induce micronuclei in human peripheral blood lymphocytes in vitro (Woods, 2023d, e, f).

***3-Keto-carbofuran***

***Figure 6. 3-Keto-carbofuran***

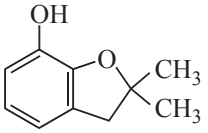


Metabolite 3-keto-carbofuran is an AChe inhibitor. The acute oral $LD_{50}$ of 3-keto-carbofuran (purity 98%) in SD rats was 107 mg/kg bw (Freeman, 1984b). In a second acute oral toxicity study (not GLP) in SD rats the $LD_{50}$ for 3-keto-carbofuran (purity not stated) was 69.0 mg/kg bw (Schoenig, 1967b). In both studies the predominant clinical signs (hyperpnea, muscular weakness, tremor, fibrillary action, exophthalmus, salivation, lacrimation, chewing motion, hypoactivity and frothing at the mouth, abdominogenital staining, chromodacryorrhea, chromorhinorrhea), were consistent with a cholinergic crisis.

No genotoxicity data on 3-keto-carbofuran was submitted. Quantitative structure–activity relationship (QSAR) evaluation of 3-keto-carbofuran (including its predicted in vitro and in vivo metabolites) using OECD ToolBox v. 4.5 yielded no predicted bacterial reverse mutation modes of action compared with its parent molecule carbofuran. Based on the QSAR findings, 3-keto-carbofuran is likely to present a similar genotoxicity hazard as its parent, carbofuran.

***7-Phenol-carbofuran***

***Figure 7. Chemical structure of 7-Phenol-carbofuran***

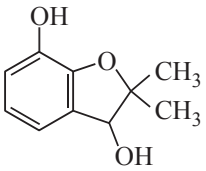


The acute oral $LD_{50}$ of 7-phenol-carbofuran (purity 98%) in SD rats was 2450 mg/kg bw in males and 1743 mg/kg bw in females (Freeman, 1984c). In a second study (not GLP) the acute oral $LD_{50}$ of 7-phenol-carbofuran (purity 97.8%) was 2200 mg/kg bw for the undiluted test material, 1800 mg/kg bw for a 25% corn oil solution, and 1800 mg/kg bw for a 75% polyethylene glycol (PEG) solution (Schoenig, 1967c).

No genotoxicity data on 7-phenol-carbofuran was submitted. A QSAR evaluation of 7-keto-carbofuran (including its predicted in vitro and in vivo metabolites) using OECD ToolBox v. 4.5 yielded no predicted bacterial reverse mutation modes of action compared with its parent molecule carbofuran. Based on the QSAR findings 7-phenol-carbofuran is likely to present a similar genotoxicity hazard as its parent, carbofuran.

***3-Hydroxy-7-phenol-carbofuran***

***Figure 8. 3-Hydroxy-7-phenol-carbofuran***

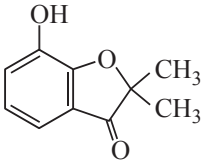



The acute oral $LD_{50}$ of 20% (w/w) 3-hydroxy-7-phenol-carbofuran (purity 98%) in corn oil was 1916 mg/kg bw in males and 1654 mg/kg bw in females (Freeman, 1984d). In a second study (not GLP) the acute oral $LD_{50}$ of 3-hydroxy-7-phenol-carbofuran (purity not stated) in SD rats was 1350 mg/kg bw (Schoenig, 1967d).

No genotoxicity data on 3-hydroxy-7-phenol-carbofuran was submitted. A QSAR evaluation of 3-hydroxy-7-phenol-carbofuran (including its predicted in vitro and in vivo metabolites) using OECD ToolBox v. 4.5 yielded no predicted bacterial reverse mutation modes of action compared with its parent molecule, carbofuran. Based on the QSAR findings 3-hydroxy-7-phenol-carbofuran is likely to present a similar genotoxicity hazard as the carbofuran.

***3-Keto-7-phenol-carbofuran***

***Figure 9. 3-Keto-7-phenol-carbofuran***




The acute oral $LD_{50}$ of 3-keto-7-phenol-carbofuran (purity 98%) in SD rats was greater than 800 mg/kg bw (Freeman, 1984e). In a second study (not GLP) the acute oral $LD_{50}$ of 5% (w/w) 3-keto-7-phenol-carbofuran (purity not stated) in corn oil in SD rats was 295.1 mg/kg bw (Schoenig, 1967d).

No genotoxicity data on 3-keto-7-phenol-carbofuran was submitted. A QSAR evaluation of 3-keto-7-phenol-carbofuran (including its predicted in vitro and in vivo metabolites) using OECD ToolBox v. 4.5 yielded no predicted bacterial reverse mutation modes of action compared with its parent molecule carbofuran. Based on the QSAR findings 3-keto-7-phenol-carbofuran is likely to present a similar genotoxicity hazard as the carbofuran.

In a one-generation reproduction study (not GLP) 3-keto-7-phenol-carbofuran was administered in the diet to eight male and 16 female albino rats of each sex per group at dose levels of 0, 10 or 50 ppm (equivalent to 0, 0.5 and 2.5 mg/kg bw per day) for one reproductive generation (two litters). No mortalities were recorded among female animals or among control or 10 ppm males. At 50 ppm, two males died due to severe respiratory infection. There were no adverse effects observed on the parental generation with respect to growth, mortality, food consumption, behaviour or with respect to gross and histological examinations of tissues and organs. Reproduction indices were unaffected, as were the survival and viability indices in the progeny. A dose-dependent decrease in mating index was noted at both doses for the F1b litters (by 16.9% at 10 ppm and 17.8% at 50 ppm), however, this was not the case for F1a litters, in which corresponding values were reduced by 13.6% at 10 ppm and 8% at 50 ppm. No effects were noted on the number of pups delivered, still births, survival, or growth. No gross abnormalities were observed in progeny over the course of the study. The parental, reproductive and offspring NOAELs were all 50 ppm (equivalent to 2.5 mg/kg bw per day), the highest dose tested (Arnold, 1969). There is no evidence that 3-keto-7-phenol-carbofuran acts as an AChE inhibitor.

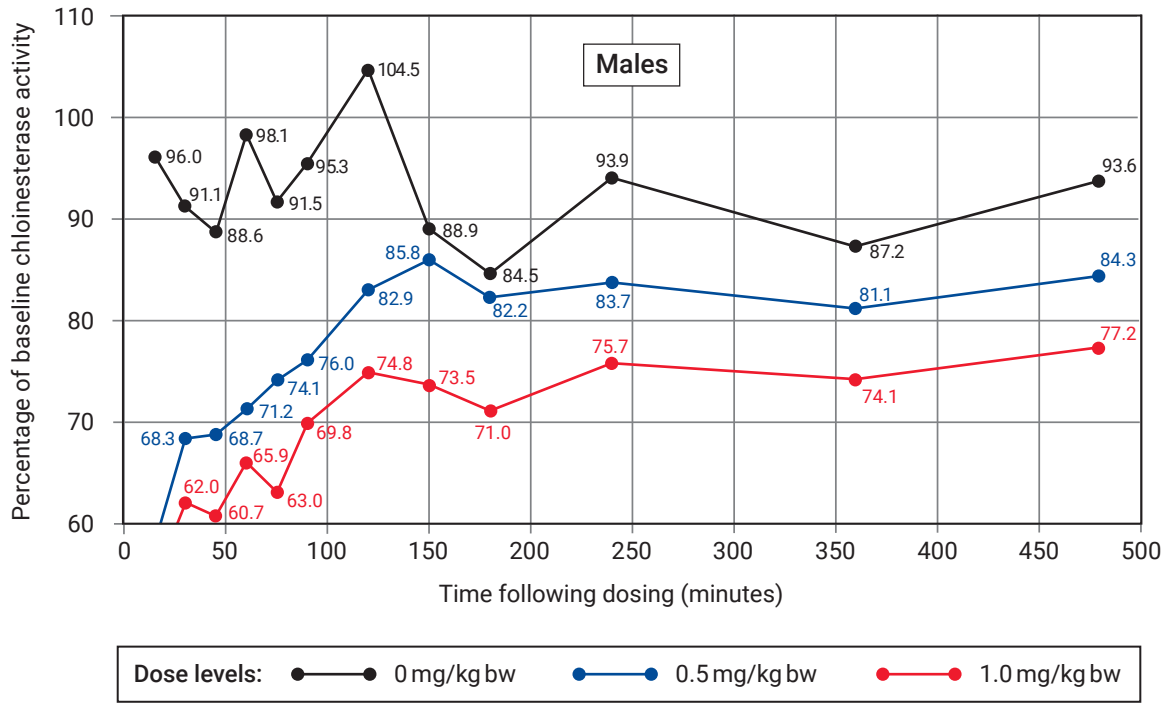
### (d) Kinetics of peripheral blood cholinesterase inhibition in adult rats

This study had previously been evaluated by JMPR in 2002. In a preliminary dose-ranging study CD rats (three of each sex per dose) were orally (gavage) dosed with 0, 0.5, 1.0, 2.0 or 4.0 mg/kg bw of carbofuran (purity 98%). Peripheral blood samples were collected before dosing and then at one hour following dosing. Plasma and erythrocyte AChE activity was measured in a preliminary dose-ranging study. In the definitive experiment, jugular-cannulated CD rats (up to eight of each sex per dose; some samples missing due to cannula obstruction) were orally (gavage) dosed with 0, 0.5 and 1.0 mg/kg bw. Blood samples were then collected over an eight-hour post dose period. Time to maximal inhibition and time to full recovery of plasma and erythrocyte AChE activity was measured.

In the preliminary study, clinical signs consistent with cholinesterase toxicity (tremor, excessive salivation, hindlimb splay, gasping respiration) occurred at 2.0 mg/kg bw and above within 15 minutes of dosing. Three animals in the high-dose groups were killed for animal welfare reasons at 30 minutes following dosing. At one hour following dosing erythrocyte AChE levels were significantly reduced ($p < 0.05$) in both males and females at 1.0 mg/kg bw and above, and in males only at 0.5 mg/kg bw. Significant plasma cholinesterase inhibition ($p < 0.05$) only occurred in males dosed at 2 mg/kg bw. Based on these findings, doses of 0, 0.5 and 1.0 mg/kg bw were selected for the definitive study.

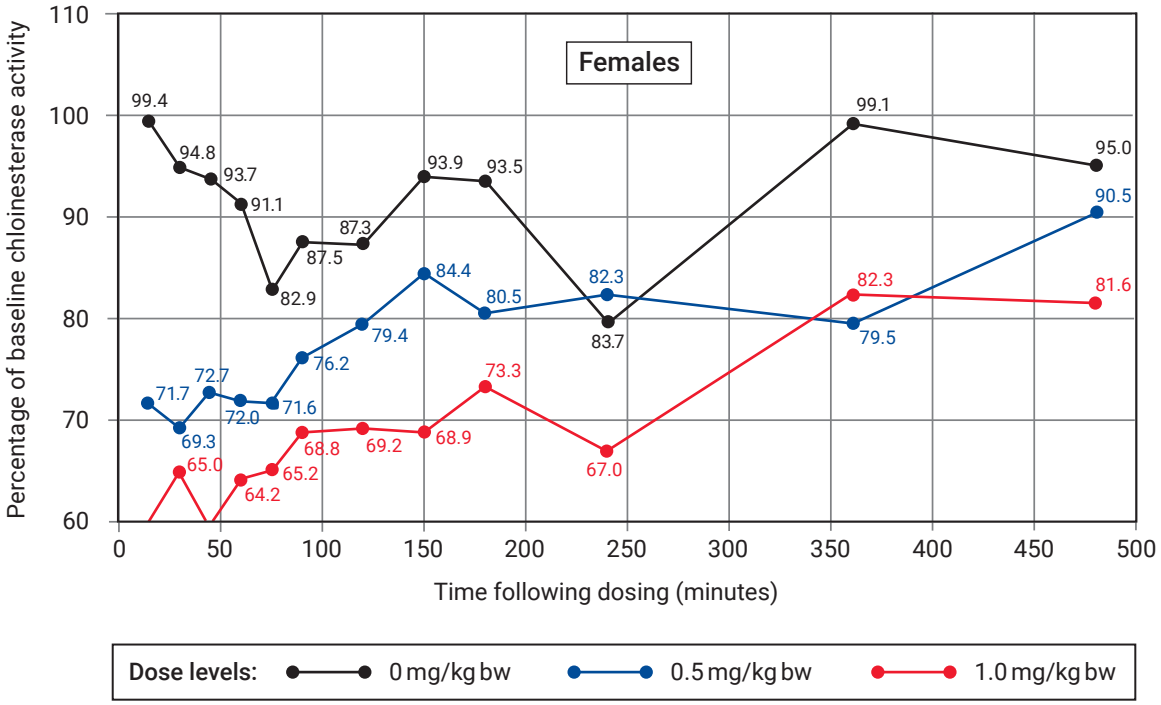
In the definitive study, significant inhibition of erythrocyte cholinesterase ($p < 0.05$) occurred at 0.5 mg/kg bw and above. Based on the percentage of baseline erythrocyte AChE activity as the key metric (used to account for the inherent variability seen in the control values) the time after dosing at 0.5 mg/kg bw to recover to under 20% inhibition was 120 minutes or less in males (Fig. 10a) and 150 minutes or less in females (Fig. 10b). Following dosing at 1.0 mg/kg bw this 20% recovery time was more than 480 minutes or more in males and 360 minutes or more in females. Plasma AChE was not significantly inhibited at either dose level. Consistent with the JMPR evaluation in 2002, no NOAEL was identified (Anderson, 2000).

***Figure 10a. Kinetics of peripheral erythrocyte cholinesterase inhibition in male rats***




(Redrawn from Anderson, 2000)

*Figure 10b. Kinetics of peripheral erythrocyte cholinesterase inhibition in female rats*




(Redrawn from Anderson, 2000)

**(e) Acute carbofuran-induced cholinesterase inhibition at different life stages**

The current evaluation supports the previous benchmark dose analysis of data on acute carbofuran-induced brain AChE inhibition in postnatal day 11 rats (Hoberman, 2007c; Moser, McDaniel & Philips, 2007a; Tyl et al., 2005c) conducted by JMPR in 2008. The $BMD_{10}$ for this effect was 0.04 mg/kg bw and the $BMDL_{10}$ was 0.03 mg/kg bw.

***Study of Tyl, Marr & Myers (2005a), using postnatal day 11 rats***

In a dose-ranging study, carbofuran (purity 99%) was administered to postnatal day 11 SD rats (three of each sex per dose) as a single, oral gavage dose at 0.3, 0.6 or 1.0 mg/kg bw. The pups were examined for clinical signs of toxicity at approximately 15, 30, 60, 90, 120, 240 and 360 minutes post dosing. At 360 minutes post dosing (or within l0 minutes of the time point), each pup was weighed, examined and euthanized. Blood and brain were collected for measurement of AChE activity. A substantial limitation of this study was that no negative control group was evaluated. The timing of sample collection for AChE measurements was also after the time of peak inhibition post dose.

There was no mortality or moribundity in any pup of either sex at any dose or time point up to termination at six hours post dosing. Clinical signs (fine tremor, whole body tremor, large head tremors) consistent with cholinergic toxicity occurred at all dose levels with maximum severity and incidence at 10–19 minutes following dosing. These clinical signs were not present at 60 minutes post dosing. The severity, incidence and duration of clinical signs were dose-dependent, with no evidence of sex differences within dose groups. Erythrocyte AChE activity in both sexes and brain AChE activity in female pups at termination (six hours post dose) were unaffected at 0.6 and 1.0 mg/kg bw using the 0.3 mg/kg bw group as the reference dose in place of the missing 0 mg/kg bw controls. Male pup brain AChE activity at termination was significantly reduced ($p < 0.05$) at doses of 0.6 and 1.0 mg/kg bw, to 91.2% and 70.7% respectively of the values at 0.3 mg/kg bw. Due to the limitations of the study design a NOAEL was not determined.

*Table 14. Mean body weight gain in the sub-chronic neurotoxicity study in rats*

| | Dose (mg/kg bw per day) | | | | | |
|---|---|---|---|---|---|---|
| | Males | | | Females | | |
| **Finding** | **0.3** | **0.6** | **1.0** | **0.3** | **0.6** | **1.0** |
| Body weight at dosing; mean ± SD (g) | 27.34 ±1.55 | 28.44 ±2.24 | 25.05 ±1.21 | 24.70 ±0.69 | 28.80 ±2.02 | 25.40 ±1.18 |
| Body weight at termination; mean ± SD (g) | 29.03 ±2.00 | 28.67 ±2.61 | 24.72 ±1.61 | 25.06 ±0.53 | 29.32 ±1.99 | 24.88 ±1.03 |
| Brain weight; mean ± SD (g) | 1.0516 ±0.0254 | 1.0285 ±0.0441 | 0.9526 ±0.0730 | 0.8726 ±0.0365 | 0.9629 ±0.454 | 0.9589 ±0.0256 |
| **Incidence of tremors:** | | | | | | |
| Number of pups affected | 3 | 2 | 3 | 3 | 2 | 3 |
| Time point pup 1 | 8, 18 | 9 | 8, 31, 54 | 15 | 7.20 | 10 |
| effect (minutes) pup 2 | 2, 15 | 8, 30 | 13, 14, 19 | 21 | 15 | 8, 12, 35, 60 |
| was observed pup 3 | 42 | - | 2, 7, 10, 14, 16 | 8 | - | 13, 19 |
| Erythrocyte AChE activity; mean ± SD (U/L) | 3307 ±202 | 3353 ±217 | 3400 ±171 | 2870 ±17 | 3393 ±267 | 3020 ±40 |
| Brain AChE activity; mean ± SD (U/g) | 7.74 ±0.03 | 7.06 ±0.17** | 5.47 ±0.09*** | 7.01 ±0.79 | 7.45 ±0.28 | 6.37 ±0.14 |

AChE: Acetylcholinesterase; SD: Standard deviation; Source: Tyl, Marr & Myers, 2005a

Significantly different from control group at: ** $p < 0.01$; *** $p < 0.001$

***Study of Hoberman , (2007a)***

Groups of three male and three female Crl:CD(SD) rats were given a single oral gavage dose of carbofuran (purity 98.9%) in corn oil by oral gavage at doses of 0, 0.03, 0.1 or 0.3 mg/kg bw on PND 11. Clinical observations were recorded prior to dosage, 1–9 minutes post dose and/or 15 minutes post dose. Body weights were recorded once during the predose period and on the day of dosage. Following administration, whole blood samples were collected ca 15 minutes post dosage via decapitation. Blood samples were processed for erythrocyte AChE levels within 15 minutes of blood collection and analysis began within one hour of collection. Immediately after blood sample collection the brain was excised, weighed, and assayed for AChE activity.

There were no deaths. Two of the five males and two of the five females in the 0.3 mg/kg bw dose group had slight to moderate whole-body tremors after dosing, just prior to sacrifice. All other pups survived dosing with no adverse clinical observations. Body weights between groups were comparable for both the male and female pups. No statistically significant reductions in erythrocyte AChE values occurred in either male or female pups.

*Table 15. Summary of pup erythrocyte and brain cholinesterase results*

| | Dose (mg/kg bw per day) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Parameter** | **0** | **0.03** | **0.1** | **0.3** | **0** | **0.03** | **0.1** | **0.3** |
| Mean brain AChe activity (U/g) | 7.173 | 6.487 | 1.758* | 2.841* | 7.066 | 6.303 | 4.620* | 3.784* |
| Percentage of control value | 100 | 90 | 66 | 40 | 100 | 89 | 65 | 53.5 |

* Significantly different from control group at $p < 0.05$; AChE: Acetylcholinesterase; Source: Hoberman, 2007a

Consistent with JMPR 2008, the NOAEL was 0.03 mg/kg bw based on inhibition of brain AChE activity by more than 20% at the next higher dose of 0.1 mg/kg bw. Critically, in this study the use of inhibition of erythrocyte AChE is insufficiently sensitive (that is, no inhibition was detected at any dose level in either sex) for it to be used as a health protective surrogate measure of carbofuran-induced inhibition of brain AChE.

***Study of Tyl, Marr & Myers (2005b)***

In an acute time-course study, groups of adult and PND 11 pup Crl:CD(SD) rats (35 per sex) were administered by oral gavage 0.6 mg/kg bw of carbofuran (purity 99%). Clinical observations were recorded before dosing and at ca 15, 30, 60, 90, 120, 240 and 360 minutes after dosing. At termination, a blood sample and the brain were collected from five each of the male and female pups, and male and female adults for each of the time points 15, 30, 60, 90, 120, 240 and 360 minutes after dosing. Also collected from the same number of pups and adults were samples from the untreated control groups at times 0 and 360 minutes after dosing. Based on the JMPR 2008 evaluation, the erythrocyte AChE measurements in this study were regarded as unreliable.

There was no mortality or moribundity in any pup or adult of either sex at 0.6 mg/kg bw at any time-point up to termination six hours after dosing. In the pups at PND 11, treatment-related clinical signs (whole-body tremors) were observed initially at two minutes after dosing. Few or no treatment-related clinical observations (tremors) were recorded after 28 minutes unless a rat was handled, which triggered tremors that persisted in pups for up to 119 minutes after dosing. The clinical observations were limited to fine tremors initially, then whole-body tremors, and then large head tremors. In adults, the earliest reports of tremors (face) were at 6–7 minutes after dosing. The incidences and severities were highest at 15 minutes and no tremors were observed 60 minutes after dosing. Brain AChE activity in male and female pups aged 11 days was inhibited (statistically significant) at all time points examined, with maximum inhibition at 15 minutes after dosing. Full recovery of brain AChE activity was not achieved in PND 11 pups by six hours after dosing, with a single oral dose of carbofuran at 0.6 mg/kg bw. In adult rats, brain AChE activity was significantly reduced at most time points and the time of maximum inhibition was at 15 minutes after dosing. Recovery of brain AChE activity was achieved in males by 360 minutes after dosing and in females by 240 minutes after dosing. Based on these results, the time of maximum inhibition of brain AChE activity was determined to be 15 minutes after dosing, in pups and adults.

***Table 16. Brain acetylcholinesterase activity in adult rats and pups after dosing with carbofuran***

| Mean brain AChE activity (U/g) | | Time of sacrifice (minutes after dosing) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **0** | 15 | 30 | 60 | 90 | 120 | 240 | 360 |
| Pups: | males | 7.33 | 3.50* | 4.02* | 4.29* | 5.31* | 4.62* | 5.54* | 6.18* |
| | females | 8.38 | 3.43* | 4.45* | 4.13* | 4.71* | 4.99* | 6.87* | 6.36* |
| Adults: | males | 12.98 | 8.04* | 8.47* | 11.28* | 10.74* | 11.68* | 11.78* | 12.00 |
| | females | 13.06 | 8.67 | 9.18 | 11.38 | 11.18 | 12.12 | 13.04 | 12.24 |

AChE: Acetylcholinesterase; Source: Tyl, Marr & Myers, 2005b

* Significantly different from control group at $p < 0.05$

***Study of Hoberman (2007b)***

In an acute time-course study groups of adult and PND 11 pup Crl:CD(SD) rats were assigned to two dosage groups, 60 rats of each sex per group, for both the adults and the pups. The test substance carbofuran (purity 98.8%) or vehicle (corn oil) were administered via oral gavage once on day 1 of the study at doses of 0 or 0.1 mg/kg bw. The groups of 60 rats were assigned to one of six time points (time between dose administration and sacrifice): 15 and 30 minutes, one, two, four and six hours, with 10 rats of each sex per time point. The adults and pups were examined for routine clinical observations within 15 minutes prior to dosage, and then at the time points specified above following dose administration. Following euthanasia, Blood and brain samples were processed and analyzed for AChE levels within two hours of collection.

The time of peak effect for brain AChE levels was 30 minutes after dose administration for adult males and females. Although erythrocyte AChE levels were increased at 15 minutes, for adult males the peak effect for erythrocyte AChE was 30 minutes after dosing. For pups the time of peak effect for erythrocyte AChE was between 15 minutes and one hour after dosing. The time of maximum AChE inhibition in pup brains was one hour after administration, although in females the values for time points 30 minutes and one hour are nearly the same.

*Table 17. Mean brain and red blood cell AChE activity in adult rats and pups after dosing with carbofuran*

| Group | | Time point (hours after dosing) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | **0** | 15 | 30 | 60 | 120 | 240 | 360 |
| | | **Adults (percentage of control value)** | | | | | | |
| Males: | erythrocyte | 100 | 88.0 | 78.3* | 99.7 | 114.6 | 81.9* | 89.2 |
| | brain | 100 | 83.4* | 79.1* | 83.8* | 88.4* | 91.4* | 95.1 |
| Females: | erythrocyte | 100 | 83.4 | 102.1 | 89.9 | 88.9 | 88.2 | 114.3 |
| | brain | 100 | 92.1 | 82.2 | 97.3 | 90.7* | 94.5* | 97.8 |
| | | **Pups (percentage of control value)** | | | | | | |
| Males: | erythrocyte | 100 | 70.7* | 81.7 | 79.1* | 91.0 | 103.2 | 123.2 |
| | brain | 100 | 79.7* | 78.7* | 73.1* | 99.3 | 101.3 | 98.5 |
| Females: | erythrocyte | 100 | 83.9 | 92.2 | 85.1 | 105.2 | 135.4 | 102.5 |
| | brain | 100 | 82.5* | 67.7* | 67.1* | 82.5* | 100.4 | 98.5 |

AChE: Acetylcholinesterase; Source: Hoberman, 2007b

* Significantly different from control group at $p < 0.05$

***Study of Tyl, Marr & Myers (2005c)***

In a dose response study, groups of PND 11 CD rat pups and adult rats (10 of each sex) were given a single dose of carbofuran (purity 99%) at 0, 0.3, 0.6 or 1.0 mg/kg bw by gavage and terminated ca 15 minutes or ca 720 minutes after dosing. Pups and adults were examined for clinical signs of toxicity at 0, 15, 30, 60, 120, 240 and 720 minutes after dosing. At 15 minutes and 720 minutes after dosing, groups of 10 male and 10 female pups per time-point, and the same sampling of adults, were taken for blood collection and brain excision. Based on the JMPR 2008 evaluation, the erythrocyte AChE measurements in this study were regarded as unreliable.

In pups, clinical signs were initially observed 5–6 minutes after dosing and by six minutes after dosing there was a clear dose–response relationship for incidence of tremors. The maximum incidence and severity ranged from nine minutes to 15–30 minutes after dosing. Treatment-related clinical signs were present at 1.0 mg/kg bw 120 minutes after dosing. For adults, clinical signs were observed initially 6–15 minutes after dosing. The highest incidence and severity of tremors ranged from 15–30 minutes after dosing. Tremors were present at 60 minutes after dosing at doses of 0.3, 0.6, and 1.0 mg/kg bw.

The LOAEL for both pups and adults was 0.3 mg/kg bw, the lowest dose tested, based on a greater than 20% inhibition of brain AChE activity at 15 minutes post dose. In both pups and adults, the effects on brain AChE were essentially completely reversed by 720 minutes following dosing.

*Table 18. Mean body weight gain in the subchronic neurotoxicity study in rats*

| Finding | | Dose (mg/kg bw per day) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | Females | | | |
| | | **0** | **0.3** | **0.6** | **1.0** | **0** | **0.3** | **0.6** | **1.0** |
| | | **Sacrifice at 15 minutes after dosing** | | | | | | | |
| **Pups** | | | | | | | | | |
| Number of pups with clinical signs | | 0 | 10 | 10 | 10 | 0 | 10 | 10 | 10 |
| Mean brain AChE activity | (U/g) | 6.62 | 3.47* | 3.13* | 2.53* | 6.90 | 3.56* | 3.27* | 2.42* |
| | (% control value) | 100 | 52 | 47 | 38 | 100 | 52 | 47 | 35 |
| **Adults** | | | | | | | | | |
| Number of pups with clinical signs | | 0 | 0 | 5 | 9 | 0 | 0 | 3 | 9 |
| Mean brain AChE activity | (U/g) | 12.46 | 9.22* | 8.44* | 7.01* | 12.88 | 9.84* | 8.54* | 7.16* |
| | (% control value) | 100 | 74 | 68 | 56 | 100 | 76 | 66 | 56 |

| | Dose (mg/kg bw per day) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Finding** | **0** | **0.3** | **0.6** | **1.0** | **0** | **0.3** | **0.6** | **1.0** |
| | **Sacrifice at 720 minutes after dosing** | | | | | | | |
| **Pups** | | | | | | | | |
| Number of pups with clinical signs | 0 | 10 | 10 | 10 | 0 | 10 | 8[a] | 10 |
| Mean brain AChE activity (U/g) | 7.03 | 6.49 | 6.55 | 6.53 | 7.17 | 6.87 | 7.10 | 7.20 |
| (% control value) | 100 | 92 | 93 | 93 | 100 | 96 | 99 | 100 |
| **Adults** | | | | | | | | |
| Number of pups with clinical signs | 3[b] | 5 | 8 | 10 | 0 | 5 | 6 | 10 |
| Mean brain AChE activity (U/g) | 13.00 | 12.29 | 12.41 | 12.18 | 13.29 | 12.74 | 12.62 | 12.55 |
| (% control value) | 100 | 95 | 95 | 94 | 100 | 96 | 95 | 94 |

[a] One pup was removed owing to a dosing error; AChE: Acetylcholinesterase; Source: Tyl, Marr & Myers, 2005c

[b] Piloerection, rough coat; * Significantly different from control group at $p < 0.05$

***Study of Hoberman (2007c)***

In a dose response study PND 11 and adult Crl:CD(SD) rats were orally (gavage) administered carbofuran (purity 98.8%) at 0, 0.03, 0.1 or 0.3 mg/kg bw. Ten adults and ten pups of each sex per dose were assigned to blood and brain collection and sacrificed at 30 minutes post dose or four hours post dose. Adults were examined for clinical observations before, and ca 15 minutes after dose administration, and prior to sacrifice, at either 30 minutes or four hours post dose. Pups were subject to clinical observations prior to and at 15 and 30 minutes, one and two hours after dose administration. Body weights were recorded on the day of dose administration. Motor activity was evaluated 30 minutes after dose administration for the adult rats, but only those assigned to the four-hour sacrifice. Following sacrifice, blood and brain samples were processed and analyzed for AChE levels within 2.5 hours of collection.

There were no mortalities or test substance-related clinical observations. There were no effects on body weight, brain weight, or motor activity for either adults or pups.

Based on the results of this study, adult erythrocyte AChE levels were reduced in a dosage-dependent pattern at 30 minutes post dose, and returned to control levels by four hours post dose. Erythrocyte AChE measurement was less sensitive in pups than adults and did not show significant differences from control values ($p < 0.05$) at 30 minutes or four hours post dose at any dose level. Pup and adult brain AChE values were reduced in a dosage-dependent pattern at 30 minutes and were essentially recovered by four hours post dose, except for male and female pups in the 0.1 and 0.3 mg/kg bw dose groups. Statistically significant brain AChE inhibition in the 0.03 mg/kg bw group pups recovered to control levels by four hours after dose administration. The NOAEL for adults was 0.03 mg/kg bw due to greater than 20% inhibition of erythrocyte and brain AChE activity at 0.1 mg/kg bw at 30 minutes after dosing. The NOAEL for pups was 0.03 mg/kg bw due to greater than 20% inhibition of brain AChE activity at 0.1 mg/kg bw at 30 minutes following dosing.

***Table 19. Cholinesterase activity in carbofuran-treated adult rats and pups 30 and 240 minutes after dosing***

| | Mean red blood cell cholinesterase values (units/mL) | | | | | |
|---|---|---|---|---|---|---|
| **Dose (mg/kg bw per day)** | **Number** | **At 30 minutes** | **Percentage of control** | **Number** | **At 240 minutes** | **Percentage of control** |
| | | | **Male pups** | | | |
| 0 | 7 | 0.826 | NA | 9 | 0.518 | NA |
| 0.03 | 6 | 0.889 | 107.6 | 10 | 0.468 | 90.3 |
| 0.1 | 8 | 0.708 | 85.7 | 8 | 0.655 | 126.3 |
| 0.3 | 9 | 0.617 | 74.8 | 9 | 0.532 | 102.6 |

| Mean red blood cell cholinesterase values (units/mL) | | | | | | |
|---|---|---|---|---|---|---|
| Dose (mg/kg bw per day) | Number | At 30 minutes | Percentage of control | Number | At 240 minutes | Percentage of control |
| | | | Female pups | | | |
| 0 | 8 | 0.965 | NA | 8 | 0.612 | NA |
| 0.03 | 9 | 0.808 | 83.8 | 9 | 0.601 | 98.1 |
| 0.1 | 8 | 0.609 | 63.1 | 9 | 0.589 | 96.2 |
| 0.3 | 9 | 0.585 | 60.6 | 9 | 0.515 | 84.1 |
| | | | Male adults | | | |
| 0 | 10 | 1.149 | NA | 10 | 1.034 | NA |
| 0.03 | 10 | 1.066 | 92.8 | 10 | 1.079 | 104.4 |
| 0.1 | 10 | 0.815* | 71.0 | 10 | 1.167 | 112.9 |
| 0.3 | 10 | 0.707* | 61.6 | 10 | 1.126 | 108.9 |
| | | | Female adults | | | |
| 0 | 10 | 1.077 | NA | 10 | 1.162 | NA |
| 0.03 | 10 | 0906* | 84.2 | 10 | 1.190 | 102.4 |
| 0.1 | 10 | 0.854* | 79.4 | 10 | 1.151 | 99.0 |
| 0.3 | 10 | 0.664* | 61.6 | 10 | 1.114 | 95.9 |

| Mean brain cholinesterase values (units/mL) | | | | | | |
|---|---|---|---|---|---|---|
| Dose (mg/kg bw per day) | Number | At 30 minutes | Percentage of control | Number | At 240 minutes | Percentage of control |
| | | | Male pups | | | |
| 0 | 9 | 5.996 | NA | 10 | 6.226 | NA |
| 0.03 | 10 | 5.192* | 86.6 | 10 | 5.681 | 91.2 |
| 0.1 | 10 | 3.881* | 64.7 | 10 | 5.035* | 80.9 |
| 0.3 | 10 | 2.771* | 46.2 | 10 | 2.935* | 47.1 |
| | | | Female pups | | | |
| 0 | 10 | 6.257 | NA | 10 | 6.374 | NA |
| 0.03 | 10 | 5.020* | 80.2 | 10 | 6.049 | 94.9 |
| 0.1 | 10 | 3.322* | 53.1 | 10 | 4.957* | 77.8 |
| 0.3 | 10 | 2.668* | 42.6 | 10 | 3.385* | 53.1 |
| | | | Male adults | | | |
| 0 | 10 | 14.835 | NA | 10 | 15.679 | NA |
| 0.03 | 10 | 13.049* | 88.0 | 10 | 14.577 | 93.0 |
| 0.1 | 10 | 10.039* | 67.7 | 10 | 13.592* | 86.7 |
| 0.3 | 10 | 7.504* | 50.6 | 10 | 13.137* | 83.8 |
| | | | Female adults | | | |
| 0 | 9 | 15.090 | NA | 10 | 15.908 | NA |
| 0.03 | 10 | 15.124 | 100.2 | 10 | 15.151 | 95.2 |
| 0.1 | 10 | 12.050* | 79.9 | 10 | 14.897 | 93.6 |
| 0.3 | 9 | 8.985* | 59.5 | 10 | 14.538 | 91.4 |

NA: Not applicable;

Source: Hoberman, 2007c

* Significantly different from control group at $p < 0.05$

***Study of Moser, McDaniel & Philips, (2007b)***

The purpose of this nonGLP study was to compare the sensitivity of inhibition of AChE activity in juvenile and adult rats. Groups of 10 male pups (PND 17) and groups of 10 male adult rats were given a single dose, in corn oil by gavage, of carbofuran (purity 99%) at 0, 0.1, 0.3, 0.6 or 1.0 mg/kg bw for the pups, or 0, 0.1, 0.3, 0.5, 0.75 or 1.5 mg/kg bw for the adults (doses derived from data collected from a separate study carried out under similar conditions). At 15 minutes after dosing motor activity was assessed, and at approximately 40 minutes after dosing, upon termination, blood was collected and brains excised. No statistical analyses were performed.

There were no unexpected deaths or cases of severe toxicity, however no further details on clinical signs attributed to treatment were reported. For the pups, brain and erythrocyte AChE activity was significantly decreased at all doses, while motor activity was decreased only at doses of 0.3 mg/kg bw and above. Erythrocyte AChE activity was inhibited to a greater degree than brain AChE activity. When compared with adult rats, pups aged 17 days showed greater inhibition of AChE activity, even at the lowest dose tested. The effect was slightly greater in erythrocytes than in the brain. There was no age-related difference in the inhibition of motor activity by carbofuran at lower doses. Pearson correlation coefficients of within-subject data were higher for brain AChE and motor activity when compared with erythrocyte AChE activity. This result was in general agreement with the overall conclusion of a comparison of acute neurobehavioural (motor activity) and AChE inhibitory effects conducted for a range of *N*-methyl carbamate pesticides (that includes carbofuran) in rats. The LOAEL was 0.1 mg/kg bw, the lowest dose tested, on the basis of a greater than 20% inhibition of brain AChE at this dose.

***Table 20. Inhibition of AChE activity and motor activity in 17-day-old rat pups***

| | Dose (mg/kg bw per day) | | | | |
|---|---|---|---|---|---|
| **Finding** | **0** | 0.1 | 0.3 | 0.6 | 1.0 |
| Erythrocyte AChE activity (mmol/min/mL)<br>[% of control] | 0.886<br>[100] | 0.446<br>[50] | 0.243<br>[27] | 0.171<br>[19] | 0.153<br>[17] |
| Brain AChE activity (mmol/min/mL)<br>[% of control] | 5.096<br>[100] | 3.686<br>[72] | 2.626<br>[52] | 2.311<br>[45] | 1.812<br>[36] |
| Motor activity (counts/20 minutes)<br>[% of control] | 80.5<br>[100] | 93.7<br>[116] | 47.8<br>[59] | 28.6<br>[36] | 3.7<br>[5] |

AChE: Acetylcholinesterase; Source: Moser, McDaniel & Philips, 2007b

**(f) Screening studies for endocrine effects**

Carbofuran is unlikely to act cause toxicity via effects on testosterone/estradiol synthesis, androgen/estrogen receptor binding, aromatase, androgen/estrogen effects on end-organs and tissues, or on the thyroid at levels of exposure that are likely to occur in the human diet. Relevant studies evaluated are summarized below in Table 21.

*Table 21. Summary of in vitro and in vivo studies on the endocrine effects of carbofuran*

| Study type, Reference | Methods | Results |
|---|---|---|
| | **In vitro** | |
| Effects of carbofuran on metabolism of testosterone and estradiol in vitro<br>Usmani, Hodgson & Rose, 2004. | See in vitro metabolism section above | Co-incubations testosterone concentrations (4–100 µM) with carbofuran (0–1600 µM) in pooled human liver microsomes resulted in increased 6β-hydroxytestosterone. The greatest level of activation (ca 30-fold) was observed at the lowest testosterone concentration (4 µM) in combination with a 1600 µM carbofuran.<br>With CYP3A4 preparations the activation of testosterone metabolism by carbofuran followed a similar pattern, but levels of activation were lower than with human liver microsomes. Greatest activation (ca 9.6-fold) was observed at the lowest testosterone concentration (4 µM) in combination with 1600 µM carbofuran. In contrast to the observed activation of testosterone metabolism by carbofuran, co-incubations of various concentrations of carbofuran with increasing concentrations of testosterone with pooled human liver microsomes and with CYP3A4 did not result in increased metabolism of carbofuran to 3-hydroxy-carbofuran, except at the highest concentrations of testosterone. Similarly, no activation or inhibition of varying concentrations of estradiol on increasing concentrations of carbofuran, or varying concentrations of carbofuran on increasing concentrations of estradiol, were observed.<br>Critically, the effects of carbofuran on testosterone metabolism occurred in vitro at relatively extreme concentrations that are very unlikely occur due to consumption of carbofuran and its residues in the diet. |
| Androgen receptor binding (rat prostate cytosol)<br>Willoughby, 2011a | Rat prostate-derived androgen receptor competitive binding assay. Based on a preliminary investigation of precipitation the final carbofuran concentrations assessed were $10^{-10}$, $10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$, $10^{-5}$, $10^{-4}$ and $10^{-3}$ M in three valid independent runs, each in triplicate. The positive control and the weak positive control (dexamethasone) were run for all assays. Solvent control tubes (six replicates) were also prepared to assess total binding. The duration of incubation at approximately 40°C was 16–20 hours. | In all three valid independent runs the mean specific binding for carbofuran was greater than 75% at every soluble concentration tested, leading to classification as a "non-binder" for each run. As carbofuran was classified as a "non-binder" for all three runs a mean relative binding affinity could not be calculated. Carbofuran was classified as a "non-binder" with respect to the androgen receptor. |

| Study type, Reference | Methods | Results |
|---|---|---|
| Estrogen receptor binding (rat uterine cytosol)<br>Willoughby, 2011b | The assay measures the conversion of androgen to estrogen in microsomes isolated from various target tissues or cell lines containing aromatase, or recombinant aromatase respectively, and cytochrome P450 reductase. Radioactive substrate ($^3$H-androstenedione) and NADPH are added to microsomes containing the aromatase (CYP19) and reductase complex. Tritiated water is released during the conversion of androstenedione to estrone and this can be quantified as a direct measurement of aromatase activity per unit reaction time. In this study human CYP19 (aromatase) and P450 reductase Supersomes™ were used; 4-hydroxyandrostenedione (4-OH-ASDN) was used as the positive control. Based on a preliminary investigation of precipitation, the final carbofuran concentrations assessed were $10^{-10}$, $10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$, $10^{-5}$, $10^{-4}$ and $10^{-3}$ M in three valid independent runs. All concentrations were tested in triplicate. | Increasing concentrations of carbofuran produced a negligible decrease in aromatase activity. The mean aromatase activity of the three runs was 99.0% (± 9.2% standard deviation) of control activity at the highest test concentration of $10^{-3}$ M. Carbofuran was therefore classified as a noninhibitor of aromatase. |
| Estrogen receptor transcriptional activation (human cell line, HeLa-9903)<br>Willoughby, 2011c | The assay is used to indicate binding of the estrogen receptor with a ligand. Following ligand binding, the receptor–ligand complex translocates to the nucleus where it binds specific DNA response elements and transactivates a firefly luciferase reporter gene, resulting in increased cellular expression of luciferase enzyme and eventually to a bioluminescent product that can be measured quantitatively with a luminometer. Data interpretation for this assay is based upon whether the maximum response level induced by a test chemical equals or exceeds an agonist response equal to 10% of that induced by a maximally inducing (1 nM) concentration of the positive control 17β-estradiol (that is, the $PC_{10}$).<br>Based on a preliminary assessment of precipitation, the final carbofuran concentrations assessed were $10^{-10}$, $10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$, $10^{-5}$, $10^{-4}$ and $10^{-3}$ M in two valid independent runs each of six replicates. In addition, for each concentration, two replicates per plate were prepared that incorporated the hERα antagonist ICI 182,780 to allow for the identification of non-specific (non-hERα-mediated) induction of the luciferase gene. The duration of exposure was 24 hours. Complete concentration response curves for each of four reference compounds (17β-estradiol, 17α-estradiol, corticosterone and 17α-methyltestosterone) were run each time the transcriptional activation assay was performed | Cytotoxicity (a 20% or greater reduction in cell viability) was observed at $10^{-4}$ M in both valid runs and therefore data were not evaluated at this concentration.<br>In two independent runs of the transcriptional activation assay, carbofuran did not result in an increase in luciferase activity relative to positive control (RPC) at any of the viable concentrations tested ($RPC_{max}$ less than 10%). The conclusion of the study was therefore that carbofuran was not an agonist of human hERα in the HeLa-9903 model system. |
| H295R steroido-genesis assay<br>Wagner, 2011 | The concentrations assessed were 0.000 1, 0.001, 0.01, 0.1, 1, 10 and 100 µM. Three valid independent runs of the steroidogenesis assay were conducted. Carbofuran, the reference substances and solvent controls were tested in triplicate. The duration of exposure was 48 ± 2 hours. Cell viability was assessed after this exposure time using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Testosterone and estradiol levels were measured using tandem high-performance liquid chromatography/mass spectrometry (HPLC-MS/MS). A statistically significant inhibition of testosterone production was observed at one concentration of carbofuran (100 µM) in one of the three runs, however inhibition was not observed at any other concentration or in the other two runs. Statistically significant induction of estradiol production was observed at one concentration of carbofuran (100 µM) in all three runs, with no clear dose–response relationship in any one of them. | It was concluded that carbofuran showed a slight (0.92-fold) statistically significant ( $p < 0.05$) inhibition of testosterone production at a concentration of 100 µM in one of three runs. A statistically significant ( $p < 0.05$; 1.45- to 1.55-fold) increase of estradiol was observed at 100 µM in all three assays.<br>Systemic exposure of the relevant components of the endocrine system to a carbofuran concentration of 100 µM is unlikely to occur in humans following dietary exposure to carbofuran and its residues. |

| Study type, Reference | Methods | Results |
|---|---|---|
| Hershberger assay of carbofuran administered orally to peripubertal orchido-epididy-mecto-mized rats<br>Herberth, 2011a | Carbofuran was administered orally by gavage once daily to three groups (Groups 4-6) of orchidoepididymectomized male Crl:CD(SD) rats at 0.03, 0.1, and 0.3 mg/kg bw per day. Testosterone propionate (TP) was administered at 0.2 mg/kg bw per day via subcutaneous injection to Group 2 (the androgenic comparison group). The test substance and TP were co-administered to Groups 7, 8, and 9 at dose levels of 0.03/0.2, 0.1/0.2, and 0.3/02 mg/kg bw per day (carbofuran/TP). Group 3 males received TP comparably to the Group 2 males, in conjunction with 3 mg/kg bw per day of flutamide (the anti-androgenic comparison group) by daily oral gavage. A concurrent control group (Group 1) received only the vehicle, orally by gavage. All groups were composed of six orchidoepididymectomized males. Rats were dosed for 10 consecutive days (study days 0–9), with the following exception. Three males in the vehicle control group (Group 1) received an additional dose on study day –1. For AChE determinations, 10 additional male rats in the vehicle control and 0.3 mg/kg bw per day groups (Groups 1 and 6, respectively) were administered the vehicle or test substance on the same regimen as the main study phase males. From this phase, five rats per group were euthanized by carbon dioxide inhalation approximately 45 minutes after dose administration on study days 0 and 9. Erythrocyte and brain homogenates were analyzed for AChE activity. | No evidence of androgen agonism or antagonism was noted at any dose level.<br>In the 0.3 mg/kg bw per day group (Group 6), erythrocyte AChE activity was reduced by 87.3% and 45.0% on study days 0 and 9, respectively. Brain AChE activity in this group was lowered by 40.4% and 32.1% compared to the vehicle control group values on study days 0 and 9, respectively.<br>Measured on study days 0 and 9, mean brain weights in the 0.3 mg/kg bw per day group (Group 6) were like those of the vehicle control group (Group 1). |
| Uterotrophic assay of carbofuran administered orally to ovari-ectomized rats<br>Herberth, 2011b | Carbofuran, was administered orally by gavage to three groups of six ovariectomized female Crl:CD(SD) rats, once daily for three consecutive days. Dosage levels were 0.03, 0.1 and 0.3 mg/kg bw per day. A positive control group composed of six ovariectomized females received the estrogenic positive control agent 17α-ethynylestradiol in corn oil via subcutaneous injection at a dosage level of 0.003 mg/kg bw per day. A concurrent vehicle control group composed of six ovariectomized females received the vehicle on a comparable regimen. For AChE evaluation 10 additional ovariectomized female Crl:CD(SD) rats in the vehicle control and 0.3 mg/kg bw per day groups were administered the vehicle or test substance on a regimen comparable to the main phase females. Five rats per group were euthanized by carbon dioxide inhalation approximately 45 minutes following dose administration on study days 0 and 2. Erythrocyte and brain homogenates were analyzed for AChE activity. | There were no carbofuran-associated effects on body weight, clinical findings, or macroscopic uterine findings. In the 0.3 mg/kg bw per day group on study days 0 and 2, erythrocyte AChE activity was reduced by 73.3% and 69.0% respectively, and whole brain AChE activity was reduced by 44.1% and 43.2% respectively. |

| Study type, Reference | Methods | Results |
|---|---|---|
| Pubertal development and thyroid function assay of carbofuran administered orally to intact juvenile/ peripubertal male rats<br>Herberth, 2012a | Carbofuran was administered orally by gavage once daily during PNDs 23–53 to three groups of 15 juvenile/peripubertal male Crl:CD(SD) rats, obtained from non-treated, time-mated females. Dose levels were 0.03, 0.1 and 0.3 mg/kg bw per day. A concurrent control group composed of 15 juvenile/peripubertal males received the vehicle on a comparable regimen. All males were observed daily (beginning on PND 30) for balanopreputial separation. A complete necropsy was conducted on all rats at scheduled euthanasia on PND 53. At termination selected organs were weighed and preserved. Hormone (T4, TSH, and testosterone) and clinical pathology evaluations (serum chemistry) were conducted on all animals on PND 53. In addition, histopathological evaluation of the thyroid, kidney, testis and epididymis was performed. For AChE determinations, 10 additional male rats in the vehicle control and 0.3 mg/kg bw per day groups were administered the vehicle or test substance on a regimen like the main phase males. From this phase, five rats per group were euthanized by carbon dioxide inhalation approximately 45 minutes following dose administration on PND 23 or 53. Erythrocyte and brain homogenates were analyzed for AChE activity. | No test substance-related effects were noted on organ weights, clinical pathology parameters, or serum thyroid hormone levels at dosage levels of 0.03, 0.1 or 0.3 mg/kg bw per day.<br>At the scheduled necropsies, no test substance-related macroscopic findings were noted at any dose level. In addition, no test substance-related differences in the incidence of microscopic changes were noted at any dose level when compared to the control group.<br>In the 0.3 mg/kg bw per day group, erythrocyte AChE activity was lower by 69.6% and 62.2% than the control group on PNDs 23 and 53 respectively. Whole brain AChE activity in this group was lower by 55.9% and 40.5% than in controls on PNDs 23 and 53 respectively.<br>On PNDs 23 and 53 mean brain weights for the 0.3 mg/kg bw per day group were like those of the control group. |
| Pubertal development and thyroid function assay of carbofuran administered orally to intact juvenile/ peripubertal female rats<br>Herberth, 2012b | Carbofuran was administered orally by gavage to three groups of 15 juvenile/peripubertal female Sprague Dawley rats, once daily on PNDs 22–42. Dose levels were 0.03, 0.1 and 0.3 mg/kg bw per day. A concurrent control group that received the vehicle on a comparable regimen. The study included an additional phase for AChE evaluation where 10 female rats in the control and highest dose groups were given the vehicle or carbofuran on a regimen comparable to the main phase females; five rats were euthanized ca 45 minutes after dosing on PND 22 or 42; blood samples and whole brains were collected and weighed, and erythrocyte and brain homogenates analyzed for AChE activity. All females were observed daily for vaginal opening. Once observed, daily vaginal lavages were performed on each female to determine the stage of estrous cycle. Complete necropsy was conducted on all rats found dead or reaching scheduled euthanasia on PND 42, when selected organs were weighed and preserved. Hormone (T4 and TSH) and clinical pathology evaluations (serum chemistry) were conducted on all surviving animals on PND 42. In addition, histopathological evaluation of the thyroid, kidney, ovary and uterus was performed. | Except for AChE inhibition there were no adverse findings. In the 0.3 mg/kg bw per day group, erythrocyte AChE activity was reduced by 59.7% and 59.2% on PNDs 22 and 42 respectively. Brain AChE activity was reduced by 43.8% and 29.2% on PNDs 22 and 42 respectively. |

T4: Thyroxine; TSH: Thyroid-stimulating hormone; PND: Postnatal day; AChE: Cholinesterase

## 3. Microbiological aspects

There was no information available in the public domain and no experimental data were submitted that addressed the possible impact of isotianil residues on the human intestinal microbiome.

## 4. Observations in humans

Regular and routine occupational medical surveillance of workers involved in the production of carbofuran have not demonstrated any adverse effects on clinical chemistry, lung function testing, electrocardiograms or audiometry. No cases of human poisoning have been reported or published in the peer-reviewed scientific literature.

# Comments

## Biochemical aspects

No new results from mass balance studies were supplied. Based on rat data previously evaluated by JMPR, carbofuran is rapidly absorbed and subsequently metabolized by hydroxylation, hydrolysis and conjugation. Radioactivity derived from carbofuran is mostly excreted in urine (up to 92% of the administered radioactivity, with most being eliminated within 24 hours following dosing). Based on the mass balance of radioactivity derived from carbofuran, oral absorption is up to 92%; carbofuran is unlikely to persist in the body. The major urinary metabolites found in rats following oral dosing were 3-hydroxy-carbofuran, 3-keto-7-phenol-carbofuran, 3-hydroxy-N-hydroxymethyl-carbofuran, 3-hydroxymethyl-carbofuran, and carbofuran-phenol. Based on in vitro data the predominant catalyst for the detoxification of carbofuran is CYP3A4 (Abass et al., 2023). The hepatic microsomal metabolism of carbofuran in humans, rabbits and mice was slower than that in rats and dogs when they were compared.

## Toxicological data

In male and female rats, the acute oral median lethal dose ($LD_{50}$) of pure carbofuran ranged from 5.2 to 14.9 mg/kg bw (Norvell, 1983a, b; Sabol, 1979a, b). The $LD_{50}$ in neonatal and weanling rats was 7.3–8.1 mg/kg bw (Rucci, Becci & Parent, 1980). Under occlusive conditions, the dermal $LD_{50}$ values for pure carbofuran in male and female rabbits were ca 2700 and ca 2000 mg/kg bw respectively when tested with abraded skin, whilst in males with intact skin the $LD_{50}$ was ca 4400 mg/kg bw (Mehta, 1998). No acute inhalation toxicity or dermal irritation studies were supplied. A single instillation of 5 mg of technical grade carbofuran per rabbit eye was minimally irritant and induced a systemic cholinergic toxicity (Palazzola, 1963a). Carbofuran was not a skin sensitizer in the Guinea pig and was not a skin irritant in the study submitted (Schoenig, 1967a).

In repeat-dose toxicity studies on mice, rats, rabbits and dogs, the main effect was the inhibition of AChE activity, clinical signs consistent with the AChE inhibition and decreased body weight.

In a 13-week dietary toxicity study in dogs carbofuran was administered at concentrations of 0, 10, 70 or 500/250 ppm (equal to 0, 0.43, 3.1 and 11 mg/kg bw per day for males and females combined). A no-observed-adverse-effect level (NOAEL) was not identified. The lowest-observed-adverse-effect level (LOAEL) was 10 ppm (equal to 0.43 mg/kg bw per day), the lowest dose tested, based on inhibition of erythrocyte AChE activity, excessive salivation and hyperaemia (Bloch et al., 2005b).

In a one-year dietary toxicity study in dogs (not GLP), carbofuran was administered at dietary concentrations of 0, 10, 20 or 500 ppm (equivalent to 0, 0.25, 0.5 and 12 mg/kg bw per day). The NOAEL was 20 ppm (equivalent to 0.5 mg/kg bw per day) based on inhibition of erythrocyte AChE activity at 500 ppm (equivalent to 12 mg/kg bw per day) (Taylor, 1983).

In a two-year combined carcinogenicity and toxicity study in mice (not GLP), carbofuran (purity 95.6%) was administered at dietary concentrations of 0, 20, 125 or 500 ppm (equal to 0, 2.8, 18 and 70 mg/kg bw per day). The NOAEL for toxicity was 20 ppm (equal to 2.8 mg/kg bw per day) based on inhibition of brain AChE activity at 125 ppm (equal to 18 mg/kg bw per day). The NOAEL for carcinogenicity was 500 ppm (equal to 70 mg/kg bw per day), the highest dose tested (Goldenthal, 1983).

In a two-year combined carcinogenicity and toxicity study in rats (not GLP), carbofuran (purity 95.6%) was administered at dietary concentrations of 0, 10, 20 or 100 ppm (equivalent to 0, 0.5, 1 and 5 mg/kg bw per day). The NOAEL for toxicity was 20 ppm (equivalent to 1 mg/kg bw per day), based on inhibition of brain and erythrocyte AChE activity at 100 ppm (equivalent to 5 mg/kg bw per day). The NOAEL for carcinogenicity was 100 ppm (equivalent to 5 mg/kg bw per day), the highest dose tested (Case & Wilson, 1979; Goldenthal, 1982a).

The Meeting concluded that carbofuran is not carcinogenic in mice or rats.

Carbofuran was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found with high (99.8%) purity carbofuran. Some lower purity carbofuran batches (purity 99% or less) were genotoxic (Farrow, 1983a, b, c; Haworth et al., 1983d, f, h; Kirby, 1983a, b). The US EPA has reported the presence of genotoxic impurities in carbofuran

(Gahli, 1997). No data on genotoxic impurities in carbofuran was supplied.

The Meeting concluded that high purity carbofuran (purity 99.8%) is unlikely to be genotoxic. However, some batches of lower purity technical grade carbofuran (purity less than 99.8%) were genotoxic.

In view of the lack of genotoxicity of high purity carbofuran (purity 99.8%), and the absence of carcinogenicity in mice and rats, the Meeting concluded that high purity carbofuran (purity 99.8%) is unlikely to be carcinogenic to humans.

In a three generation reproductive toxicity study in rats (not GLP), carbofuran was administered at dietary concentrations of 0, 20 or 100 ppm (equal to 0, 1.2 and 6 mg/kg bw per day for males, 0, 1.9 and 9.7 mg/kg bw per day for females). The NOAEL for reproductive toxicity was 100 ppm (equal to 6 mg/kg bw per day), the highest dose tested. The offspring NOAEL was 20 ppm (equal to 1.2 mg/kg bw per day), based on reduced growth and survival of pups at 100 ppm (equal to 6 mg/kg bw per day). The parental NOAEL was 100 ppm (equal to 6 mg/kg bw per day), the highest dose tested (Goldenthal, 1982b).

In a developmental toxicity study in rats (not GLP), carbofuran was administered by gavage at dose levels of 0, 0.1, 0.3 or 1.0 mg/kg bw per day. The maternal NOAEL was 0.1 mg/kg bw per day, based on increased incidence of cholinergic clinical signs at 0.3 mg/kg bw per day. The embryo/fetal NOAEL was 1 mg/kg bw per day, the highest dose tested (Rao, 1978a).

In a second developmental toxicity study in rats (not GLP), carbofuran was administered by gavage at dose levels of 0, 0.25, 0.50 or 1.20 mg/kg bw per day. The maternal and embryo/fetal NOAELs were 1.20 mg/kg bw per day, the highest dose tested (Rodwell, 1980).

In a third developmental toxicity study in rats (not GLP), carbofuran was administered at dietary concentrations of 0, 20, 60 or 160 ppm (equal to 0, 1.5, 4.4 and 11 mg/kg bw per day). The maternal NOAEL was 20 ppm (equal to 1.5 mg/kg bw per day), based on reduced body weight gain at 60 ppm (equal to 4.4 mg/kg bw per day). The embryo/fetal NOAEL was 60 ppm (equal to 4.4 mg/kg bw per day) based on reduced weight of pups at 160 ppm (equal to 11 mg/kg bw per day) (Rodwell, 1981).

In a developmental toxicity study in rabbits, carbofuran was administered by gavage at dose levels of 0, 0.2, 0.6 or 2 mg/kg bw per day. The maternal NOAEL was 0.6 mg/kg bw per day, based on clinical signs consistent with cholinergic toxicity at 2 mg/kg bw per day. The embryo/fetal NOAEL was 2 mg/kg bw per day, the highest dose tested (Rao, 1978b).

The Meeting concluded that carbofuran is not teratogenic.

In a 13-week neurotoxicity study in rats, carbofuran was administered at dietary concentrations of 0, 50, 500 or 1000 ppm (equal to 0, 2.4, 27.3 and 55.3 mg/kg bw per day for males, 0, 3.1, 35.3 and 64.4 mg/kg bw per day for females). No NOAELs were identified. The LOAEL for neurotoxicity was 50 ppm, (equal to 3.1 mg/kg bw per day) the lowest dose tested, based on increased landing foot splay in females. The LOAEL for systemic toxicity was 50 ppm (equal to 2.4 mg/kg bw per day) the lowest dose tested, based on reduced body weight gain (Freeman, 1994).

In a developmental neurotoxicity study in rats, carbofuran (purity 99.1%) was administered at dietary concentrations of 0, 20, 75 or 300 ppm (equal to 0, 1.7, 5.0 and 8.6 mg/kg bw per day). The maternal NOAEL was 20 ppm (equal to 1.7 mg/kg bw per day) based on adverse reduction in body weight parameters at 75 ppm (equal to 5.0 mg/kg bw per day). The developmental NOAEL was 20 ppm (equal to 1.7 mg/kg bw per day), based on adverse effects on pup body weight parameters, pup viability and weaning indices, and pup developmental delay at 75 ppm (equal to 5.0 mg/kg bw per day) (Ponnock, 1994).

To evaluate possible life stage-related differences in susceptibility to acute neurotoxicity, carbofuran was administered by gavage at dose levels of 0, 0.3, 0.6 or 1.0 mg/kg bw to rat pups on postnatal day (PND) 11 and to adult rats. The LOAEL was 0.3 mg/kg bw, the lowest dose tested, based on inhibition of brain AChE activity in pups and adults (Tyl, Marr & Myers, 2005c).

In a second study, carbofuran was administered by gavage at dose levels of 0, 0.03, 0.1 or 0.3 mg/kg bw to pups on PND 11. The NOAEL was 0.03 mg/kg bw, due to inhibition of brain AChE activity at 0.1 mg/kg bw (Hoberman, 2007a).

In a third study, carbofuran was administered by gavage at dose levels of 0, 0.03, 0.1 or 0.3 mg/kg bw to pups on PND 11, and to adult rats. The NOAEL for adults was 0.03 mg/kg bw based on inhibition of erythrocyte and brain AChE at 0.1 mg/kg bw 30 minutes after dosing. The NOAEL for pups was 0.03 mg/kg bw due to inhibition of brain AChE at 0.1 mg/kg bw (Hoberman, 2007c).

In an acute toxicity study in young rats (not GLP), carbofuran was administered by gavage to male pups on PND 17, at dose levels of 0, 0.1, 0.3, 0.6 or 1.0 mg/kg bw, and to adult males at 0, 0.1, 0.3, 0.5, 0.75 or 1.5 mg/kg bw. In pups the LOAEL was 0.1 mg/kg bw, the lowest dose tested, based on inhibition of brain AChE (Moser, McDaniel & Philips, 2007b). Re-evaluation of this study and the similar one reported in the previous paragraph supported JMPR's 2008 value for a combined lower confidence limit benchmark dose at 10% response ($BMDL_{10}$) for rat pups of 0.03 mg/kg bw, which is equal to the lowest NOAEL of 0.03 mg/kg bw for both adults and pups, proposed in the study reported here (Hoberman, 2007c).

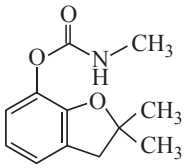
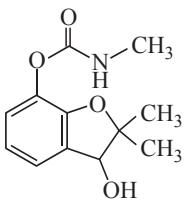
The Meeting concluded that carbofuran is neurotoxic. No studies on delayed neurotoxic potential were submitted. However, experimental data with several carbamates showed that their interaction with the target for delayed polyneuropathy (neuropathy target esterase) protects from, rather than causes, any such neurotoxic effect.

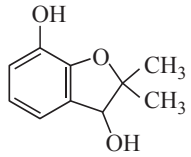
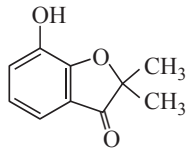
Based on the results of a combined in vitro and in vivo endocrine disruption screening study battery, carbofuran is unlikely to cause toxicity via effects on testosterone/estradiol synthesis, androgen/estrogen receptor binding, aromatase, androgen/estrogen effects on end-organs and tissues, or effects on the thyroid at the levels of exposure that are likely to occur in the human diet (Herberth, 2011a, b, 2012a, b; Usmani, Hodgson & Rose, 2004; Wagner, 2011; Wilga, 2011; Willoughby, 2011a, b, c).

## Toxicological data on metabolites and/or degradates

A summary of the results for plant and livestock metabolites of carbofuran is shown in Table 27, below.

***Table 22. Summary of toxicological characterization of plant/livestock metabolites***

| **Compound, codes and structure** | **Major rat metabolite** (>10% AD)[a] | **Genotoxicity assessment** (data, QSAR, read across) | **General toxicity** | **Toxicological reference values** |
|---|---|---|---|---|
| **Carbofuran**  | Parent | Not genotoxic provided purity 99.8% or greater | Full data set | ADI[b]: 0–0.001 mg/kg bw<br>ARfD[b]: 0.001 mg/kg bw |
| **3-Hydroxy-carbofuran**  | Uncertain | Not genotoxic in vitro[c] | Rat oral $LD_{50}$ 17.9–21.9 mg/kg bw per day<br>Acts as an anticholinesterase, with similar potency to parent, based on acute toxicity | Parent ADI and ARfD |

| Compound, codes and structure | Major rat metabolite (> 10% AD)[a] | Genotoxicity assessment (data, QSAR, read across) | General toxicity | Toxicological reference values |
|---|---|---|---|---|
| **3-Hydroxy-7-phenol-carbofuran**  | Uncertain | Negative QSAR | Rat oral $LD_{50}$ for 20% 3-hydroxy-7-phenol was 1654 mg/kg bw<br>There was no evidence of clinical signs consistent with anticholinesterase effects in the acute toxicology studies in rats | Parent ADI and ARfD |
| **3-Keto-7-phenol-carbofuran**  | Uncertain | Negative QSAR | Acute oral $LD_{50}$ of 5% (w/w) 3-keto-7-phenol-carbofuran in rats was 295.1 mg/kg bw<br>Acute oral $LD_{50}$ of 10% (w/w) 3-keto-7-phenol-carbofuran in rats was > 800 mg/kg bw<br>NOAEL for parental and offspring toxicity in a one-generation reproduction study was 2.5 mg/kg bw per day, (highest dose tested); no evidence of toxicity associated with the inhibition of AChE was observed. | Parent ADI and ARfD |

QSAR: Quantitative structure–activity relationship; TTC: Threshold of toxicological concern; AD: Administered dose ADI: Acceptable daily intake; ARfD: Acute reference dose; AChE: Acetylcholinesterase;

[a] Based on 1996 evaluation (JMPR, 1997);

[b] Applies only to sources of carbofuran of purity 99.8% or greater;

[c] 3-Hydroxy-carbofuran (purity 99.1%) was not genotoxic in a bacterial reverse mutation assay, did not induce forward mutations in the Chinese hamster ovary HPRT test, and did not induce micronuclei in human peripheral blood lymphocytes in vitro (Woods, 2023d, e, f);

[d] In male and female rats, the $LD_{50}$ values for 3-hydroxy-carbofuran (purity not stated and/or 96% pure) ranged from 8.3 to 21.9 mg/kg bw, with clinical signs consistent with a cholinergic crisis (Schoenig, 1967b; Freeman, 1984a)

## Microbiological aspects

There was no information available in the public domain and no experimental data were submitted that addressed the possible impact of carbofuran residues on the human intestinal microbiome.

## Human data

No workplace surveillance and/or poisoning reports were submitted.

The Meeting concluded that the existing database on carbofuran was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

## Toxicological evaluation

The Meeting re-affirmed JMPR's current ADI of 0–0.001 mg/kg bw, based on the overall NOAEL of 0.03 mg/kg bw per day from acute neurotoxicity studies in rats and using a safety factor of 25. This ADI only applies to sources of carbofuran that have a purity of 99.8% or greater.

The Meeting re-affirmed JMPR's current ARfD of 0.001 mg/kg bw, based on the overall NOAEL of 0.03 mg/kg bw per day from acute neurotoxicity studies in rats, and using a safety factor of 25. This ARfD only applies to sources of carbofuran that have a purity of 99.8% or greater.

***Levels relevant to risk assessment of carbofuran***

| Species | Study | Effect | NOAEL | LOAEL |
|---|---|---|---|---|
| **Mouse** | Two-year study of toxicity and carcinogenicity[a] | Toxicity | 20 ppm, equal to 2.8 mg/kg bw per day | 125 ppm, equal to 18 mg/kg bw per day |
| | | Carcinogenicity | 500 ppm, equal to 70 mg/kg bw per day[b] | - |
| Rat | Acute neurotoxicity studies in pups and adults[d,e] | Neurotoxicity | 0.03 mg/kg bw | 0.1 mg/kg bw |
| | 13-week study of neurotoxicity[a] | Neurotoxicity | - | 50 ppm, equal to 3.1 mg/kg bw per day[c] |
| | | Systemic toxicity | - | 50 ppm, equal to 2.4 mg/kg bw per day[c] |
| | Developmental neurotoxicity study[a] | Maternal toxicity | 20 ppm, equal to 1.7 mg/kg bw per day | 75 ppm, equal to 5.0 mg/kg bw per day |
| | | Embryo/fetal toxicity | 20 ppm, equal to 1.7 mg/kg bw per day | 75 ppm, equal to 5.0 mg/kg bw per day |
| | Two-year study of toxicity and carcinogenicity[a] | Toxicity | 20 ppm, equivalent to 1 mg/kg bw per day | 100 ppm, equivalent to 5 mg/kg bw per day |
| | | Carcinogenicity | 100 ppm, equivalent to 5 mg/kg bw per day[b] | - |
| | Three-generation study of reproductive toxicity[a] | Reproductive toxicity | 100 ppm equal to 6 mg/kg bw per day[b] | - |
| | | Parental toxicity | 100 ppm equal to 6 mg/kg bw per day[b] | - |
| | | Offspring toxicity | 20 ppm, equal to 1.2 mg/kg bw per day | 100 ppm equal to 6 mg/kg bw per day |
| | Developmental toxicity study[d] | Maternal toxicity | 0.1 mg/kg bw per day | 0.3 mg/kg bw per day |
| | | Embryo/fetal toxicity | 1 mg/kg bw per day[b] | - |
| | Developmental toxicity study[d] | Maternal toxicity | 1.2 mg/kg bw per day[b] | - |
| | | Embryo/fetal toxicity | 1.2 mg/kg bw per day[b] | - |
| | Developmental toxicity study[a] | Maternal toxicity | 20 ppm, equal to 1.5 mg/kg bw per day | 60 ppm, equal to 4.4 mg/kg bw per day |
| | | Embryo/fetal toxicity | 60 ppm, equal to 4.4 mg/kg bw per day | 160 ppm, equal to 11 mg/kg bw per day |
| Rabbit | Developmental toxicity study[d] | Maternal toxicity | 0.6 mg/kg bw per day | 2 mg/kg bw per day |
| | | Embryo/fetal toxicity | 2 mg/kg bw per day[b] | - |
| Dog | 13-week study of toxicity[a] | Toxicity | - | 10 ppm, equal to 0.43 mg/kg bw per day[c] |
| | One-year study of toxicity[a] | Toxicity | 20 ppm, equal to 0.5 mg/kg bw per day | 500 ppm, equal to 12 mg/kg bw per day |

| Species | Study | Effect | NOAEL | LOAEL |
|---|---|---|---|---|
| **3-Keto-7-phenol-carbofuran** | | | | |
| Rat | One-generation study of reproductive toxicity [a] | Reproductive toxicity | 50 ppm, equal to 2.5 mg/kg bw per day [b] | - |
| | | Parental toxicity | 50 ppm, equal to 2.5 mg/kg bw per day [b] | - |
| | | Offspring toxicity | 50 ppm, equal to 2.5 mg/kg bw per day [b] | - |

[a] Dietary administration; [b] Highest dose tested; [c] Lowest dose tested; [d] Gavage administration; [e] Two or more studies combined

*Acceptable daily intake (ADI) for carbofuran**

*0–0.001 mg/kg bw*

*An acute reference dose (ARfD) for carbofuran**

*0.001 mg/kg bw*

* Applies to carbofuran plus 3-hydroxy-carbofuran, 3-hydroxy-7-phenol-carbofuran and 3-keto-7-phenol-carbofuran, expressed as carbofuran.
The ADI and ARfD apply only to sources of carbofuran of purity 99.8% or greater.

## Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure.

***Critical end-points for setting guidance values for exposure to carbofuran***

| | |
|---|---|
| **Absorption, distribution, excretion and metabolism in mammals** | |
| Rate and extent of oral absorption | Rapid and extensive |
| Dermal absorption | No data |
| Distribution | Wide; highest concentration in liver |
| Potential for accumulation | Low |
| Rate and extent of excretion | Rapid with majority of the absorbed radiolabel being excreted in urine within 24 hours |
| Metabolism in animals | Primarily by CYP3A4-mediated hydroxylation and hydrolysis with subsequent conjugation; 3-hydroxy-carbofuran, 3-keto-carbofuran phenol, 3-hydroxy-N-hydroxymethyl-carbofuran, 3-hydroxymethyl-carbofuran, and carbofuran-phenol were the major metabolites in urine |
| Toxicologically significant compounds in animals and plants | Carbofuran, 3-hydroxy-carbofuran, 3-hydroxy-7-phenol-carbofuran and 3-keto-7-phenol-carbofuran |
| **Acute toxicity** | |
| Rat, $LD_{50}$, oral | 5.2 to 14.9 mg/kg bw |
| Rat, $LD_{50}$, dermal | No data |
| Rat, $LC_{50}$, inhalation | No data |
| Rabbit, dermal irritation | Not irritating |
| Rabbit, ocular irritation | Minimally irritant (at 5 mg per eye; expected to be lethal at higher, untested doses) |
| Mouse, dermal sensitization | No data |
| Guinea pig, dermal sensitization | Not sensitizing (Draize) |

| | |
|---|---|
| **Short-term studies of toxicity** | |
| Target/critical effect | Inhibition of acetylcholinesterase activity (rabbit, dog) |
| Lowest relevant oral NOAEL | 0.43 mg/kg bw per day (dog) |
| Lowest relevant dermal NOAEL | 1000 mg/kg bw per day, highest dose tested (rabbit) |
| Lowest relevant inhalation NOAEC | No data |
| **Long-term studies of toxicity and carcinogenicity** | |
| Target/critical effect | Inhibition of AChE (mice, rats and dogs)<br>Clinical signs of cholinergic toxicity (rats, rabbits)<br>Decreased body weight /body weight gain (rats) |
| Lowest relevant NOAEL | 1 mg/kg bw per day (rat) |
| Carcinogenicity | Not carcinogenic [a] |
| **Genotoxicity** | Unlikely to be genotoxic if purity is 99.8% or higher [a] |
| **Reproductive toxicity** | |
| Target/critical effect | Reduced pup growth and survival (rat) |
| Lowest relevant parental NOAEL | 6 mg/kg bw per day (rat) |
| Lowest relevant offspring NOAEL | 1.2 mg/kg bw per day (rat) |
| Lowest relevant reproductive NOAEL | 6 mg/kg bw per day (rat) |
| **Developmental toxicity** | |
| Target/critical effect | Reduced maternal body weight gain (rat)<br>Reduced fetal body weight (rat),<br>Maternal signs of cholinergic crisis (rat, rabbit) |
| Lowest relevant maternal NOAEL | 0.1 mg/kg bw per day (rat) |
| Lowest relevant embryo/fetal NOAEL | 1 mg/kg bw per day (rat) |
| **Neurotoxicity** | |
| Acute neurotoxicity NOAEL | 0.03 mg/kg bw (rat, adults and pups 11–17 days old) |
| Subchronic neurotoxicity NOAEL | 3.1 mg/kg bw per day [b] (rat) |
| Developmental neurotoxicity NOAEL | 1.7 mg/kg bw per day (rat) |
| **Other toxicological studies** | |
| Immunotoxicity | No data; unlikely to be immunotoxic |
| **Studies on toxicologically relevant metabolites** | |
| **3-Hydroxy-carbofuran** | Acute oral $LD_{50}$: 8.3 to 21.9 mg/kg bw (rat)<br>Not genotoxic in in vitro assays<br>Acts as cholinesterase inhibitor (potency likely similar to parent) |
| **Microbiological aspects** | No data submitted |
| **Human data** | No reports of clinical cases or poisoning incidents have been submitted |

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

[b] Lowest dose tested

## Summary

| | Value | Study | Safety factor |
|---|---|---|---|
| ADI | 0–0.001 mg/kg bw [a] | Acute neurotoxicity studies (rat) | 25 |
| ARfD | 0.001 mg/kg bw [a] | Acute neurotoxicity studies (rat) | 25 |

[a] Applies to carbofuran, 3-hydroxy-carbofuran, 3-hydroxy-7-phenol-carbofuran and 3-keto-7-phenol-carbofuran, expressed as carbofuran.

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# Carbosulfan

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## Explanation

Carbosulfan is the International Organization for Standardization (ISO)–approved common name for (2,2-dimethyl-3*H*-1-benzofuran-7-yl)-*N*-(dibutylamino)sulfanyl-*N*-methylcarbamate, with the Chemical Abstracts Service (CAS) number 55285-14-8. Carbosulfan is a procarbamate (having an *N*-(dibutylamino)sulfanyl group instead of the simple amino group of carbofuran) insecticide that, when converted to carbofuran (by removal of the *N*-(dibutylamino)sulfanyl group) acts by reversibly inhibiting the activity of acetylcholinesterase (AChE) thus, inhibiting the breakdown of the neurotransmitter acetylcholine (ACh). This results in increasing both the level and duration of the neurotransmitter action at acetylcholine receptors.

Carbosulfan has been previously evaluated at FAO/WHO Joint Meeting on Pesticide Residues (JMPR) meetings in 1984, 1986 and 2003. In 2003, JMPR established an acceptable daily intake (ADI) for carbosulfan of 0–0.01 mg/kg body weight (bw) based on a NOAEL of 1 mg/kg bw per day, due to pathological changes in the eye, inhibition of brain and erythrocyte AChE activity and body weight reduction in the two-year study in rats, with a safety factor of 100. A lower safety factor, dependent upon the maximum concentration ($C_{max}$) could not be justified because pathological changes seen in the eye could not definitely be attributed to inhibition of AChE. In 2003 JMPR established an acute reference dose (ARfD) for carbosulfan of 0.02 mg/kg bw based on inhibition of brain AChE activity in a study of acute neurotoxicity in rats, and a safety factor of 25 as the relevant toxic effects were dependent on the $C_{max}$. Carbosulfan was reviewed by the present Meeting at the request of CCPR as part of their periodic review program.

A major metabolite, spontaneous degradant and impurity of carbosulfan is carbofuran, which is also a pesticide active ingredient in its own right. Based on the 1995 FAO/WHO Joint Meeting on Pesticide Specifications chemical specification, technical grade carbosulfan may contain up to 2% carbofuran. In JMPR 2008 established an ADI of 0–0.001 mg/kg bw for carbofuran (JMPR, 2010), based on the overall no-observed-adverse-effect level (NOAEL) of 0.03 mg/kg bw per day identified for inhibition of brain AChE activity in rat pups aged 11 days and a safety factor of 25 [1]. Accordingly, based on a comparison of the points of departure used to establish the relevant ADIs, carbofuran is substantially more toxic than carbosulfan. For this reason, currently marketed technical grade carbosulfan contains a small amount of epoxidized vegetable oils or some other water scavenger to reduce the rate of spontaneous degradation of carbosulfan to carbofuran. These additives act as chemical stabilizers and shelf-life extenders. Stabilized technical grade carbosulfan typically contains around 92% or more carbosulfan. Notably some studies submitted for evaluation used non-stabilized carbosulfan as the test article. This potentially resulted in dosing with a mixture of carbosulfan and carbofuran in unspecified proportions. Such test articles may be more toxic than currently marketed, stabilized, technical grade carbosulfan.

New acute toxicity studies (seven oral, two dermal and three inhalation acute studies), irritation studies (two ocular and two dermal irritation studies, all in rabbits), sensitization studies (three dermal sensitization studies), and in vitro and in vivo genotoxicity studies were submitted for evaluation. Twelve, relevant, peer-reviewed, published scientific papers were identified from a literature search using US National Institutes of Health, National Library of Medicine PubMed, using the search term "carbosulfan". A total of 166 peer-reviewed scientific publications were retrieved. While all these publications were examined, only those of value in assessing the compound and of appropriate quality have been summarized here. Biomonitoring data for workers involved in manufacturing/formulating carbosulfan in 2017 and 2018 was also submitted for evaluation.

Where appropriate, summaries of studies previously evaluated by JMPR 2003 (JMPR, 2004) have been included in the monograph. Studies using formulated carbosulfan products or outdated test methods of uncertain regulatory value were evaluated, but not summarized since they contributed no additionally useful information. Many studies were not conducted in accordance with current national or international test guidelines. These studies were evaluated, however only those studies considered to be of acceptable quality and of value in the assessment are summarized in this monograph. Unless

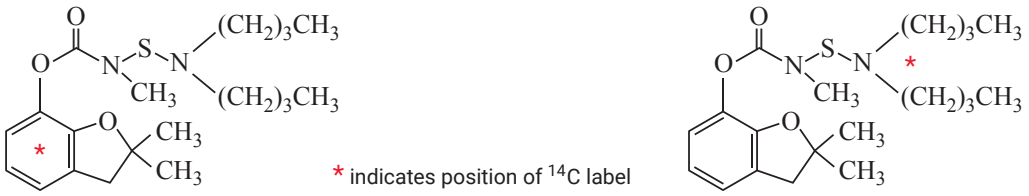
1 This NOAEL was supported by the $BMDL_{10}$ of 0.03 mg/kg bw extrapolated from data on inhibition of brain AChE activity from three studies in rat pups aged 11 days. A safety factor of 25 was considered to be appropriate because the acute toxic effects of carbofuran are dependent on $C_{max}$ rather than AUC, and data indicated that the sensitivity of humans and laboratory animals (rats, dogs) to inhibition of AChE activity by carbofuran was similar

otherwise stated, all critical studies were conducted in compliance with good laboratory practice (GLP) and statements of quality assurance were provided.

# Evaluation for acceptable daily intake

## 1 Biochemical aspects

***Figure 1. Chemical structure of carbosulfan, showing the alternative $^{14}C$ labelling positions***




[*Phenyl*-$^{14}$C]carbosulfan

[*Dibutylamino*-$^{14}$C]carbosulfan

### 1.1. Absorption, distribution and excretion

**(a) Oral route**

***Rat***

In the in vivo arm of a non-GLP, peer-reviewed published paper, three fasted female rats (strain not stated) were orally (gavage) dosed with 30 mg/kg bw of [*carbonyl*-$^{14}$C]carbosulfan. The rats (one per time point) were killed at 15, 35 and 80 minutes following dosing, the stomach and its contents were immediately removed, and the contents extracted with a phosphate buffered ether and water mixture. The ether fraction (following drying over anhydrous sodium sulfate) and the aqueous fraction were then analyzed for radioactivity by reverse phase thin layer chromatography (TLC). In the in vitro arm of the study [*carbonyl*-$^{14}$C]carbosulfan (1.36 mg/mL) was incubated with hydrochloric acid (pH 2.0 or pH 2.95) at 40°C for 24 hours. Samples for analysis were collected at 20 minutes, then 1.5, 4 and 24 hours after the commencement of the study. Following ether:water extraction the ether and aqueous fractions were analyzed as described above.

In the in vivo arm of the study 60%, 46.8% and 49.5% of the administered radioactivity (AR) was recovered from the stomach contents in the form of carbosulfan at 15, 35 and 80 minutes respectively following dosing. The predominant degradant present in the stomach contents (14.6%, 20.3% and 15.8% of AR at 15, 35 and 80 minutes, respectively) was carbofuran; this represents a toxication reaction. A second major degradant (11.1%, 17.0%, 18.4% or AR at 15, 35 and 80 minutes, respectively following dosing) co-eluted with biscarbofuran-*N*,*N*′-disulfide (a detoxification reaction).

In the in vitro arm of the study the concentration of carbosulfan progressively declined at pH 2.95, with 82.2%, 60.3%, 41.7% and 40.6% of AR remaining at 20 minutes, 1.5, 4 and 24 hours following the start of the study, respectively. The major degradant was carbofuran (representing 12.1%, 31.0%, 45.8% and 49.3% of AR at 20 minutes, 1.5, 4 and 24 hours following the start of the study, respectively. At pH 2.0, about 48% of the carbosulfan dose was degraded by 1.5 hours following the start of the study. The major degradant was carbofuran, at 38.7% of the administered dose (AD). Overall the study demonstrated that:

- carbosulfan (about 20% of AD) undergoes substantial presystemic degradation to carbofuran (toxication) within 35 minutes of oral, gavage dosing;
- carbosulfan (about 20% of AD) undergoes substantial presystemic degradation to biscarbofuran-*N*,*N*′-disulfide (detoxification) within 35 minutes of oral, dosing;
- carbosulfan is more stable in the rat stomach than in simple hydrochloric acid solutions.

The study findings are consistent with the lower oral toxicity of carbosulfan compared to carbofuran being in part due to the conversion of carbosulfan to less toxic polysulfide derivatives, rather than toxication to carbofuran and, in part, due to the stability of carbosulfan in the rat stomach (Umetsu & Fukuto, 1982).

In a mass balance, absorption, distribution, metabolism and excretion study (not GLP) male and female Sprague Dawley rats were orally (gavage) dosed with either phenyl- or dibutylamino-labelled [$^{14}$C]carbosulfan in corn oil. Thirty-six rats of each sex were divided into a preliminary phase consisting of group A (two of each sex per label) and a definitive phase consisting of groups B (two controls of each sex, no label), C, D, E (five of each sex per label). Groups A and C each received a single oral (gavage) dose of 4 mg/kg bw. Group D received multiple unlabelled oral doses of 4 mg/kg bw per day for two weeks, followed by a single radiolabelled dose (4 mg/kg bw). Group E received one single oral high dose of 30 mg/kg bw. Based on the results of group A, 90%–101 % of AR was recovered, with most $^{14}$C residues being excreted in urine in the case of both labels. Exhaled labelled carbon dioxide (7%–10% of AR) was found only in the group receiving [*dibutylamino*-$^{14}$C]carbosulfan. Therefore in the definitive study Roth metabolism cages were used, and expired air was collected for the groups dosed with [*dibutylamino*-$^{14}$C]carbosulfan.

Overall recoveries of the administered dose in groups C, D and E were 90%–98% for both labels. The majority (80%–90%) of the $^{14}$C label was eliminated within 48 hours for the 4 mg/kg bw cohorts and 72 hours for the 30 mg/kg bw cohorts. The main excretory route was in urine, with 72%–88% of the dose excreted for the phenyl label and 65%–71% for the dibutylamino label. Excretion in faeces accounted for 5%–22% of the dose in the case of both labels. Excretion of radiolabel in the urine tended to be slightly higher (phenyl group: 79%–88%, dibutylamino group 71%) in rats receiving repeated low doses of carbosulfan than in rats treated with a single dose at 4 mg/kg bw, while excretion in faeces tended to be lower (5%–15%) in these animals. Furthermore, in repeatedly exposed rats the rate of excretion of radiolabel was slightly increased with 80%–87% of the radiolabel excreted within 24 hours in rats treated with repeated low doses, compared with 72%–78% in 24 hours for those treated with a single low dose. When rats received dibutylamino-labelled carbosulfan, an additional 10%–17% of the dose was eliminated mainly as expired carbon dioxide.

The levels of $^{14}$C residues in the blood and most of the tissues examined following administration of phenyl-labelled carbosulfan were below the level of detection, while in heart, kidney, muscle, lung and residual carcass the levels ranged from 0.001 to 0.036 mg/kg tissue for the low-dose groups and 0.010 to 0.541 mg/kg tissue for the high-dose groups. In rats dosed with dibutylamine-labelled carbosulfan, the $^{14}$C residue levels in blood, tissues, and carcass ranged from 0.005 to 0.132 mg/kg tissue for the low-dose groups and 0.083 to 0.861 mg/kg tissue for the high-dose group. Portions of the metabolic intermediate acids in the dibutylamine-labelled carbosulfan can elongate to fatty acids and be stored as natural products. This may explain the relatively higher tissue residues with the dibutylamine label compared to the phenyl label.

At 168 hours after dosing, less than 2% of the dose was retained in the carcass and in any of the tissues/organs, including blood, bone, brain, fat, ovaries, testes, heart, liver, kidneys, lungs, muscle, spleen, skin and uterus. Accordingly, no further analysis of the tissues/organs was performed. No biologically important differences in the absorption, distribution or excretion were noted either between sexes or across the different study groups. There was no evidence indicative of bioaccumulation or bioretention. Overall more than 82% of the AD was biotransformed and eliminated in excreta (or expired as carbon dioxide in the case of dibutylamine-labelled carbosulfan), indicating extensive absorption and degradation of carbosulfan after oral administration. The metabolism arm of the study is summarized in section 1.2 Biotransformation, below (Fang & El Naggar, 1995).

In the single-dose arm of a mass balance, absorption, distribution, metabolism and excretion study (not GLP), [*phenyl*-$^{14}$C]carbosulfan was orally (gavage) administered to fasted SD rats (five of each sex per dose) at nominal doses of 3 or 30 mg/kg bw. In the multiple-dose arm of the study carbosulfan was orally (gavage) administered to fasted SD rats (five of each sex per dose) at a nominal dose of 3 mg/kg bw per day for 14 consecutive days, followed by a single oral (gavage) dose of 3 mg/kg bw of [*phenyl*-$^{14}$C]carbosulfan. All rats in the single oral 30 mg/kg bw dose cohort displayed clinical signs consistent with a cholinergic crisis (increased salivation, frequent urination, tremor). In both sexes an average of 90.4% to 91.4% of AR was excreted in urine, and an average of 8.6% to 9.6% was excreted in faeces within the 120 hours following dosing. Oral absorption was estimated to be near complete. There were no meaningful sex differences in the patterns of excretion. The level of radioactivity in most organs and tissues was very low; less than twice the background. Values consistently higher than twice the background level were detected in the skin and liver of most animals. In four out of 10 animals, slightly

higher than twice the background values were measured in adipose tissue. In the 3 mg/kg bw single oral dose cohort, both sexes excreted 87.7% to 91.6% of AR in urine and 8.4% to 12.3% in faeces. There were no meaningful sex differences in the pattern of excretion. The level of radioactivity in the organs and tissues was very low (less than twice the background), except for one animal that had slightly higher than background values in the skin and adipose tissue. Repeated daily dosing at 3 mg/kg bw per day had no meaningful effects on the pattern of excretion or distribution. There was no evidence of retention of carbosulfan-derived radioactivity in the body. In all study cohorts most of the administered radioactivity was excreted by 50 hours after dosing, with peak excretion in the urine and faeces occurring around 20–25 hours post dose (Selim, 1982).

In the single oral (gavage) dose arm of a balance, absorption, distribution, and metabolism study (not GLP), SD rats (five of each sex per dose level) were administered either 3 or 30 mg/kg bw of [*phenyl*-$^{14}$C]carbosulfan. In the repeat-dose study arm rats (five of each sex) were orally (gavage) administered 3 mg/kg bw per day of unlabelled carbosulfan for 14 days, followed by a single oral dose of [*phenyl*-$^{14}$C]carbosulfan on study day 15. In both study arms excretion of radioactivity in urine and faeces was monitored for five days following the (final) dose after which the rats were killed, and tissue radioactive residue levels were determined. The chemical structure of excreted metabolites collected in the first 24 hours following dosing was determined by high-performance liquid chromatography (HPLC) using isotopic dilution with known metabolite standards. In all dosing regimens, most of the radioactivity was excreted in urine and faeces with 91.7% or more of AR being eliminated within five days of dosing. In all study cohorts 71.7% or more of AD was eliminated over the first 24 hours post dosing. Irrespective of dosing regimen, 80.4% or more of AR was eliminated in urine and 5.8% or more of AR was eliminated in the faeces. In all study cohorts only minimal levels of radioactivity were detected at five days post dosing; the highest levels being in liver and skin (Wu, 1982a).

A second study identical in design to Wu, 1982a (above) was conducted, except that [*dibutylamine*-$^{14}$C]carbosulfan was the test article. Rats, irrespective of dose level, excreted most of the radioactive dose in urine and faeces with the majority being via urine. Elimination as expired $^{14}$C carbon dioxide over the first 48 hours was less than 0.4% of AD. By seven days after dosing male rats administered a single oral low dose excreted a total of 92.2% of the radioactivity, with 80.1% in urine and 12.1% in faeces, while females excreted 80.4% in urine and 11.3% in faeces, a total of 91.7%. For male rats receiving the single, oral, high dose, a total of 86.5% of AR was excreted with 78.7% in urine and 7.8% in faeces. The male rats on the multiple oral low dose regime excreted 83.7% of the radioactivity in urine and 7.8% in faeces, making a total of 89.3%. At five days after dosing 1.1% of AD remained in the combined tissues and carcass in the case of both single and multiple oral low dose rats. For the single, oral, high dose the $^{14}$C-containing residues remaining in the tissues and carcass amounted to 2.6% (Wu, 1982b).

In a whole-body autoradiography and disposition study (not GLP), a single oral (gavage) dose (30 mg/kg bw) of either [*dibutylamine*-$^{14}$C]carbosulfan or [*phenyl*-$^{14}$C]carbosulfan was administered to fasted male SD rats. Groups of six rats were killed at 0.5, 1, 2, 6 and 24 hours (phenyl label) or 0.5, 2, 6, 24 and 48 hours (dibutylamine label) following dosing. Two rats from each group were used in the whole-body autoradiography study arm and four rats from each group were used in the disposition study arm. No adverse clinical signs were observed. Based on autoradiography, most of the administered radioactivity was localized primarily in the gastrointestinal (GI) tract, irrespective of label position. At 24 and 48 hours following dosing much of the administered radioactivity remained in the GI tract. High levels of radioactivity occurred in the kidney between 30 minutes and 24 hours after dosing. Systemic absorption was higher than with the dibutylamine label than the phenyl label, with absorbed radioactivity derived from [*dibutylamine*-$^{14}$C]carbosulfan concentrating in the central nervous system, muscle, glandular tissue, testes, lung, blood and bone marrow. Peak tissue radioactivity levels occurred at six hours following dosing. Moderate radioactivity levels were seen in the liver during the first six hours in rats administered [*phenyl*-$^{14}$C]carbosulfan, and during the first 24 hours in those dosed with the [*dibutylamine*-$^{14}$C]carbosulfan. By 24 hours after dosing, radioactivity from the dibutylamine label was minimal in most tissues except the liver, stomach contents salivary glands, parotid glands, ethmoturbinate epithelium, and in faeces and/or urine. By 24 hours (for the phenyl label) and 48 hours (for the dibutylamine label) following dosing levels of radioactivity in the liver were just above the background. Radioactivity from the DBA label concentrated in lipid-rich tissue (salivary glands, thymus,

Harderian glands). Radioactivity derived from both labels entered the CNS (localizing uniformly in the cerebrum, cerebellum and spinal cord) with higher levels occurring with the dibutylamine-labelled carbosulfan. The time to maximum concentration ($T_{max}$) in the central nervous system was six hours.

In the disposition study arm, there was a lack of coincidence between the concentrations of the different labels, implying that metabolism involving the separation of the phenyl group from the dibutylamino side-chain had occurred. With the phenyl label, radioactivity in the liver and kidney declined from 0.5 hours after dosing and then rose to a secondary peak at six hours. With the dibutylamine label the liver and kidney $T_{max}$ was six hours post dosing, and more radioactivity was retained in the liver than in the kidney (Liss et al. 1981).

In the tissue distribution arm of a peer reviewed published study (not GLP), fasted female SD rats (number not stated) were orally (gavage) dosed with 30 mg/kg bw of carbonyl-, phenyl- or dibutylamine-labelled [$^{14}$C]carbosulfan. The rats in this study arm were killed for tissue analysis at 48 hours (carbonyl label) and 96 hours (phenyl and dibutylamine label) following dosing. Residue analysis was performed by TLC using relevant authentic chemical standards. Irrespective of label position or sampling time the highest levels of radioactivity occurred the blood, liver, kidney, heart and spleen (Marsden, Ruwano & Fukuto, 1982).

**(b) Dermal route**

In a peer-reviewed published paper (not GLP), the dermal penetration rate of technical grade carbosulfan (purity 99.3%; final concentration 2 or 15 mg applied to an area of 3.14 cm$^2$) was determined using clipped male SD rat abdominal skin in vitro. The method employed static, sealed Franz diffusion cells incubated at 32°C for 48 hours (eight sampling time points) with shaking. Samples were stored at –20°C until analyzed by HPLC. Only carbofuran was detected in the receptor solution. Spontaneous hydrolysis of carbosulfan to carbofuran did not occur in the absence of a rat skin barrier, implying that carbosulfan was converted by rat abdominal skin to carbofuran in vitro. The mean carbosulfan flux rate at the 2 mg dose was 1.05 µg/cm$^2$ per hour, and at the 15 mg dose it was 1.52 µg/cm$^2$ per hour (Liu & Kim, 2003).

## 1.2 Biotransformation

**(a) In vitro**

In a peer-reviewed published paper (not GLP) the in vitro metabolism of carbosulfan was evaluated with pooled hepatic microsomal preparations derived from humans (six male and four female donors), SD rats, DBA/2 mice, beagle dogs, New Zealand White rabbits, Gottingen minipigs and cynomolgus monkeys (10 male donors for each of the nonhuman species). The incubation mixture contained 100 µM carbosulfan, pooled liver microsomal protein (0.15 mg for nonhuman incubations and 0.14 mg in the human studies), in a final volume of 200 µl of 0.1 M phosphate buffer (pH 7.4). After a two-minute pre-incubation at 37°C in a shaking incubator block the reaction was started by adding 1 mM NADPH. The mixture was incubated at 37°C for 20 and 60 minutes. The reaction was stopped with 600 µL of ice-cold acetonitrile containing a carbaryl (1-naphthylmethylcarbamate) internal standard. All incubations were carried out in triplicate. After centrifugation at 10 000 *g* for 15 minutes the supernatant was collected and stored at −20°C until analyzed. Analysis and metabolite identification was performed using HPLC–time-of-flight mass spectroscopy.

Following the reactions, the parent compound and eight metabolites were detected:

carbofuran, 3-hydroxy-carbofuran, 3-hydroxy-7-phenol-carbofuran, 3-keto-carbofuran, 3-keto-7-phenol-carbofuran, 7-phenol-carbofuran, carbosulfan sulfinamide (presumptive), and dibutylamine).

At 20 minute incubations, mouse microsomes had the highest formation rate of carbofuran, whereas with 60 minutes incubation, human microsomes showed the highest rate of formation (2.5 and 1.9 nmol/mg protein per minute, respectively). Metabolite 7-phenol-carbofuran was detected only with rabbit microsomes (at both 20 and 60 minutes; 0.18 and 0.004 nmol/mg protein per minute, respectively). The highest formation rate of 3-hydroxycarbofuran was detected in monkeys at 20 minutes (15.2 nmol/mg protein per minute) and minipigs at 60 minutes (5.4 nmol/mg protein per minute). Among the different species tested, minipig microsomes were the most active in carbosulfan biotransformation

to 3-ketocarbofuran (0.6 and 0.4 nmol/mg protein per minute) and 3-keto-7-phenolcarbofuran (0.6 and 0.3 nmol/mg protein per minute) for both incubation times. The corresponding rates of carbosulfan sulfinamide formed with dog preparations, and were the highest at 10.2 and 2.0 nmol/(mg protein per minute) for the two incubation times. Humans showed the highest metabolite formation rate for dibutylamine after 20 minutes, while dogs had the highest after 60 minutes of incubation. Metabolite 3-hydroxy-7-phenol-carbofuran was not detected in rats or mice. No phase II enzyme-associated metabolites were observed after incubation with human hepatic homogenates fortified with appropriate cofactors; uridine diphosphate glucuronic acid (UDPGA), 3′-phosphoadenosine 5′-phosphosulfate (PAPS), glutathione). A proposed in vitro metabolism pathway is shown in Fig. 2 below. Overall, two main in vitro hepatic microsomal metabolic pathways were detected:

- an initial oxidation of sulfur to carbosulfan sulfinamide (the "sulfur oxidation pathway" or detoxification pathway);
- the cleavage of the nitrogen–sulfur bond to give carbofuran and/or dibutylamine (the "carbofuran pathway" or toxication pathway).

Carbofuran was subsequently further metabolized.

The disappearance or depletion of carbosulfan was quantified by triple quadrupole mass spectrometry from incubations of varying concentrations (from 2.5 to 150 µM) examining all seven mammalian liver microsome preparations (Fig. 3). Carbosulfan NADPH-dependent depletion was fastest in dogs, and the depletion curve in this species was close to linear over the concentration range tested. The lowest rate of NADPH-dependent depletion was in mice. Notably, the carbosulfan NADPH-dependent depletion rate and extent was considerably greater in humans compared to mice and greater than in rats.

Carbosulfan biotransformation by mammalian liver microsomes followed Michaelis–Menten kinetics as demonstrated by Eadie–Hofstee plots. The kinetic parameters for the two primary pathways of carbosulfan metabolism, (sulfur oxidation and nitrogen–sulfur bond cleavage) in liver microsomes were determined using a wide concentration range (2.5–300 µM) of carbosulfan (Table 1). Mouse microsomes had the highest affinity, corresponding with the lowest $K_M$ (89.2 µM), and the lowest capacity, corresponding to the lowest $V_{max}$ (2.9 nmol carbosulfan sulfinamide/mg protein per minute). Cynomolgus monkey microsomes displayed the lowest affinity (316.3 µM), whereas dogs microsomes exhibited the highest capacity (13.5 nmol carbosulfan sulfinamide/mg protein per minute). Dog microsomes also had the highest intrinsic clearance ($CL_{int}$) rate, whereas humans and rats displayed the lowest $CL_{int}$ rate (51.2, 18.7 and 11.9 µL/minute per mg protein respectively).

Species differences in relation to metabolism of carbosulfan to carbosulfan sulfinamide (detoxification) were also apparent (Table 1). Mice exhibited the highest affinity, corresponding to the lowest $K_M$ (89.2 µM), and the lowest capacity, corresponding to the lowest $V_{max}$ (2.9 nmol carbosulfan sulfinamide/mg protein per minute). Cynomolgus monkeys had the lowest affinity (316.3 µM), whereas dogs had the highest capacity (13.5 nmol carbosulfan sulfinamide/mg protein per minute). Dogs also had highest $CL_{int}$ rate, whereas humans and rats had the lowest $CL_{int}$ rates (51.2, 18.7 and 11.9 µL/minute per mg protein respectively).

The cleavage of the nitrogen–sulfur bond of carbosulfan yields dibutylamine and carbofuran and thence its more distal metabolites. Species differences were apparent in dibutylamine formation. Rats, mice and humans had the highest affinities (22.0, 31.8, and 32.1 µM respectively) and lowest capacities (2.5, 2.5 and 2.0 nmol dibutylamine/mg protein per minute, respectively). Based on the $CL_{int}$ rates, rats were the most efficient at metabolizing carbosulfan to dibutylamine, and rabbits were the least efficient (114.5 and 18.3 µL/minute per mg protein respectively) (Abbas et al., 2009).

*Table 1a. Kinetic parameters of carbosulfan metabolite formation by hepatic microsomes in vitro*

| Species[a] | $V_{max}$ (nmol/mg protein per minute) | $K_M$ (µM) | $CL_{int}$ (µM/min per mg protein) | Interspecies differences[b] |
|---|---|---|---|---|
| Human | 5.0 | 268.6 | 18.7 | Not applicable |
| Rat | 3.2 | 264.8 | 11.9 | 0.64 |
| Mouse | 2.9 | 89.2 | 33.0 | 1.74 |
| Dog | 13.5 | 262.6 | 51.2 | 2.74 |
| Rabbit | 8.8 | 229.4 | 38.2 | 2.04 |
| Minipig | 4.5 | 177.4 | 25.2 | 1.35 |
| Monkey | 8.6 | 316.3 | 27.3 | 1.46 |

[a] Each value represents the mean of three determinations; Source: Abass et al., 2009

[b] Interspecies differences represents animal to human fold differences in toxicokinetics

*Table 1b. Kinetic parameters of dibutylamine formation obtained with different mammalian liver microsomes*

| Species[a] | $V_{max}$ (nmol/mg protein per minute) | $K_M$ (µM) | $CL_{int}$ (µM/min per mg protein) | Interspecies differences[b] |
|---|---|---|---|---|
| Human | 2.0 | 32.1 | 63.2 | Not applicable |
| Rat | 2.5 | 22.0 | 114.5 | 1.81 |
| Mouse | 2.5 | 31.8 | 80.0 | 1.27 |
| Dog | 8.7 | 100.3 | 86.7 | 1.37 |
| Rabbit | 2.8 | 150.5 | 18.3 | 0.29 |
| Minipig | 7.9 | 137.3 | 57.4 | 0.91 |
| Monkey | 8.1 | 169.9 | 47.4 | 0.75 |

[a] Each value represents the mean of three determinations; Source: Abass et al., 2009

[b] Interspecies differences represents animal to human fold differences in toxicokinetics

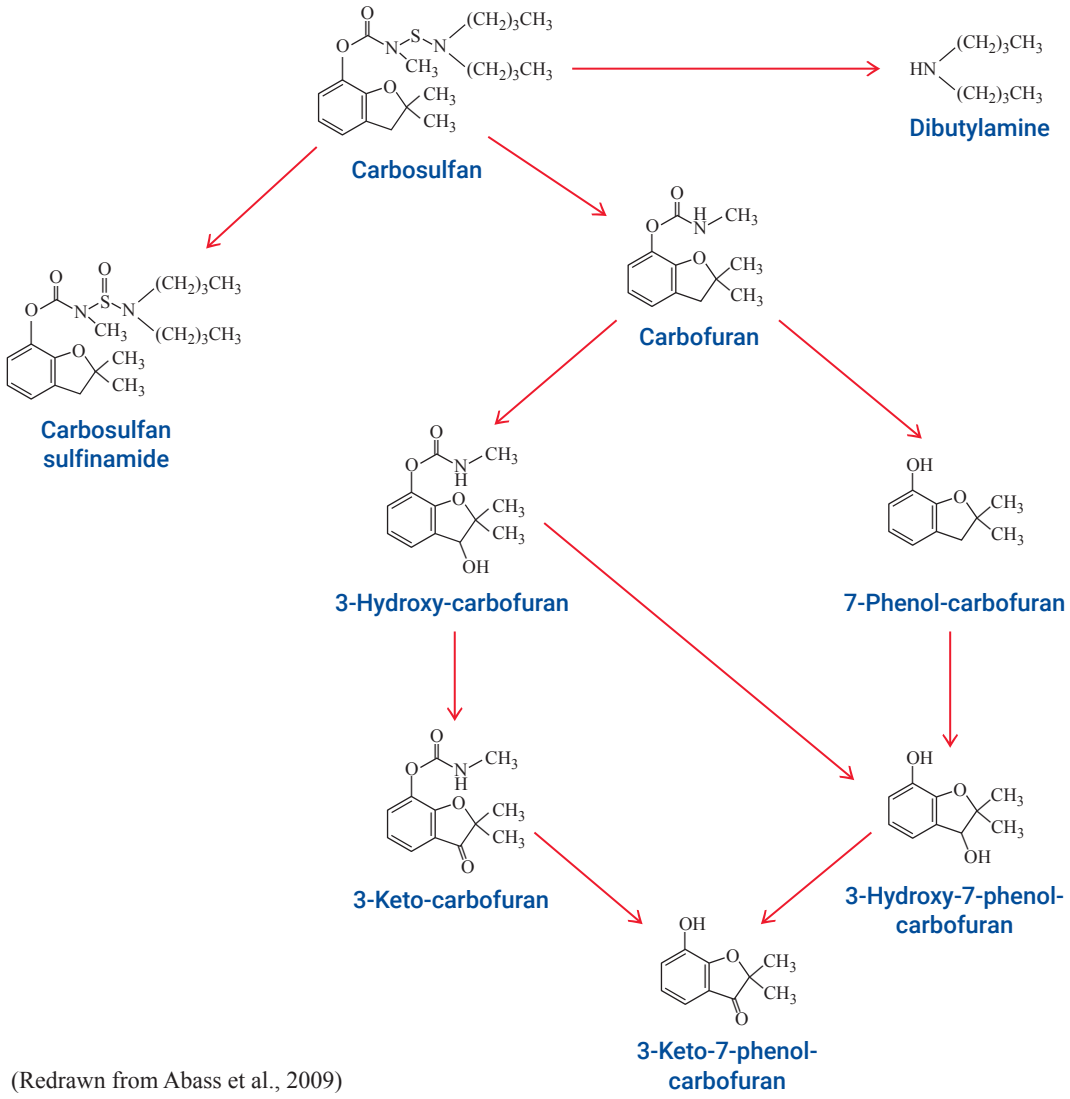
*Table 1c. Kinetic parameters of the carbofuran metabolic pathway obtained with different mammalian liver microsomes*

| Species[a] | $V_{max}$ (nmol/mg protein per minute) | $K_M$ (µM) | $CL_{int}$ (µM/min per mg protein) | Interspecies differences[b] |
|---|---|---|---|---|
| Human | 15.6 | 34.4 | 454.9 | Not applicable |
| Rat | 5.5 | 16.9 | 326.6 | 0.72 |
| Mouse | 3.4 | 13.2 | 253.2 | 0.56 |
| Dog | 16.2 | 72.4 | 223.9 | 0.49 |
| Rabbit | 8.5 | 25.2 | 335.7 | 0.74 |
| Minipig | 13.7 | 29.0 | 471.4 | 1.03 |
| Monkey | 17.2 | 38.0 | 450.8 | 0.99 |

[a] Each value represents the mean of three determinations; Source: Abass et al., 2009

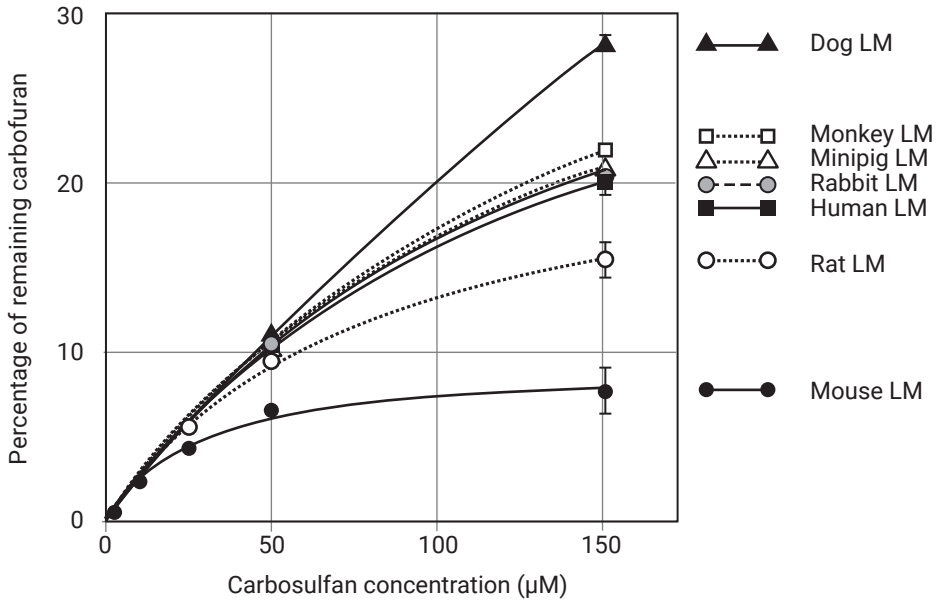
[b] Interspecies differences represents animal to human fold differences in toxicokinetics

*Figure 2. Proposed in vitro hepatic microsomal metabolic pathway of carbosulfan*




(Redrawn from Abass et al., 2009)

*Figure 3. Concentration-dependent depletion rate of carbosulfan in mammalian liver microsomes*




(Redrawn from Abass et al., 2009)

Species differences in the carbofuran toxication pathway were also detected. Humans and cynomolgus monkeys displayed similar affinity ($K_M$ values 34.4 and 38.0 µM respectively) and capacity ($V_{max}$ values 15.6 and 17.2 nmol/mg protein per minute respectively). Dogs had the lowest affinity corresponding to the highest $K_M$ value (72.4 µM), while mice and rats had the highest affinity (3.4 and 5.5 nmol/[mg protein . minute] ). Cynomolgus monkeys, dogs, and human liver microsomes had the highest capacity corresponding to the highest $V_{max}$, whist the reverse was true for mice, rats, and rabbits. The catalysis efficiency ($CL_{int}$) for minipigs (471.411.9 µL/minute per mg protein) was higher than those for other mammalian liver microsomes, whereas dogs had the lowest efficiency (223.911.9 µL/minute per mg protein). The rank order of the $CL_{int}$ values in different species for the carbofuran (toxication) pathway was: dog < mouse < rat < rabbit < monkey < human < minipig.

Based on in vitro metabolite formation rates, mice and rats had the lowest 3-hydroxycarbofuran formation rates, whilst dogs, monkeys, humans and minipigs exhibited the faster formation rates. Because of their low 3-hydroxy- and 3-keto-carbofuran formation rates, the distal metabolites (3-hydroxy and 3-keto-7-phenolcarbofuran) in mice and rats were not detected at any carbosulfan concentration. Generally, with all the different species, the amounts of 3-keto-7-phenolcarbofuran decreased with increasing carbosulfan concentration. Only rabbit microsomes produced 7-phenolcarbofuran at a maximal formation rate of 0.19 nmol/mg protein per minute.

Maximum interspecies difference (between animal and human microsomes) in intrinsic clearance to carbosulfan sulfinamide, carbofuran and dibutylamine was typically less than two-fold (but 2.7-fold difference in dog to human clearance to carbosulfan sulfinamide, and 0.29-fold for rabbit to human clearance to dibutylamine) (Abass et al., 2009).

The objectives of a second, peer-reviewed published paper (not GLP) were:

- to characterize the specific human CYP enzymes involved in carbosulfan biotransformation;
- to determine the in vitro interindividual variability of enzyme kinetic parameters of carbosulfan bioactivation by human hepatic microsomal preparations from a panel of ten donors, six male and four female.

The inhibitory effects of known CYP3A4-selective inhibitors on the formation of carbofuran and carbosulfan sulfinamide were evaluated. Formation rates of metabolites were determined from reaction mixtures (as described above in Abass et al., 2009) incubated in the presence or absence of ketoconazole at 100 µM. The effects of carbosulfan on ethoxyresorufin-*O*-deethylation (EROD) (CYP1A1/2), penthoxyresorufin-*O*-depenthylation (PROD) (CYP2B), 7-ethoxycoumarin-*O*-deethylation (ECOD) (multiple CYPs; predominantly CYP2A6), coumarin-7-hydroxylation (COH) (CYP2A6), bupropion hydroxylation (CYP2B6), amodiaquine deethylation (CYP2C8), tolbutamide methylhydroxylation (CYP2C9), dextromethorphan-*O*-demethylation (CYP2D6), chlorzoxazone-6-hydroxylation (CYP2E1), midazolam-1′-hydroxylation (CYP3A4); omeprazole-5-hydroxylation (CYP2C19) and sulfoxidation (CYP3A4) were also determined. The $IC_{50}$ values for inhibitors (concentration causing 50% reduction of control activity) were determined from duplicate incubations by linear regression analysis from the plot of the logarithm of inhibitor concentration versus percentage of the activity remaining after inhibition. Enzyme kinetic parameters were determined for both recombinantly expressed P450 enzymes (CYP; baculovirus insect cell-expressed human CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, 3A7 and 4A11) and human hepatic microsomal preparations. Carbosulfan concentrations were investigated in the range 2.5–300 µM. In vitro screening assays for carbosulfan metabolites were conducted using human hepatic microsomal preparations as in Abass et al., 2009, described above. Metabolite quantification and identification was accomplished by a combination of HPLC and time-of-flight mass spectroscopy.

Metabolites generated by human hepatic microsomes in vitro (7-phenol-carbofuran, carbofuran, 3-keto-7-phenol-carbofuran, 3-keto-carbofuran, 3-hydroxy-carbofuran, 3-hydroxy-7-phenol-carbofuran and dibutylamine) were consistent with the findings of Abass et al., 2009. The relevant kinetic parameters of the carbosulfan to carbofuran metabolic pathway are shown in Table 2. Across the liver donor population there was a 7.1-fold variation in $V_{max}$, a 5.9-fold difference in $K_M$, and a 6.6-fold difference in $CL_{int}$ (see Table 2). Interindividual variability also occurred in the formation of distal carbofuran metabolites. The formation rate of 3-hydroxy-carbofuran varied from 1.0 to 14.9 nmol/minute per mg protein at 300 µM carbosulfan, displaying a mean value of 5.9 nmol/minute per mg protein and a 15.7-fold variation. The rates of 3-keto-carbofuran formation ranged from 0.02 to 1.1 nmol/min per mg protein,

displaying a mean value of 0.3 nmol/min per mg protein and 73-fold variation.

***Table 2. Kinetic parameters of carbofuran (toxication) metabolic pathway obtained with liver microsome preparations from ten human donors***

| Human liver microsomes[a] | $V_{max}$ (nmol/mg protein per min) | $K_M$ (μM) | $CL_{int}$ (μM/min per mg protein) |
|---|---|---|---|
| HLM20 | 6.8 | 17.3 | 391.2 |
| HLM21 | 8.7 | 33.1 | 264.4 |
| HLM22 | 27.7 | 76.0 | 364.8 |
| HLM23 | 6.2 | 16.4 | 380.7 |
| HLM24 | 8.6 | 12.8 | 670.8 |
| HLM28 | 26.8 | 63.9 | 420.1 |
| HLM29 | 11.5 | 29.3 | 394.2 |
| HLM30 | 11.6 | 24.5 | 472.9 |
| HLM31 | 3.9 | 38.8 | 101.1 |
| HLM32 | 7.5 | 21.1 | 353.9 |
| Variation (fold)[b] | 7.10 | 5.93 | 381.4 |
| Interindividual variability (fold)[c] | Data not supplied | 1.76 | 6.64 |

[a] Each value represents the mean of three determinations; Source: Abass et al., 2010
Combined formation rates for all metabolites of the carbofuran pathway were used to calculate the kinetic parameters;

[b] Differences between the maximum and minimum observed values;

[c] Interindividual variability represents fold differences between mean and the highest value

The CYP3A subfamily was the most metabolically active for carbosulfan metabolism via both the carbofuran metabolic pathway and the carbosulfan sulfinamide metabolic pathway (Table 3). The importance of CYP3A in carbosulfan metabolism was confirmed in a ketoconazole CYP3A inhibition assay where ketoconazole inhibited formation rates of the carbofuran pathway by 90%–97% and, to a lesser extent carbosulfan sulfinamide formation (by 47%–94%). CYP2C8, CYP2C18, and CYP2C19 were associated with the carbofuran metabolic pathway, whereas all tested CYP2C subfamily members were involved in carbosulfan sulfinamide formation. CYP1A1 and CYP2B6 catalyzed both carbosulfan metabolic pathways. CYP1B1 and CYP2E1 did not have detectable activity toward carbosulfan. The relevant kinetic parameters are shown in Table 3. Carbosulfan was not a biologically important inhibitor of CYPs 1A1/2, 1A2, 2A6, 2B, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, or 3A4 in vitro ($IC_{50}$ > 17 μM, and above 100 μM in most cases) (Abass et al., 2010).

***Table 3. Kinetic parameters for the formation of carbosulfan metabolites obtained using human recombinant P450s***

| P450 isoforms | Vmax[a] (nmol/nmol P450 per minute) | Km (μM) | $CL_{int}$ (μL/nmol P450 per minute) | Relative contribution |
|---|---|---|---|---|
| | | **Carbofuran metabolic pathway[b]** | | |
| CYP1A1 | 53.5 | 14.4 | 3709.6 | Not determined |
| CYP1A2 | 42.8 | 68.2 | 626.8 | 0.22 |
| CYP2A6 | 92.2 | 178.3 | 515.9 | 0.60 |
| CYP2B6 | 31.0 | 40.9 | 757.9 | 0.27 |
| CYP2C8 | 61.7 | 106.4 | 580.3 | 0.45 |
| CYP2C18 | 84.7 | 174.9 | 484.4 | Not determined |
| CYP2C19 | 72.9 | 80.8 | 902.8 | 0.41 |
| CYP3A4 | 816.1 | 29.9 | 27330.9 | 98.1 |
| CYP3A5 | 133.5 | 7.6 | 17528.9 | Not determined |
| CYP3A7 | 40.2 | 10.2 | 3952.8 | Not determined |

| P450 isoforms | Vmax[a] (nmol/nmol P450 per minute) | Km (μM) | $CL_{int}$ (μL/nmol P450 per minute) | Relative contribution |
|---|---|---|---|---|
| | | **Carbosulfan sulfinamide metabolic pathway** | | |
| CYP1A1 | 29.2 | 38.6 | 756.4 | Not determined |
| CYP2B6 | 26.9 | 28.0 | 961.4 | 36.8 |
| CYP2C19 | 11.6 | 93.4 | 123.7 | 6.0 |
| CYP3A4 | 25.3 | 170.5 | 148.4 | 57.2 |
| CYP3A5 | 39.9 | 29.7 | 1346.3 | Not determined |
| CYP3A7 | 5.6 | 30.5 | 185.2 | Not determined |

[a] $V_{max}$ and $K_M$ values represent the means of three determination; Source: Abass et al., 2010

[b] Combined formation rates for all metabolites of the carbofuran pathway were used to calculate the kinetic parameters

**(b) In vivo**

Metabolites in the excreta samples collected in Fang & El Naggar (1995) were analyzed using HPLC and TLC, and where possible the identities of the metabolites were subsequently confirmed by gas chromatography–mass spectrometry (GC-MS) or liquid chromatography–mass spectrometry (LC-MS) (see Table 4). The identified metabolites totalled 74%–86% of the dose for the phenyl label and 79%–86% for the dibutylamine label.

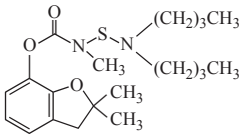
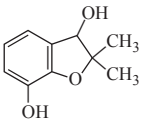
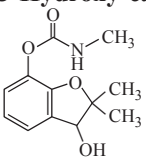
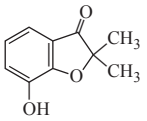
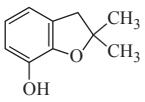
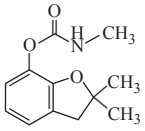
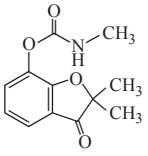
The majority of the applied phenyl-labelled carbosulfan was biotransformed to 3-keto-7-phenol-carbofuran, 3-hydroxy-7-phenol-carbofuran, 7-phenol-carbofuran and 3-hydroxy-carbofuran, with less than 5% remaining as the parent compound. This suggests that carbosulfan mainly undergoes hydrolysis at the C7 ester bond to form 7-phenol-carbofuran (detoxification pathway) and/or at the nitrogen–sulfur bond to form carbofuran (toxication pathway) products. In addition to the hydrolysis, both 7-phenol-carbofuran and carbofuran can undergo oxidation at the C3 position to generate 3-hydroxy metabolites which can be further oxidized to 3-keto metabolites. These hydrolysis and oxidation metabolites were excreted mainly as their sulfate/glucuronide conjugates in rat urine.

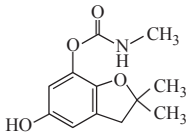
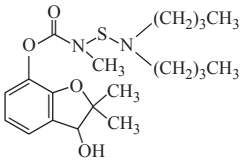
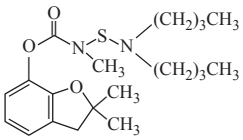
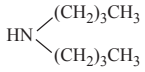
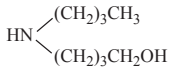
In the case of the dibutylamine-labelled carbosulfan, the major metabolites were dibutylamine, hydroxy-dibutylamines (two structural isomers), and carbon dioxide, with 4%–8% remaining as the parent. Dibutylamine, which results from the nitrogen–sulfur bond hydrolysis, is either eliminated intact in urine and faeces or undergoes oxidation to hydroxy-dibutylamines followed by elimination in urine. The ω-3 oxidation of dibutylamine leads to *N*-dealkylation/deamination and further oxidation to form acids, and finally carbon dioxide via β-oxidation of the intermediate acids.

In the rats treated with single low or high doses of carbosulfan, the relative quantities of metabolites were similar. Compared with rats treated with single doses, the rats treated repeatedly with a low dose of carbosulfan contained more 3-hydroxy-7-phenol-carbofuran and 3-hydroxy-carbofuran, and less 3-keto-7-phenol-carbofuran and 7-phenol-carbofuran.

Overall, carbosulfan metabolism was extensive affecting more than 80% of AR and primarily involving hydrolysis at the C7 ester bond to form 7-phenol-carbofuran and at the nitrogen–sulfur bond to form carbofuran and dibutylamine, which can then be further metabolized. Both 7-phenol-carbofuran and carbofuran can undergo oxidation to generate 3-hydroxy-carbofuran and 3-keto-carbofuran, which are conjugated and eliminated via urine. (Fang & El Naggar, 1995).

*Table 4. Quantitative excretion profile of carbosulfan and its metabolites; results expressed as percentage of administered radioactivity*

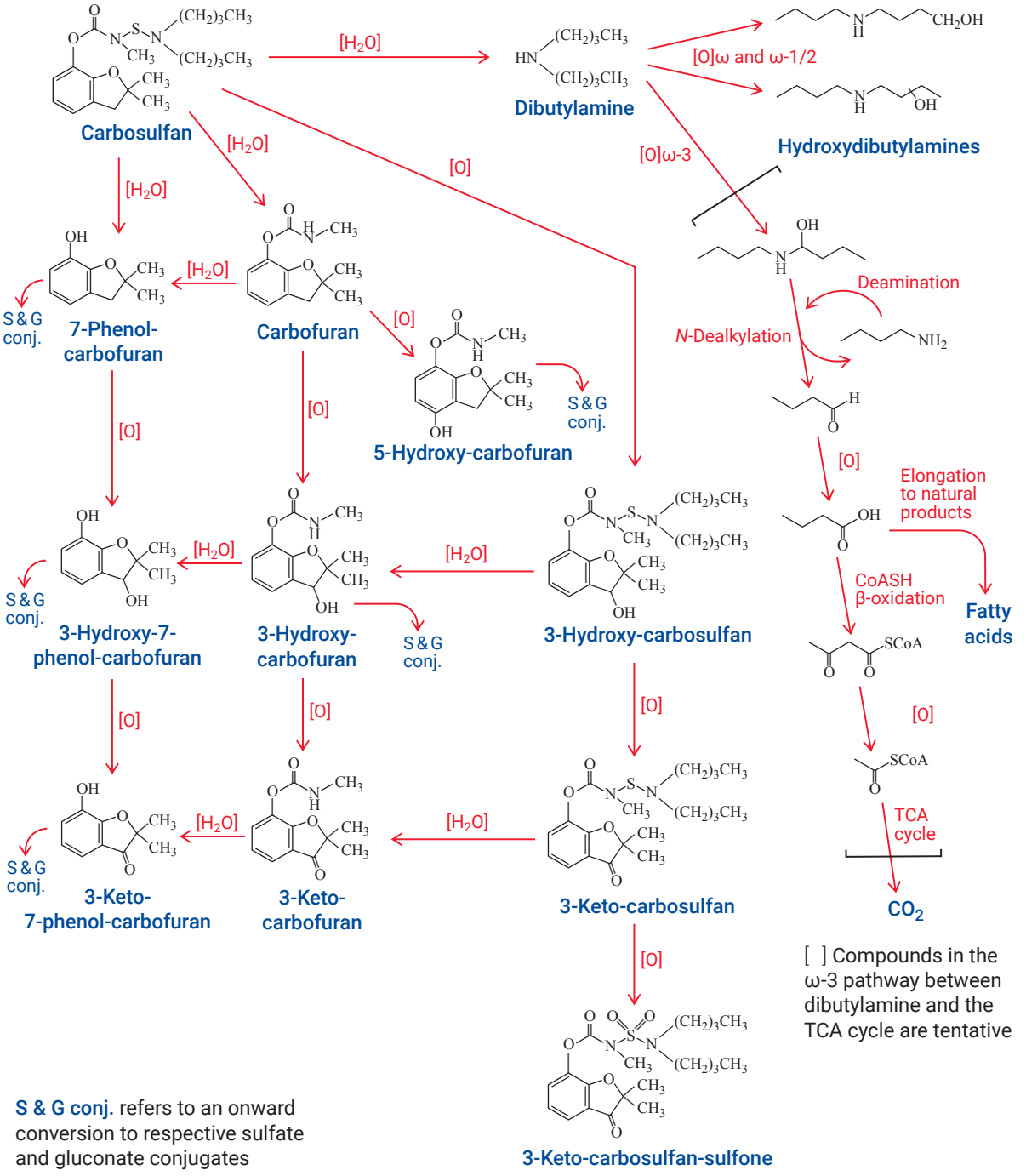
| | Dose (mg/kg bw) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 4 (single dose) | | | | 4 (repeated doses) | | | | 30 (single dose) | | | |
| | Urine | | Faeces | | Urine | | Faeces | | Urine | | Faeces | |
| | M | F | M | F | M | F | M | F | M | F | M | F |
| | Metabolites with $^{14}$C-phenyl label | | | | | | | | | | | |
| **Carbosulfan**  | - | - | 2.5 | 3.4 | - | - | 0.9 | 0.4 | - | 0.3 | 1.7 | 4.4 |
| **3-Hydroxy-7-phenol-carbofuran**[a]  | 14.2 | 12.7 | 1.2 | 0.7 | 25.3 | 25.6 | 0.9 | 0.3 | 17.9 | 17.9 | 0.6 | 0.5 |
| **3-Hydroxy-carbofuran**[a]  | 11.0 | 10.0 | 6.4 | 3.8 | 17.6 | 20.1 | 4.5 | 1.0 | 16.0 | 11.5 | 1.3 | 1.9 |
| **3-Keto-7-phenol-carbofuran**[a]  | 20.3 | 26.3 | 0.4 | 0.3 | 14.4 | 20.7 | 0.2 | 0.1 | 25.7 | 23.9 | 0.2 | 0.4 |
| **7-Phenol-carbofuran**[a]  | 23.5 | 22.8 | 0.4 | 0.3 | 8.8 | 11.6 | 0.1 | 0.0 | 7.3 | 4.8 | 0.1 | 0.3 |
| Carbofuran  | 0.1 | - | 2.7 | 2.6 | 0.1 | - | 2.2 | 0.9 | - | 0.3 | 1.8 | 3.1 |
| 3-Keto-carbofuran  | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 | 0.1 | 0.1 |

| | Dose (mg/kg bw) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 4 (single dose) | | | | 4 (repeated doses) | | | | 30 (single dose) | | | |
| | Urine | | Faeces | | Urine | | Faeces | | Urine | | Faeces | |
| | M | F | M | F | M | F | M | F | M | F | M | F |
| 5-Hydroxy-carbofuran  | 1.9 | 1.8 | 0.3 | 0.2 | - | - | 0.2 | 0.1 | - | 0.0 | 0.3 | 0.7 |
| 3-Hydroxy-carbosulfan  | - | - | 0.3 | 0.2 | - | - | 0.2 | 0.1 | - | 0.0 | 0.3 | 0.7 |
| | **Metabolites with $^{14}$C-dibutylamino label** | | | | | | | | | | | |
| Carbosulfan  | - | - | 6.3 | 8.3 | - | - | 3.5 | 4.2 | - | - | 4.1 | 6.9 |
| **Dibutylamine**[a]  | 33.9 | 36.2 | 4.5 | 6.3 | 37.5 | 42.2 | 3.4 | 2.5 | 36.3 | 46.5 | 2.7 | 4.3 |
| **Hydroxy-dibutylamine**[a]  | 23.8 | 22.5 | - | - | 25.8 | 24.7 | - | - | 23.5 | 17.1 | - | - |

[a] Major metabolites are shown in bold typeface; Source: Fang & El Naggar, 1995

A proposed metabolic pathway is shown in Fig. 4, below.

*Figure 4. Proposed in vivo metabolic pathway of carbosulfan*




(Redrawn from Fang & El Naggar, 1995)

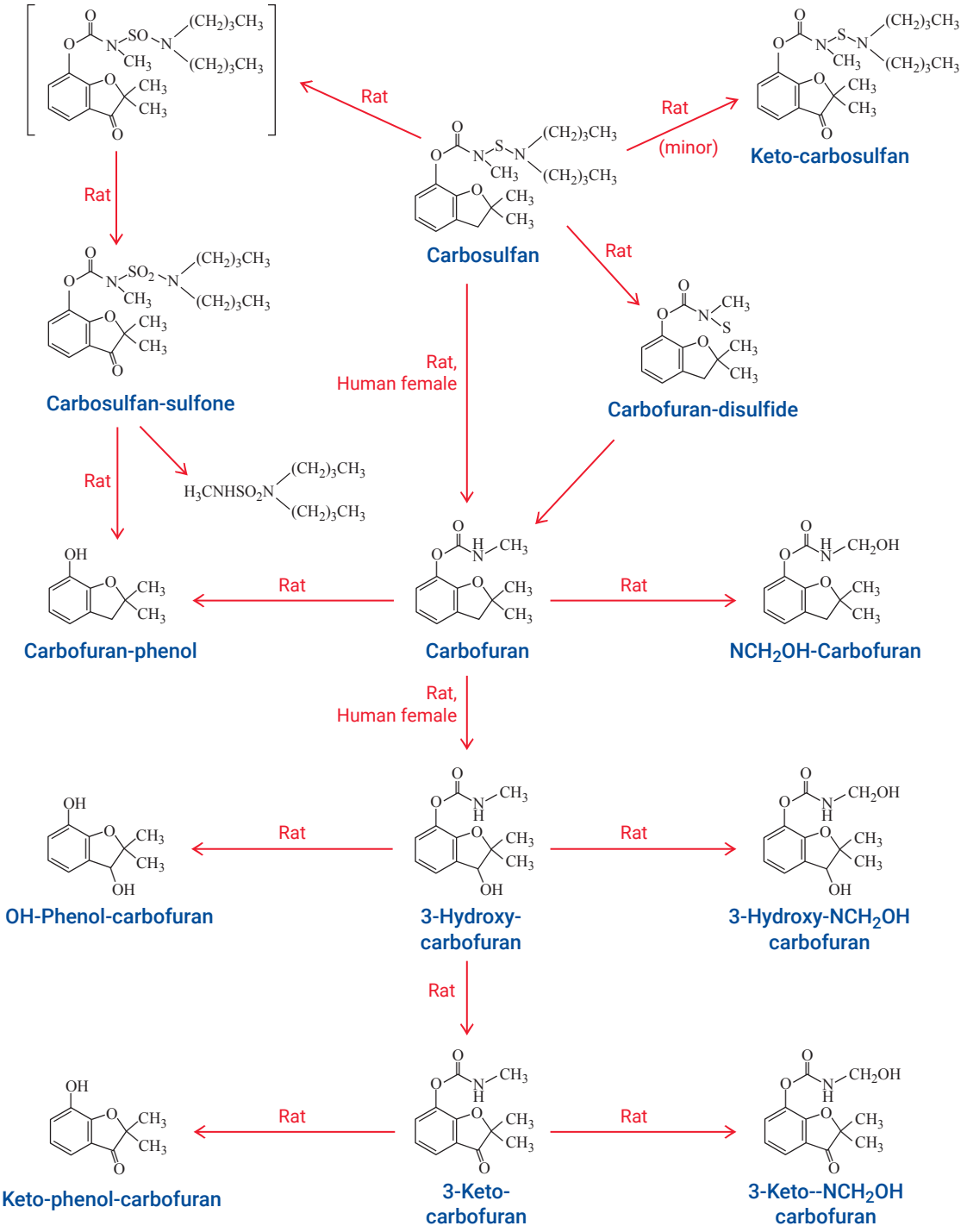
Based on excreta samples collected from Wu, 1982a, the major metabolites (representing 10% or more of AR) in urine included 3-hydroxy-carbofuran (up to 18.2% of the dose), 3-keto-7-phenol-carbofuran (up to 41.8% of the dose), 3-hydroxy-7-phenol-carbofuran (up to 10.3% of the dose) and 7-phenol-carbofuran (up to 11.9% of the dose). Minor metabolites (less than 10% of AR) identified in urine included 3-hydroxy-*N*-hydroxymethyl-carbofuran, 3-keto-carbofuran, and carbofuran. Major metabolites in faeces included 3-hydroxy-carbofuran (up to 30.4% of the dose) and carbofuran (up to 12.8% of the dose) (Wu, 1982a).

In the excreta residue arm of a peer-reviewed published paper (not GLP), fasted female SD rats (number not stated) were orally (gavage) dosed with 30 mg/kg bw of carbonyl-, phenyl- or

dibutylamino-labelled $^{14}C$ carbosulfan (label positions not shown). Excreta and volatile metabolites were collected over 24-hour intervals up to 96 hours post dosing.

Total recovery for all labels exceeded 95.5% of AR. Of the administered radioactivity, 65%–80% was eliminated as carbon dioxide or as urinary and faecal residues. Irrespective of label position, elimination of administered radioactivity was largely complete by 48 hours following dosing, with most of the radioactivity being eliminated within the first 24 hours. For all label positions, urine was a major route of elimination (27.1%, 79.4%, and 49.6% of radioactivity respectively being eliminated in urine with carbonyl, phenyl and dibutylamine-labelled carbosulfan by 24 hours following dosing). With the carbonyl label, 38.2% of AR was exhaled as carbon dioxide, and with the dibutylamine label, 14.4% of AR was exhaled as carbon dioxide or excreted as dibutylamine. None of the radioactivity derived from the phenyl-labelled test article was exhaled. For all label positions, elimination in faeces was a secondary route of elimination. An in vivo metabolic pathway was proposed and is shown in Fig. 3 (Marsden, Ruwano & Fukuto, 1982).

*Figure 5. Proposed in vivo metabolic pathway of carbosulfan*




(Redrawn from Marsden, Ruwano & Fukuto, 1982)

## 1.3 Effects on enzymes and other biochemical parameters

Carbosulfan was not a biologically important inhibitor of CYPs 1A1/2, 1A2, 2A6, 2B, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 or 3A4 in vitro; $IC_{50}$ values were greater than 17 µM, and above 100 µM in most cases (Abass et al., 2010).

# 2. Toxicological studies

## 2.1 Acute toxicity

### (a) Lethal doses

Studies to determine lethal doses for carbosulfan are summarized in Table 5.

***Table 5. Acute oral and dermal toxicity of carbosulfan*** [a]

| Route | Purity | Species | Sex | Result | Comments | References |
|---|---|---|---|---|---|---|
| Oral | 92.5% | Rat | F | $LD_{50}$: > 50–< 300 mg/kg bw | - | Madurapathi, 2014a |
| | 86.4% | Rat | F | $LD_{50}$: > 25–< 300 mg/kg bw | Tremors occurred at ≥ 5 mg/kg bw | Costa Vieira, 2004a |
| | 89.5%–97.6% | Rat | F | $LD_{50}$: 44.4–79.0 mg/kg bw | Unstabilized carbosulfan | Lowe, 2014a |
| | 94.6% | Rat | F | $LD_{50}$: 13.4 mg/kg bw | Unstabilized carbosulfan<br>Tremors occurred at ≥ 7 mg/kg bw | Lowe, 2014b |
| | 94.6% | Rat | F | $LD_{50}$: ≤ 7.9 mg/kg bw | Unstabilized carbosulfan<br>Tremors occurred at ≥ 7.9 mg/kg bw | Lowe, 2014c |
| | 93.1%–95.0% | Rat | F | $LD_{50}$: 44–79 mg/kg bw | Unstabilized carbosulfan<br>Tremors occurred at ≥ 25 mg/kg bw | Lowe, 2015 |
| | 91.6% | Rat | F | $LD_{50}$: 25 mg/kg bw | Cholinergic toxicity occurred at ≥ 5 mg/kg bw | |
| | 92.2% | Rat | M, F | $LD_{50}$: 180 mg/kg bw | Stabilized carbosulfan containing 1.2% carbofuran<br>Clinical signs consistent with cholinergic toxicity occurred at ≥ 180 mg/kg bw | Durando, 2007 |
| | 92.5% | Mouse | F | $LD_{50}$: > 50–< 300 mg/kg bw | - | Madurapathi, 2014b |
| Dermal (occlusive) | 92.5% | Rat | F | $LD_{50}$: > 2000 mg/kg bw | - | Madurapathi, 2014c |
| | 86.4% | Rat | F | $LD_{50}$: > 4000 mg/kg bw | - | Costa Vieira, 2004b |
| | 92.5% | Rat | M, F | $LD_{50}$: 2000 > mg/kg bw | Unstabilized carbosulfan | Lowe, 2007a |
| Inhalation, nose only; aerosol four-hour exposure | 92.5% | Rat | M, F | $LD_{50}$: 0.38 mg/L | MMAD 2.21–2.30 µm, GSD 21.5–2.22 µm | Subashini, 2014 |
| | 86.4% | Rat | M, F | $LD_{50}$: 0.05 mg/L | MMAD and GSD not stated<br>No data on stabilization<br>Epistaxis and ocular mucosal haemorrhage occurred at ≥ 0.0186 mg/L<br>Tremors occurred at 0.1395 mg/L | Penteado, 2005a |
| | 92.2% | Rat | M, F | 100% mortality at ≥ 0.47 mg/L | Unstabilized carbofuran<br>MMAD 2.1–2.4 µm<br>GSD 1.69–1.91 µm | Lowe, 2007b |

| Route | Purity | Species | Sex | Result | Comments | References |
|---|---|---|---|---|---|---|
| Acute; nose only; aerosol one-hour exposure | - | Rat | M, F | > 2.98 mg/L<br>1/5 M deaths at 2.98 mg/L<br>2/5 F deaths at 2.98 mg/L<br>No deaths at 0.3 mg/L | Unstabilized carbofuran<br>MMAD 2.8 µm<br>GSD 2.89 µm | Lowe, 2007c |

M: Male; F: Female; MMAD: Mass median aerodynamic diameter; GSD: Geometric standard deviation; $LD_{50}$: Median lethal dose

[a] Acute studies previously evaluated by JMPR used formulated carbosulfan products rather than technical grade carbosulfan; because of this key limitation these studies have not been summarized in this monograph

**(b) Dermal irritation**

Dermal irritation studies previously evaluated by JMPR used formulated carbosulfan products rather than technical grade carbosulfan. These studies have not been summarized in this monograph.

Semi-occlusive topical dermal exposure of female New Zealand White rabbits to 0.5 mL of carbosulfan (purity 92.5%) did not result in any irritation or systemic toxicity (Madurapathi, 2014d). Likewise, semi-occlusive topical dermal exposure of female New Zealand White rabbits to 0.5 mL of carbosulfan (purity 86.4%) was not irritant in male and female New Zealand White rabbits (Verdolin Milo Collor, 2004b). However, semi-occlusive topical dermal exposure of female New Zealand White rabbits to 0.5 mL of nonstabilized carbosulfan (purity 92.2%) was slightly irritating (primary dermal irritation index of 0.9) in male New Zealand White rabbits (Lowe, 2007e).

**(c) Ocular irritation**

Ocular irritation studies previously evaluated by JMPR used formulated carbosulfan products rather than technical grade carbosulfan. These studies have not been summarized in this monograph.

Topical ocular exposure to 0.1 mL of unstabilized carbosulfan (92.2% purity) without post-treatment rinsing was slightly irritant in male New Zealand White rabbits. Post application rinsing of the eye decreased the level of irritancy (Lowe, 2007d). In a second eye irritation study, topical exposure of the eye to 0.1 mL of carbosulfan (86.4% purity) was slightly irritating (Grade 1 hyperaemia and secretion only at one hour after treatment) in male and female New Zealand White rabbits (Verdolin Milo Collor, 2004a).

**(d) Dermal sensitization**

Dermal sensitization studies using formulated carbosulfan products rather than technical grade carbosulfan have not been summarized in this monograph. Topical exposure to 100% carbosulfan (purity 86.4%) did not induce delayed hypersensitivity in Guinea pigs in a Magnusson & Kligman assay (Penteado, 2005b). Nonstabilized carbofuran (purity 92.2%) diluted 1:1000 in corn oil was not a skin sensitizer in Guinea pigs in a Buehler assay (Lowe, 2007f).

## 2.2 Short-term studies of toxicity

**(a) Oral administration**

***Rat***

In a preGLP, repeat oral (dietary) exposure study, groups of 25rats of each sex per concentration were fed diets for 90 days containing carbosulfan (purity not stated; stabilization unknown) at 0, 10, 20 or 500 ppm (equal to 0, 0.25, 0.50 and 13.6 mg/kg bw per day for males, 0, 0.19, 0.38 and 9.8 mg/kg bw per day for females) . Statistical analyses, when performed, used t-test comparisons which are inappropriate for the study design. No statistical analysis of AChE levels was performed. Dietary concentrations were between 83% and 119% of the target values over the course of the study. There were no adverse test article-related effects on clinical signs, body weight parameters, ophthalmological findings, haematology, serum chemistry, urinalysis findings, organ weights or anatomic pathology findings. One animal per sex in the high-dose cohort died prematurely. Erythrocyte AChE activities were consistently reduced by

more than 20% in both sexes in the high-dose cohort on study days 30, 60 and 90. Brain AChE activity at 500 ppm also reduced by more than 40% or 50 % compared with the control cohort (Table 6). The no observed effect level (NOEL) was 20 ppm (equal to 0.38 mg/kg bw per day) due to inhibition of brain AChE activity by more than 20% at 500 ppm (equal to 9.8 mg/kg bw per day). Benchmark dose (BMD) and BMD lower confidence limit (BMDL) values could not be calculated due to a lack of adequate model fit (Marshall et al., 1979).

***Table 6. Mean erythrocyte and brain acetylcholinesterase activity in rats treated with carbosulfan for 90 days***

| | Dietary concentration (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Study day** | **0** | **10** | **20** | **500** | **0** | **10** | **20** | **500** |
| **Erythrocyte AChE activity (units of activity)** | | | | | | | | |
| 30 | 15.9 | 15.7 | 14.6 | 11.8 | 14.9 | 14.8 | 13.6 | 10.9 |
| 60 | 14.9 | 14.3 | 14.1 | 10.7 | 14.2 | 14.5 | 13.5 | 10.0 |
| 90 | 12.6 | 13.6 | 14.3 | 10.8 | 15.4 | 14.9 | 13.3 | 10.6 |
| BMD[a] | No suitable model | | | | No suitable model | | | |
| **Brain AChE activity (units of activity)** | | | | | | | | |
| 90 | 11.4 | 11.1 | 12.0 | 7.0 | 12.8 | 12.0 | 12.5 | 5.2 |
| BMD | No suitable model | | | | No suitable model | | | |

Source: Marshall et al., 1979

[a] United States Environmental Protection Agency. (2023). BMDS-Online (Build d9394f61; Model Library Version 2023.03) [Web App]. Accessed March 20, 2023.

A second GLP, 90-day repeat oral (gavage) exposure study was conducted in male and female Wistar rats treated with carbosulfan technical at doses of 0, 5, 40 or 75 mg/kg bw per day in corn oil. Clinical signs indicative of Acetylcholinesterase inhibition, such as tremor, salivation and soft stool were observed at 75 mg/kg bw. However, as the study did not measure Acetylcholinesterase activities, the critical end-points, it provided no additional useful information. Accordingly, this study has not been summarized here (Kesavan, 2013a).

***Dog***

In a GLP study, six male and six female beagle dogs were fed diets containing carbosulfan (purity, 94.7%; stabilization unknown) for 26 weeks, at 0, 50, 500 or 2000 ppm. The high dose was reduced to 1000 ppm during study week 2 due to unexpected mortality in females. These dietary concentrations were equal to 0, 1.7, 17.0 and 31.2 mg/kg bw per day for males, 0, 2.0, 16.9 and 31.7 mg/kg bw per day for females, based on the average of the weekly mean carbosulfan intakes. Dietary concentrations were between 96.5% and 104.4% of the target values over the course of the study. Acetylcholinesterase activity in red-blood cells was measured at 1, 3, and 6 months, at termination in the brain. There were no test article-associated adverse effects on ophthalmoscopic observations, food consumption, haematology, urinalysis findings or anatomic pathology findings. Body weight gain was significantly ($p < 0.05$) reduced in the high-dose females.

Based on clinical observations there was a tendency towards an increased incidence in the number of dogs with hypersalivation, and the total animal-days it was observed at 500 ppm and above, although there was no dose–response relationship. A statistically significant reduction in erythrocyte AChE activity (by ca 20% compared to controls) occurred at 1000/2000 ppm when assayed at month 1. At 500 ppm activity was reduced (not statistically significant) by up to 13% (Table 7). Following six months of exposure, brain AChE levels in males were reduced ($p < 0.05$) by 21% at 1000/2000 ppm. In females, brain AChE levels were reduced by 25% or more at all levels of exposure, reaching statistically significance ($p < 0.05$) at 2000/1000 ppm. The lowest-observed-effect level (LOAEL) for the study was 50 ppm (equal to 2 mg/kg bw per day) due to a greater than 20% inhibition of brain AChE compared to controls in females at all dose levels. However, given the poor dose–response relationship and the lack of consistency with effects on erythrocyte AChE, this was not considered a very reliable conclusion (Nye, 1981).

*Table 7. Findings in the six-month oral (dietary) exposure study of carbosulfan in dogs*

| | | Dietary concentration (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | Females | | | |
| | | 0 | 50 | 500 | 2000/1000 | 0 | 10 | 20 | 2000/1000 |
| **Mean body weight (kg) at week 26, [as % of control]** | | | | | | | | | |
| | | 12.147 [100] | 12.683 [104] | 11.140 [92] | 11.787 [97] | 9.433 [100] | 9.023 [96] | 9.067 [96] | 7.640 [81] |
| **Acetylcholinesterase activity (units of activity), [as % of control]** | | | | | | | | | |
| Erythrocyte AChE activity | 1 month | 7.640 [81] | 11.92 [111] | 9.40 [87] | 8.55* [79] | 10.25 [100] | 11.67* [114] | 9.10 [89] | 8.50* [83] |
| | 3 months | 11.83 [100] | 13.02 [110] | 10.22 [86] | 9.65 [82] | 11.38 [100] | 12.07 [106] | 10.88 [96] | 8.55 [75] |
| | 6 months | 14.45 [100] | 14.85 [103] | 10.20 [71] | 9.52 [66] | 9.75 [100] | 11.25 [115] | 9.75 [100] | 8.64 [89] |
| | BMD[a] | | No suitable model | | | | No suitable model | | |
| Brain AChE activity | 6 months | 14.95 [100] | 16.15 [108] | 14.00 [94] | 11.75 [79] | 17.40 [100] | 13.00 [75] | 12.70 [73] | 10.70* [61] |
| | BMD[a] | | No suitable model | | | | No suitable model | | |

[a] United States Environmental Protection Agency. (2023). BMDS-Online (Build d9394f61; Model Library Version 2023.03) [Web App]. Accessed March 20, 2023

Source: Nye, 1981

* Significantly different from control group at $p < 0.05$

### (b) Dermal application

***Rabbit***

In a GLP, repeat-dose toxicity study, groups of six male and six female New Zealand White rabbits were topically (occlusive) exposed to carbosulfan (purity 91.6%; stabilization unknown) for six hours per day for 21 days at doses of 0, 50, 200 or 800 mg/kg bw per day. Plasma and erythrocyte acetylcholinesterase activities were determined before initiation of the study and during the period of exposure on days 10 and 21. Acetylcholinesterase activity in the brain was determined at termination, within two hours of exposure ending. There were no test article-associated adverse effects on mortality, body weight, food consumption, haematology, serum clinical pathology findings, organ weights or anatomic pathology findings (except for the skin at site of application).

One male and two female rabbits in the groups receiving the highest dose died during the study. Some rabbits in the group receiving the lowest dose had soft stools. Animals in the groups receiving the intermediate and highest doses frequently exhibited diarrhoea. In the latter group, one male and several females displayed decreased limb tone, and nasal discharge was observed in two females. All treatment groups showed slight erythema (incidence was dose-dependent) and oedema at the site of application. Erythrocyte AChE activity was decreased by more than 20% at 200 and 800 mg/kg bw (Table 8). Decreases in brain AChE activity were more apparent than decreases in erythrocyte AChE activity, with a more than 20% decrease in brain activity compared with controls, observed in females receiving 50 mg/kg bw per day. The decreases in erythrocyte and brain AChE activities were more severe in females than males but were not statistically significant (at $p > 0.05$) in groups receiving 200 mg/kg bw per day and below. The NOAEL for dermal repeated application in rabbits was 50 mg/kg bw per day based on significant ($p < 0.05$) inhibition of brain AChE of 20 % or more compared to controls in males and females at the next highest dose of 200 mg/kg bw per day (Goldenthal, 1990a).

***Table 8. Effects of topical (occlusive) exposure to carbosulfan on mean acetylcholinesterase activity in rabbits after dermal application***

| | | Dose (mg/kg bw) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | | 50 | | 200 | | 800 | |
| **Substrate** | | **M** | **F** | **M** | **F** | **M** | **F** | **M** | **F** |
| **AChE activity (µM/mL per minute), [as % of control]** | | | | | | | | | |
| Erythrocytes | Pretest | 2.9 | 2.8 | 2.8 [97%] | 3.0 [107%] | 2.7 [93%] | 2.7 [96%] | 3.0 [103%] | 2.7 [96%] |
| | Day 10 | 3.5 | 3.2 | 3.0 [86%] | 2.9 [91%] | 3.0 [86%] | 3.0 [94%] | 3.3 [94%] | 2.4 [75%] |
| | Terminal | 3.1 | 3.2 | 2.6 [84%] | 2.8 [88%] | 2.6 [84%] | 2.5* [78%] | 2.6 [84%] | 2.3* [72%] |
| | $BMD_{20}$[a] | | | | No suitable model | | | | |
| Brain | Terminal | 1.0 | 0.9 | 0.9 [90%] | 0.7 [78%] | 0.7 [70%] | 0.7 [78%] | 0.4** [40%] | 0.4** [44%] |
| | $BMD_{20}$[a] | | | | No suitable model | | | | |

[a] United States Environmental Protection Agency. (2023). BMDS-Online (Build d9394f61; Model Library Version 2023.03) [Web App]. Accessed March 20, 2023.

Source: Goldenthal, 1990a

Significantly different from control group at: * $p < 0.05$; ** $p < 0.01$

A second repeat dermal-exposure toxicity test in rabbits was supplied, which had previously been evaluated at JMPR 2003 as follows:

> "In a study performed in compliance with EPA guideline 82-2, groups of six male New Zealand White rabbits were given carbosulfan (purity, 91.6%) at a dose of 0, 5, 50 or 100 mg/kg bw per day for 21 consecutive days by daily dermal application, under an occlusive wrapping, to the clipped skin of the back (doses were selected on the basis of a range-finding study). The test material, being a liquid, was applied undiluted to the skin, covering < 5%, 5% and 10% of the test site in animals in the groups receiving the lowest, intermediate and highest doses, respectively. Animals in the control group were treated in the same manner, but received no test material. Each day, the test material was removed 6 h after application, by wiping the treated skin with a gauze pad moistened with methanol and subsequently rinsing it with tepid tap water. The animals were observed daily for mortality, clinical signs or signs of dermal toxicity. Body weight and food consumption were measured weekly. After 21 days, the rabbits were killed and necropsies were performed. Clinical chemistry was performed before the start of treatment and at termination. Plasma and erythrocyte AChE activity was determined before the initiation of the study and on days 10 and 21, during the 6 h period of exposure. Acetylcholinesterase activity in one-half brain sections was determined at termination, within 2 h after the end of the exposure. Statements of compliance with GLP and QA were provided. No clinical signs of toxicity were observed. Dose-related slight (all treatment groups) to moderate (highest dose) erythema and oedema were observed. Bodyweight and food consumption were not affected. No treatment-related haematological and biochemical changes were observed. Organ weight was not affected. Except for dermal effects at the site of application, no microscopic changes in the organs were observed. The effects of carbosulfan on AChE activity are summarized in Table [9].
>
> Erythrocyte AChE activity appeared to be far less sensitive to treatment with carbosulfan than plasma or brain AChE activity. On the basis of the statistically significant reduction in brain AChE activity in animals in the group receiving 50 mg/kg bw per day, the NOAEL was 5 mg/kg bw per day."

It was noted that measurements of plasma, erythrocyte and brain AChE activity in this study may have underestimated the level of inhibition induced by carbosulfan. (Goldenthal, 1990b).

***Table 9. Effects of carbosulfan on acetylcholinesterase activity[a] in male rabbits after dermal application***

| | | Dose (mg/kg bw) | | |
|---|---|---|---|---|
| **Substrate** | | **5** | **50** | **200** |
| Erythrocytes | Day10 | 81 | 86 | 74 |
| | Terminal | 93 | 100 | 90 |
| Plasma | Day10 | 89* | 67* | 67* |
| | Terminal | 75* | 63* | 63* |
| Brain | Terminal | 92 | 77* | 69* |

Source: Goldenthal, 1990b, with reference to JMPR 2003 (JMPR, 2004)

[a] As acetylcholinesterase activities varied considerably over time, AChE activity of animals in the treatment groups is expressed as percentage of the value for concurrent controls rather than as percentage of the pretest value;

* Significantly different from control

A 28-day, repeat-dose, dermal exposure study was conducted in rabbits. The study did not measure cholinesterase activity, the critical end-point. The study provided no additional useful information so has not been summarized here (Kesavan, 2013b).

**(c) Exposure by inhalation**

Groups of five male and five female Sprague Dawley rats received whole-body exposure to air containing liquid droplets of carbosulfan technical (purity 89.6%) stabilized in epoxidized soybean oil, for six hours per day, for five days. Mean actual concentrations to which the animals were exposed were 5.4, 15.6 and 47 µg/L. A control group was exposed to room air. An additional group of five rats of each sex were killed before treatment to establish AChE activity before exposure to the test material. During exposure, the groups of animals were observed regularly for clinical signs. Detailed observations of individual animals were carried out before and after exposure. Body weights were measured before exposure on day 1 and after exposure on day 5. After the exposure on day 5 the animals were killed and necropsies performed; AChE activity was determined in erythrocytes, plasma and brain. Histopathology was not performed. Statements of compliance with GLP and QA were provided. The MMAD ranged from 1.9 to 2.6 µm.

Dose-related, adverse effects were observed in all carbosulfan-exposed groups. Females tended to be more sensitive to exposure than males. During exposure, muscle fasciculation was observed in all treatment groups. After exposure, anogenital staining, decreased activity, lacrimation and irregular breathing were observed to occur in a dose-dependent manner in all groups. Additionally, salivation and tremors were observed in the group receiving the highest dose. On day 5, body weights in all treatment groups tended to be lower than those of controls; this effect reached statistical significance ($p < 0.05$) in males at the highest dose. Except for discolouration of the anogenital and nasal area, no treatment-related macroscopic changes were observed. The effects of carbosulfan on AChE activity are summarized in Table 10.

***Table 10. Effects on acetylcholinesterase activity in rats exposed of carbosulfan by inhalation for five days, (percentage of control value shown)***

| | Dose (µg/L) | | | | | |
|---|---|---|---|---|---|---|
| | **5.4** | | **15.6** | | **47** | |
| **Substrate** | **M** | **F** | **M** | **F** | **M** | **F** |
| Erythrocyte | 95 | 95 | 92* | 90* | 94 | 87* |
| Plasma | 61* | 53 | 60* | 42* | 49* | 37* |
| Brain | 59* | 52* | 54* | 52* | 38* | 40* |

* Significantly different from control; Source: Whitman, 1990, with reference to JMPR 2003 (JMPR, 2004)

No consistent sex differences in AChE inhibition were found. Owing to the reduction in brain AChE activity and the clinical signs observed during and after exposure in the groups receiving the lowest dose, a no-observed-adverse-effect concentration (NOAEC) could not be identified. The lowest-observed-adverse-effect concentration (LOAEC) was 5.4 mg/L (Whitman, 1990). Critically, the animals were killed up to three hours and 20 minutes after the end of the final exposure. The effects of carbamates are known to be transient. Accordingly, inhibition of blood and brain AChe activities may be below their peak at the time of sampling in this study.

A follow-up inhalation study (not submitted for this monograph) was evaluated by JMPR 2003:

> "Groups of 10 male and 10 female Sprague Dawley rats received whole-body exposure to air containing liquid droplets of carbosulfan technical (purity, 89.6%) stabilized in epoxidized soybean oil, 6 h per day, 5 days per week for 3 weeks. Mean actual concentrations to which the animals were exposed were 0.15, 0.65 and 5.34 µg/L. An additional group of 10 rats of each sex was killed before treatment, to establish cholinesterase activity before exposure. During exposure, the groups of animals were observed hourly for clinical signs. Detailed observations of individual animals were carried out before and after exposure. Body weights were measured before study initiation, weekly at the start of the exposure, and before and after exposure on the last day. Ophthalmological examinations were performed before study initiation and during the last week of exposure. Within 3 h 20 min after the last exposure, the animals were killed and necropsies were performed. Blood and brain tissue were collected for haematology and clinical chemistry, including determination of cholinesterase activity in erythrocytes, plasma and brain. Organs were dissected and weighed, and microscopical examination was performed on a full range of organs and tissues. Statements of compliance with GLP and QA were provided. The MMAD ranged from 1.9 to 2.0 µm. No clinical signs were observed in animals at the lowest and intermediate doses. At the highest dose, fasciculations, decreased activity, irregular and laboured breathing, salivation, lacrimation, and dried nasal discharge, anogenital staining, soft stool were observed during or after exposure. No treatment-related effects on ophthalmology and body weight were observed. Occasional findings on macroscopical and histopathological examinations, on organ weights and on blood urea nitrogen values were considered to be incidental and not related to treatment. The effects of carbosulfan on cholinesterase activity are summarized in Table [11]. Erythrocyte cholinesterase activity appeared to be far less sensitive to treatment with carbosulfan than plasma or brain cholinesterase activity. No consistent sex differences in cholinesterase inhibition were found. Although statistically significant, only small decreases in brain cholinesterase activity were observed at the intermediate dose (7% in males, 11% in females). Moreover, no clinical signs of toxicity were observed in this group. On the basis of the occurrence of clinical signs and inhibition of brain cholinesterase activity in animals at the highest dose, the NOAEC was 0.65 µg/L. The Meeting noted that the animals were killed within 5 h 52 min after the end of the final exposure. The effects of carbamates being known to be transient, plasma concentrations of carbosulfan at this time may be below peak concentrations. Therefore, measurements of plasma, erythrocyte and brain cholinesterase activity in this study may underestimate the level of inhibition induced by carbosulfan."

A 28-day repeat inhalation exposure study was conducted in male and female Wistar rats, in which animals were exposed to carbosulfan technical at concentrations of 0, 0.05, 0.14 or 0.21 mg/L for six hours per day. The study did not measure cholinesterase activity or the critical end-points, and provided no additional useful information. Accordingly, the study has not been summarized here (Subashini, 2013).

## 2.3 Long-term studies of toxicity and carcinogenicity

***Mouse***

In a GLP study groups of 100 male and 100 female Charles River CD-1 mice were given diets for 24 months containing carbosulfan (purity 94.5%–95.6%, containing 0.6%–2.4% carbofuran) at concentrations of 0, 10, 20, 500 or 2500 ppm (equal to 0, 1.3, 2.5, 62 and 320 mg/kg bw per day for males, 0, 1.5, 3.1, 72 and 337 mg/kg bw per day for females,). Dietary concentrations fell within 94%–98% of the target levels. There were no test article-associated adverse effects on mortality and survival, water consumption, haematology and clinical chemistry, organ weights, neoplasia or anatomical pathology findings.

An increased incidence of eye-related observations (corneal opacity, eccentric pupil, irregular shape, darkly coloured, white, and cloudy eyes) occurred in the high-dose cohort. Evidence of discharge from various body cavities was more frequently reported among male mice from the 500 and 2500 ppm dosage group than for the control males. Significantly ($p < 0.05$) and consistently reduced body weights

(by 10% or more compared to controls) occurred in the high-dose male cohort and in high-dose females during study weeks 1–4, 28, 36–40, 48–96, 100 and 102. Significantly lower body weights ($p<0.05$) were also noted among other treated groups: male animals receiving the 500 ppm (study weeks 1–64, 72–78 and 82–104) and the 20 ppm (study weeks 32–34, 42–50, 54–66, 70–98 and 102–104). However, the absolute mean weights were within 10% of control values. During an interval including study weeks 30–52, all female treated groups exhibited lower mean body weights than controls. However, only high-dose (2500 ppm) female body weights were depressed by more than 10% compared to control values during this interval. These effects generally correlated with reduced food intake. Significantly reduced erythrocyte and brain AChE activity ($p<0.05$; a greater than 20% reduction compared to controls) occurred in dose groups receiving 500 ppm and above.

The NOAEL for chronic toxicity in mice was 20 ppm (equal to 2.5 mg/kg bw per day) based on reduced (by at least 20%) brain AChE activity at 500 ppm. There was no evidence of carcinogenicity up to the highest dose tested, 2500 ppm (equal to 337 mg/kg bw per day) (DeProspo, Norvell & Fletcher, 1982a)

***Table 11. Key findings in the 104-week repeat-dose dietary exposure study in mice***

| | Dietary concentration (ppm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Males | | | | | Females | | | | |
| **Parameter** | **0** | **10** | **20** | **500** | **2500** | **0** | **10** | **20** | **500** | **2500** |
| **Mean body weight (g); Mean ± SD shown** | | | | | | | | | | |
| Week 104 | 41.7 | 40.6 | 39.3* | 39.5* | 35.1* | 34.5 | 37.6* | 35.7 | 34.9 | 31.6* |
| **Erythrocyte acetylcholinesterase activity (units[b]) Mean ± SD** | | | | | | | | | | |
| 6 months | 3.8 ±0.96 | 4.0 ±1.03 | 4.0 ±0.43 | 3.2 ±0.66 | 2.7* ±0.67 | 5.1 ±1.29 | 5.3 ±1.15 | 5.7 ±0.82 | 4.3 ±1.1 | 3.8* ±0.9 |
| 12 months | 4.8 ±0.75 | 4.7 ±0.96 | 4.5 ±0.65 | 3.8* ±0.79 | 3.3* ±0.56 | 5.2 ±1.07 | 5.1 ±0.7 | 5.3 ±0.9 | 4.0* ±0.74 | 3.1* ±1.04 |
| 18 months | 4.5 ±1.34 | 3.8 ±0.87 | 4.5 ±0.91 | 2.7* ±0.94 | 2.2* ±0.50 | 4.2 ±1.14 | 4.6 ±1.2 | 4.6 ±1.22 | 2.8* ±0.85 | 2.7* ±1.77 |
| 24 months | 5.0 ±2.16 | 5.5 ±2.45 | 4.5 ±2.27 | 3.4 ±1.78 | 2.3* ±1.03 | 3.9 ±1.12 | 4.1 ±1.1 | 4.2 ±1.46 | 3.4 ±0.75 | 2.7* ±0.7 |
| **Brain acetylcholinesterase activity (units[b]) Mean ± SD** | | | | | | | | | | |
| 6 months | 24.7 ±10.50 | 24.8 ±9.26 | 25.9 ±7.52 | 15.7* ±8.69 | 11.8* ±5.23 | 26.3 ±9.85 | 22.9 ±11.74 | 23.9 ±8.2 | 13.2* ±6.96 | 11.5* ±6.71 |
| 12 months | 17.9 ±2.55 | 17.9 ±2.51 | 18.6 ±3.7 | 11.4* ±3.42 | 7.6* ±3.06 | 16.3 ±3.53 | 18.4 ±3.41 | 17.9 ±2.98 | 11.3* ±3.19 | 6.9* ±1.09 |
| 18 months | 13.7 ±2.76 | 15.8 ±4.40 | 14.1 ±5.02 | 9.4* ±5.17 | 5.9* ±3.5 | 13.5 ±4.73 | 14.6 ±3.22 | 13.4 ±3.28 | 8.7* ±3.3 | 5.4* ±2.34 |
| 24 months | 37.5 ±5.22 | 39.2 ±36.4 | 36.4 ±7.34 | 26.9* ±8.74 | 18.5* ±5.38 | 38.1 ±3.39 | 36.3 ±5.5 | 37.0 ±7.9 | 23.7* ±5.98 | 21.0* ±5.84 |
| **Mean depression in acetylcholinesterase activity at 104 weeks (% depression from initial value)** | | | | | | | | | | |
| Erythrocytes | - | 2 | 8 | 36* | 48* | - | 4 | 7 | 34* | 44* |
| BMD[a] | | No suitable model | | | | | No suitable model | | | |
| Brain | - | 3.8 | 1.8 | 48.8* | 64.5* | - | 0.5 | 5.5 | 49.5* | 60.0* |
| BMD[a] | | No suitable model | | | | | No suitable model | | | |

BMD: Benchmark dose; SD: Standard deviation Source: DeProspo, Norvell & Fletcher, 1982a

[a] United States Environmental Protection Agency. (2023). BMDS-Online (Build d9394f61; Model Library Version 2023.03) [Web App]. Accessed March 20, 2023.

[b] 1 unit = 1 µMol of acetylcholine degraded/mL/minute;

* Significantly different from controls

***Rat***

In a GLP dietary exposure carcinogenesis study, groups of 90 male and 90 female CD rats were fed diets for 104 weeks containing carbosulfan (purity 94.5%–95.6%, containing 0.6%–2.4% carbofuran; stabilization unknown) at concentrations of 0, 10, 20, 500 or 2500 ppm (equal to 0, 0.5, 1.0, 26.8 and 152.8 mg/kg bw per day for males, 0, 0.6, 1.2, 34.7 and 213.3 mg/kg bw per day for females). Dietary test article concentrations were within 92%–102% of target values. There were no test article-associated adverse effects on mortality and survival, food consumption in females, water consumption, haematology and clinical chemistry findings, organ weights, neoplastic findings and non-neoplastic anatomic pathology findings (except for the eye).

Mean body weights were significantly ($p < 0.05$) and consistently decreased (by 20% or more compared to controls at week 104) in a dose-related manner at 500 ppm and above. This correlated with consistently reduced ($p < 0.05$) food consumption in males at 500 ppm and above. An increased incidence of tremors, laboured breathing, eye-related observations (corneal opacity and white coloured eyes), and various types of discharge occurred in the high-dose cohort. An increased incidence of hair loss occurred at 20 ppm and above, and the incidence of discoloured and swollen skin areas was increased at 500 ppm and above. An increased incidence of iris lesions (described as iris atrophy, iris coloboma or absence of iris tissue) occurred at 500 ppm and above. Ophthalmoscopically, the affected eyes exhibited an absence of stroma on both sides of the iris collarette and in the temporoventral iris quadrants. Histologically, the thinning of the iris appeared to spare the ciliary epithelium with the greatest loss of tissue occurring in the iris stroma. Inflammatory cells were not identified ophthalmoscopically or histologically. The female high-dose cohort had an increased incidence of pale ocular fundi and degenerative retinopathy. Both erythrocyte and brain AChE were inhibited ($p < 0.05$) by more than 20% compared to controls at 500 ppm and above.

The NOAEL for chronic toxicity in rats was 20 ppm (equal to 1.0 mg/kg bw per day) based on a greater than 20% inhibition of brain AChE activity, an increased incidence of focal iris atrophy, and greater than 20% depression in body weight compared to controls at 500 ppm (equal to 26.8 mg/kg bw per day). The NOAEL of 20 ppm was essentially equal to the $BMD_{20}$ and $BMDL_{20}$ values for inhibition of brain AChE in females. There was no evidence for carcinogenicity up to the highest dose tested, 2500 ppm (equal to 152.8 mg/kg bw per day) (DeProspo, Norvell & Fletcher, 1982b).

***Table 12. Key findings in the 104-week repeat dietary exposure study in rats***

| | Dietary concentration (ppm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Males | | | | | Females | | | | |
| **Parameter** | **0** | **10** | **20** | **500** | **2500** | **0** | **10** | **20** | **500** | **2500** |
| **Mean body weight (g)** | | | | | | | | | | |
| Week 104 | 759.6 | 753.4 | 788.1 | 598.4* | 475.8* | 490.2 | 500.8 | 490.3 | 426.6* | 299.5* |
| **Ophthalmology findings (incidence; number affected/number examined)** | | | | | | | | | | |
| Absence of iris tissue at 104 weeks | 0/37 | 0/43 | 0/45 | 5/43* | 16/39 | 0/38 | 0/39 | 0/41 | 8/45 | 26/47 |
| Focal iris atrophy | 0/56 | 0/57 | 0/57 | 9/50 | 12/50 | 0/52 | 0/58 | 0/59 | 8/55 | 17/53 |
| Degenerative retinopathy | 0/56 | 1/57 | 1/57 | 0/50 | 0/50 | 1/52 | 2/58 | 1/59 | 1/55 | 14/53 |
| Pale ocular fundi | 3/37 | 7/43 | 2/45 | 1/43 | 3/39 | 5/37 | 4/39 | 5/41 | 3/46 | 11/47 |
| **Erythrocyte acetylcholinesterase activity (units[b]) Mean ± SD** | | | | | | | | | | |
| 6 months | 5.5 ±1.57 | 5.0 ±0.69 | 4.9 ±0.60 | 3.1* ±0.62 | 2.2* ±0.37 | 5.0 ±0.80 | 4.9 ±0.88 | 4.9 ±0.88 | 3.2* ±0.60 | 2.9* ±0.75 |
| 12 months | 4.9 ±0.44 | 5.1 ±0.72 | 4.6 ±0.63 | 3.4* ±0.51 | 3.1* ±0.42 | 5.4 ±0.64 | 5.4 ±0.61 | 5.0 ±0.28 | 3.6* ±0.28 | 3.2* ±0.51 |
| 18 months | 5.1 ±.90 | 5.1 ±0.98 | 4.6 ±1.17 | 3.0* ±0.61 | 2.4* ±0.31 | 5.6 ±0.79 | 5.3 ±0.63 | 4.9 ±0.58 | 3.6* ±0.67 | 2.7* ±0.31 |
| 24 months | 4.9 ±0.74 | 4.7 ±0.84 | 4.6 ±0.85 | 3.6* ±0.60 | 2.8* ±0.69 | 5.0 ±0.96 | 44.5 ±0.46 | 4.7 ±0.57 | 3.5* ±0.50 | 3.0* ±0.60 |

| | Dietary concentration (ppm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Males | | | | | Females | | | | |
| **Parameter** | **0** | **10** | **20** | **500** | **2500** | **0** | **10** | **20** | **500** | **2500** |
| **Brain acetylcholinesterase activity (units[b]) Mean ± SD** | | | | | | | | | | |
| 6 months | 4.4 ±1.09 | 3.9 ±0.91 | 5.0 ±1.48 | 2.4* ±1.72 | 1.9* ±1.43 | 4.9 ±1.74 | 5.0 ±1.07 | 5.2 ±0.88 | 3.5 ±2.74 | 3.0* ±1.83 |
| 12 months | 4.4 ±1.29 | 4.7 ±1.08 | 4.6 ±0.82 | 1.7* ±0.23 | 1.4* ±0.13 | 4.8 ±1.11 | 4.7 ±0.74 | 4.3 ±0.99 | 1.7* ±0.49 | 1.4* ±0.25 |
| 18 months | 4.6 ±1.19 | 4.4 ±0.90 | 4.3 ±1.28 | 2.5* ±0.75 | 1.5* ±0.73 | 5.1 ±0.72 | 5.2 ±0.89 | 5.2 ±1.35 | 2.5* ±0.83 | 1.5* ±0.50 |
| 24 months | 5.6 ±0.90 | 6.0 ±1.72 | 6.1 ±2.75 | 3.2 ±0.84 | 1.9* ±0.50 | 6.4 ±1.40 | 6.5 ±0.91 | 5.6 ±1.07 | 3.0* ±0.61 | 2.6* ±1.03 |
| **Mean depression in acetylcholinesterase activity at 104 weeks (% depression from initial value)** | | | | | | | | | | |
| Erythrocytes | - | 3.3 | 8.3 | 35.8* | 48.3* | - | 4.3 | 6.8 | 33.8* | 43.8* |
| $BMD_{20}$[a] | | | 362 ppm | | | | | 212 ppm | | |
| $BMDL_{20}$[a] | | | 192 ppm | | | | | 42 ppm | | |
| Brain | - | 3.8 | 1.8 | 48.8* | 64.5* | - | 0.5 | 5.5 | 49.5* | 60.0* |
| $BMD_{20}$[a] | | | No suitable model | | | | | 21 ppm | | |
| $BMDL_{20}$[a] | | | No suitable model | | | | | 20 ppm | | |

BMD: Benchmark dose; SD: Standard deviation Source: DeProspo, Norvell & Fletcher, 1982a

[a] BMD analysis shown in Appendix 1; a $BMD_{20}$ is the predicted dose resulting a benchmark response of 20% inhibition;

[b] 1 unit = 1 µMol of acetylcholine degraded/mL/minute;

* Significantly different from controls

## 2.4 Genotoxicity

### (a) In vitro studies

Carbosulfan was evaluated in an adequate test battery of in vitro genotoxicity assays. Based on the available weight of evidence carbosulfan is not genotoxic in vitro.

***Table 13. Summary of in vitro genotoxicity studies with carbosulfan (all studies GLP unless otherwise stated)***

| End-point | Test system | Concentrations tested | Purity[a] | Results and comments | Reference |
|---|---|---|---|---|---|
| Bacterial reverse mutation | *S. typhimurium* TA98, TA100, TA102, TA1535, TA1537 | Test 1: pre-incubation with 0.079, 0.157, 0.313, 0.625, 1.25, 2.5 and 5 µL/plate ±S9<br>Test 1: plate incorporation with 0.313, 0.625, 1.25, 2.5 and 5 µL/plate ±S9 | 92.0% | Negative | Parvathi (2014a) |
| Bacterial reverse mutation | *S. typhimurium* TA97a, TA98, TA100, TA102, TA1535 | Plate incorporation with 0.001, 0.01, 0.1, 1.0 and 5.0 mg/plate ±S9 | 90.75% | Negative | Miyaji (2007) |

| | | | | | |
|---|---|---|---|---|---|
| Bacterial reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA153<br>*E. coli* WP2 uvrA | Test 1:<br>plate incorporation with 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg/plate<br>± S9<br>Test 2:<br>plate incorporation with 50, 150, 500, 1500 and 5000 µg/plate<br>± S9 | 87.2% | Negative; precipitation at 1500 µg/plate and above | Wagner & VanDyke (2006) |
| Bacterial reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538 | Test 1:<br>plate incorporation with 0.003, 0.01, 0.1, 0.3, 1.0, 3.1, 10 µL/plate<br>± S9<br>Test 2:<br>plate incorporation method with 0.1, 0.5, 2.5, 5 and 10 µL/plate<br>± S9 | 93% | Negative | Haworth et al. (1980) |
| Mammalian gene mutation | L5178Y $tk^{+/-}$ | Study 1:<br>3-hour treatment using 0.001, 0.002, 0.004 and 0.008 µg/mL<br>± S9<br>Test 2:<br>24-hour treatment using 0.001, 0.002, 0.004 and 0.008 µg/mL<br>– S9 | 92.5% | Negative | Parvathi, 2014d |
| Mammalian gene mutation | Chinese Hamster ovary cells/HGPRT locus | 5-hour treatment 20.57, 6.85, 2.28, 0.76 and 0.25 µg /mL<br>± S9 | 87.2% | Negative | Sadhu, (2008a)[b] |
| Mammalian gene mutation | Mouse lymphoma L5178Y $tk^{+/-}$ | Study 1:<br>4-hour treatment using 0.0042, 0.0075, 0.010, 0.018, 0.024 and 0.032 µL/mL<br>– S9<br>Study 2:<br>4-hour treatment using 0.0056, 0.0075, 0.01, 0.013, 0.018, 0.024, 0.032, 0.042, 0.056 and 0.075 µL/mL<br>– S9 | 93% | Negative | Kirby et al. (1981)[b] |
| Chromosomal aberration | Chinese Hamster ovary cells | 3-hour treatment (± S9) and 18-hours (– S9) both using 0.079, 0.157, 0.313 µL/mL | 92.5% | Negative Cytotoxicity ≥ 1.25 µL/mL | Parvathi, 2014c |
| Chromosomal aberration | Chinese Hamster ovary (CHO) cells | 3-hour treatment using 0.76, 2.28, 6.85, 20.57 µg/mL<br>± S9 | 87.2 | Negative | Sadhu, 2008b |

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*;
*S. typhimurium*: *Salmonella typhimurium*; *E. coli*: *Escherichia coli*;

[a] Stabilization status reported as "unknown" for all studies;

[b] OECD deleted the SCE test (former Test Guideline 479) in 2014 due to concerns regarding reliability and interpretability

### (a) In vivo studies

Carbosulfan was evaluated in an adequate test battery of in vivo genotoxicity assays. Based on the available weight of evidence carbosulfan is not genotoxic in vivo.

***Table 14. Summary of in vivo genotoxicity studies with carbosulfan (all studies GLP unless otherwise stated)***

| End-point | Test system | Concentrations tested | Purity [a] | Results and comments | Reference |
|---|---|---|---|---|---|
| Chromosomal aberration | Mouse bone marrow cells | 0, 37.5, 75, 150 mg/kg bw by oral gavage with fixation at 18- and 42-hours following dosing | 88.4% | Negative<br>Clinical signs of toxicity at all doses, | Krsmanovic, 2005 |
| Chromosomal aberration | Rat bone marrow cells | 0, 5, 12, 30 mg/kg bw administered as a single dose or as a daily dose over 5 consecutive days by oral gavage with fixation at 6, 24 and 48 hours following in the single oral dose cohort, and at 6 hours following the last dose in repeated oral dosing cohort | 93% | Negative<br>Non-GLP | Putman & Schechtman, 1981 |
| Micronucleus formation | Mouse bone marrow cells | Single oral administration of 0, 30, 60 or 90 mg/kg bw and sampling at 24 and 48 hours post treatment<br>Two oral administrations 24 hours apart 90 mg/kg bw and sampling at 24 hours post treatment | 92.52% | Negative | Parvathi, 2014b |
| Micronucleus formation | Mouse bone marrow cells | Study 1:<br>0, 43.5, 87, 174 mg/kg bw by oral gavage | 93% | Negative<br>High mortality in dose groups 87 mg/kg bw and above | Kirkhart, Jones & Skinner, 1979 |
| Dominant lethal mutation | Mice | 0, 7, 20, 60 mg/kg bw by oral gavage for five consecutive days | 93% | Negative | Preache, (1981) |

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*

[a] Stabilization status reported as "unknown" for all studies;

## 2.5 Reproductive and developmental toxicity

### (a) Multigeneration studies

***Rat***

A three-generation toxicity study was evaluated by JMPR 2003 (JMPR, 2004). A re-evaluation of this study for this monograph concurred with the findings of JMPR 2003:

> "Groups of 15 male and 30 female Charles River CD rats were given diets containing carbosulfan at a concentration of 0, 10, 20 and 250 mg/kg (equivalent to 0, 0.67, 1.3 and 16.7 mg/kg bw per day) for three generations. Two successive litters were reared from each female. General condition and behaviour were observed routinely and individual body weights were recorded throughout the study. The number of pups in each litter was examined and pups were culled to 10 per litter at age 4 days. Individual pup weights were measured on days 0, 4, 7, 14 and 21. Ten male and 10 female F1b, F2b, and F3b weanlings were randomly selected for gross necropsy and tissue collection. The F1a and F2a litters were discarded at weaning, and the F1b and F2b litters were used to produce succeeding generations.

Weanlings not selected for continuation in the study (F1a, F2a, F1b, F2b, F3a and F3b) were subjected to gross external examination and sacrificed and discarded. A statement of compliance with quality assurance was provided.

Body weights of F0 males and females at 20 and 250 mg/kg showed initial decreases. These decreases were associated with reduced food consumption, and both recovered to normal levels between week 4 and sacrifice. Body weights of parental males (F1 and F2) receiving the diet containing carbosulfan at 250 mg/kg were consistently lower than those of animals in the corresponding control group. A similar effect was observed in females at 250 mg/kg at some times during gestation and lactation, but not during the growth phases.

Mating index, gestation index and number of viable foetuses were essentially normal throughout the study, except for F2b dams at 250 mg/kg, for which values for these parameters were decreased. Litter size, pup weights and/or pup weight gains for all litter sets in the group receiving carbosulfan at 250 mg/kg were significantly lower at most age intervals between birth and weaning than for the concurrent control group. Neonatal survival at 250 mg/kg was also significantly lower for the first four litters (F1a, F1b, F2a and F2b). There were no treatment-related gross or histological changes observed among the F0, F1b and F2b adults or the F1b, F2b and F3b weanlings. Carbosulfan did not have an adverse effect on reproductive performance. Pup weight, litter size, and pup survival was decreased at 250 mg/kg.

In parental animals, the NOAEL was 20 mg/kg (equivalent to 1.3 mg/kg bw per day), on the basis of the decreases in body weight. The NOAEL for offspring was 20 mg/kg (equivalent to 1.3 mg/kg bw per day) on the basis of reductions in litter size, pup weight and pup weight gain. The NOAEL for reproductive toxicity was 250 mg/kg (equivalent to 16.7 mg/kg bw per day, the highest dose tested) (Kehoe & MacKenzie, 1982).” (Evaluated in JMPR, 2004.)

### (b) Developmental toxicity

***Rat***

A rat pre-natal developmental study was evaluated by JMPR 2003 (JMPR, 2004):

“Groups of 25 female Charles River CD rats were given carbosulfan at a dose of 0, 2, 10 and 20 mg/kg bw per day by oral gavage on days 6–19 of gestation. Necropsies were performed on surviving females on day 20 and on foetuses delivered by hysterotomy. The number and position of viable/non-viable foetuses, early/late resorptions, mean number of corpora lutea and total number of implantations were recorded. External, internal and skeletal examinations of fetuses were performed. One-third of the fetuses were evaluated for soft-tissue anomalies and the remaining two-thirds for skeletal effects. A statement of compliance with quality assurance was provided.

There was no dose-related increase in mortality in any treated group. Body tremors were reported at 20 mg/kg bw per day after administration of each dose. Clear oral discharge was observed in dams at 10 and 20 mg/kg bw per day. Mean maternal body-weight gains were slightly reduced at 10 mg/kg bw per day[1] and significantly reduced at 20 mg/kg[2] during gestation. There were no differences in number of pregnancies, early/late resorptions, viable foetuses, post implantation loss or sex distribution. The number of corpora lutea per dam was increased in all treated groups, while the mean foetal body weight was significantly reduced at 10 and 20 mg/kg bw per day[3]. There was also an increase in the number of litters with developmental variations at 20 mg/kg bw per day; these variations included reduced ossification of the skull, unossified hyoid body, unossified sternebrae 5 and 6, and undeveloped renal papilla and/or distended ureters. There were no reported effects on the number or per cent of foetuses or litters with external, internal or skeletal malformations or anomalies at any dose. On the basis of the clinical signs and reduction in body weight, the NOAEL for maternal toxicity was 2 mg/kg bw per day. On the basis of the reduction in foetal body weight, the NOAEL for fetotoxicity was 2 mg/kg bw per day. The NOAEL for developmental toxicity was 20 mg/kg bw per day (the highest dose tested) (Janes, Rodwell & Blair, 1980a).” (Evaluated in JMPR, 2004.)

The maternal and fetal body weight data for Janes, Rodwell & Blair, 1980a are shown in Table 15. No significant changes ($p > 0.05$) in uterine weight occurred. In the high-dose cohort maternal body weight was significantly reduced ($p < 0.05$) compared to controls over gestation days (GDs) 9 to 20 with

Footnotes are added data from elsewhere in JMPR 2003 evaluation:

1 by ca 2% compared to controls on GD 20

2 by about 10% compared to controls on GD 20

3 by ca 5% compared to controls at 10 mg/kg bw per day, and by ca 14% compared to control at 20 mg/kg bw per day

a reduction in body weight by 10% occurring on GD 20. Although not statistically significant at $p > 0.05$, there was a reduction by 11% compared to controls in corrected maternal body weight in this group. Body weight changes in groups dosed at 10 mg/kg bw per day and less were not biologically adverse and not significantly different from controls at $p > 0.05$. Fetal body weight was significantly ($p < 0.05$) and adversely reduced (by 14% compared to controls) in the high-dose cohort. While significantly reduced body weight ($p < 0.05$) occurred following maternal dosing at 10 mg/kg bw per day, the magnitude of the change was not regarded as adverse. Given these findings, the proposed new maternal and fetal NOAELs are 10 mg/kg bw per day due to adverse reductions in body weight at 20 mg/kg bw per day (Janes et al., 1980a).

***Table 15a. Mean maternal body weights in the rat prenatal toxicity study, mean weight (g) [percentage of control value] shown***

| Dose (mg/kg bw per day) | | Gestation day | | | | | |
|---|---|---|---|---|---|---|---|
| | Maternal body weight (g) | 0 | 6 | 9 | 12 | 16 | 20 |
| 0 | Mean maternal body weight | 245 | 277 | 285 | 302 | 330 | 384 |
| | Final maternal corrected body weight | - | - | - | - | - | 313 |
| 2 | Mean maternal body weight | 254 [104%] | 282 [102%] | 290 [102%] | 306 [101%] | 334 [101%] | 391 |
| | Final maternal corrected body weight | - | - | - | - | - | 317 [101%] |
| 10 | Mean maternal body weight | 259* [106%] | 287 [104%] | 280 [98%] | 294 [97%] | 294 [97%] | 376 |
| | Final maternal corrected body weight | - | - | - | - | - | 301 [96%] |
| 20 | Mean maternal body weight | 249 [102%] | 275 [99%] | 266* [93%] | 279* [92%] | 302* [92%] | 347* [90%] |
| | Final maternal corrected body weight | - | - | - | - | - | 278 [89%] |

No significant effects on uterine weight at GD 20 occurred; Source: Janes, Rodwell & Blair, 1980a

* Significantly different from control at $p < 0.05$

***Table 15b. Mean fetal body weights in the rat prenatal toxicity study***

| Dose (mg/kg bw per day) | Fetal body weight (g) | Fetal body weight as percentage of control value |
|---|---|---|
| 0 | 3.7 | - |
| 2 | 3.6 | 97% |
| 10 | 3.5* | 95% |
| 20 | 3.2* | 86% |

No significant effects on uterine weight at gestational day 20 occurred; Source: Janes, Rodwell & Blair, 1980a

* Significantly different from control at $p < 0.05$

***Rabbit***

A rabbit prenatal developmental study was evaluated at JMPR 2003 (JMPR, 2004). A re-evaluation of this study for this monograph concurred with the findings of JMPR 2003:

> "Groups of 16 New Zealand albino rabbits were given carbosulfan at a dose of 0, 2, 5 or 10 mg/kg bw per day by gavage on days 6–28 of gestation. Pups were delivered by caesarean section on day 29, and the number, location, and distribution of viable/nonviable fetuses, corpora lutea, early/late resorptions and total implantations were recorded. All foetuses were examined grossly, sectioned for visceral anomalies and stained for skeletal anomalies. A statement of compliance with quality assurance was provided.

No treatment-related effects on appearance and behaviour were observed in the dams. There were three deaths of animals treated at 10 mg/kg, one death in the control group and one death in the group receiving the lowest dose. A cause of death could not be established for two dams in the group receiving the highest dose and in the dam in the group receiving the lowest dose. The deaths of one of the dams at the highest dose and the dam at the lowest dose were attributed to enteritis. The numbers of dams with viable foetuses were 12, 10, 15 and 11 at 0 (control), 2, 5 and 10 mg/kg, respectively. There were no compound related effects on the number of viable foetuses, corpora lutea, foetal sex distribution or total implantations per dam. There were slight, non-significant increases in post implantation losses (6, 9, 11 and 13 at 0, 2, 5 and 10 mg/kg bw per day, respectively) and early resorption rate (3, 8, 9 and 11 at 0, 2, 5 and 10 mg/kg bw per day, respectively). Also, the mean foetal body weight was slightly decreased (7%) at the highest dose. There was a single occurrence of scoliosis in each of the three treated groups, but none was reported in the controls. There were no compound-related effects on skeletal variations, such as delayed ossification. Major vessel variations, identified primarily as left carotid arising from the innominate, were observed in 16.7, 100, 46.7 and 72.7% of the litters at 0, 2, 5, and 10 mg/kg bw per day, respectively (mean of data for historical controls in the test laboratory, 51%). The proportions of foetuses presenting this defect were 4.9, 44, 12.8 and 20.8%, respectively (mean of data for historical controls in the test laboratory, 14%). The increase in major vessel variations was not dose dependent. Furthermore, the Meeting noted that data for historical controls show that these major vessel variations are very common and occur at highly variable incidences in rabbits of this strain. Therefore, the Meeting considered these effects to be incidental. The NOAEL for maternal and fetotoxicity was 10mg/kg bw per day (the highest dose tested). Carbosulfan was not teratogenic in rabbits. The NOAEL for developmental toxicity was 10 mg/kg bw per day (the highest dose tested) (Janes et al., 1980b)." (Evaluated in JMPR, 2004.)

## 2.6 Special studies

### (a) Neurotoxicity

***Acute neurotoxicity***

An acute neurotoxicity study in rats was evaluated in JMPR 2003 (JMPR, 2004). A re-evaluation of this study for this monograph concurs with the findings in JMPR 2003:

"The neurotoxic effects of acute oral exposure to carbosulfan (purity, 88%) were assessed in groups of 27 male and 27 female Crl :CD BR rats treated by gavage in a study that complied with EPA guideline 81-8-SS. The animals received vehicle (corn oil), or carbosulfan at a dose of 0.5, 5 or 30 mg/kg bw. All animals were checked daily for viability and clinical signs. Body weights were measured on days –6, 0, 7, 14 and 15. The doses and time-points were selected on the basis of a preliminary study. Seven animals of each sex per dose were allocated to undergo neuropathological examination on day 15; this consisted of assessment of brain weight and dimensions, evaluation for gross changes, and histopathological examination of a range of central and peripheral nervous system tissues (including eyes, ganglions and nerve fibres). In groups of five animals of each sex per dose, brain, erythrocyte and plasma cholinesterase activities were measured before treatment, and 4 h after dosing on days 0, 7 and 15. Additionally, at termination the brains of animals in these four groups were weighed, and six brain regions [see Table 16] were dissected. The animals that were allocated to undergo neuropathological examination and the animals for which cholinesterase activity was measured on day 15 were also tested in a functional observational battery (FOB) and for locomotor activity before treatment, 4 h after dosing, and on days 7 and 14. Statements of compliance with GLP and QA were provided. No mortality was observed. During the first week after treatment, body-weight gain was lower in males at the highest dose. Also at the highest dose, some animals had staining of the ventral abdomen and urogenital area on days 1 and 2. Home cage observations revealed one male at the highest dose with tremors on day 0. At 4 h after dosing, body temperatures were decreased in males and females at the highest dose, a few animals with slight tremors and impaired gait were observed during open-field testing, and sensory tests revealed a slow tail-pinch response in males. Furthermore, motor activity was decreased in animals at the highest dose. Effects on brain weight, gross changes and histopathology were considered not to be treatment-related. Data on cholinesterase activity at 4 h after dosing in the groups treated with carbosulfan are summarized in Table [16]. In females, plasma cholinesterase activity appeared to be less sensitive than erythrocyte and brain cholinesterase activity to inhibition by carbosulfan. Brain and erythrocyte cholinesterase activity was reduced in males and females at the intermediate and highest doses, 4 h after administration of carbosulfan. No consistent differences in cholinesterase activity were observed before treatment or on days 7 and 14. Occasional significant differences at these time-points were considered not to be treatment-related.

On the basis of the effects on cholinesterase activity in brain and erythrocytes at the intermediate dose, the NOAEL was 0.5 mg/kg bw (Knapp, 1996)." (Evaluated in JMPR, 2004.)

***Table 16. Effects of carbosulfan on acetylcholinesterase activity[a] in rats four hours after dosing by oral gavage, (percentages of control value shown)***

| | | Dose (mg/kg bw) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0.5 | | 5 | | 30 | |
| **Substrate** | | **Males** | **Females** | **Males** | **Females** | **Males** | **Females** |
| Blood: | plasma | 96 | 135 | 76* | 116 | 61* | 88 |
| | erythrocytes | 101 | 89 | 62* | 64* | 54* | 55* |
| Brain: | hippocampus | 86 | 108 | 73 | 62* | 54* | 62* |
| | olfactory region | 93 | 88 | 66 | 66* | 52* | 58* |
| | midbrain | 97 | 92 | 64* | 61* | 53* | 53* |
| | brain stem | 84 | 94 | 56* | 59* | 48* | 52* |
| | cerebellum | 95 | 94 | 74* | 67* | 61* | 68* |
| | cortex | 88* | 94 | 62* | 66* | 52* | 51* |

* Significantly different from controls; Source: Knapp, 1996

### *Neurotoxicity after repeated doses*

A 13-week repeat dietary exposure neurotoxicity study in rats was evaluated in JMPR 2003 (JMPR, 2004). A re-evaluation of this study for this monograph concurs with the findings of JMPR 2003:

> "Groups of 10 male and 10 female Sprague Dawley CD rats were given diets containing carbosulfan (purity, 88.0%) at a concentration of 0, 20, 1000 or 2000 mg/kg (equal to 0, 1.2, 65 and 131 mg/kg bw per day in males, and 0, 1.4, 79 and 152 mg/kg bw per day in females) for 13 weeks, in a study that complied with EPA guidelines 81, 82 and 83. Clinical signs were recorded daily. Body weights and food consumption were measured weekly. The animals were subjected to FOB and motor activity tests before treatment and 4, 8 and 13 weeks after the start of treatment. At termination, the animals were killed and necropsies were performed. The nervous systems of five animals of each sex in the control group and in the group receiving the highest dose were examined for neuropathological lesions. Statements of compliance with GLP and QA were provided.
>
> At the intermediate and highest doses (dietary concentrations, 1000 or 2000 mg/kg respectively), body weight and body-weight gains were decreased in males and females. At these doses, food consumption in males was decreased throughout the study and food consumption in females was decreased during weeks 2 and 3. Chromodacryorrhoea and decreased faeces were also observed in males at these doses. Additionally, tremors, exophthalmus, chromorhinorrhoea and dehydration were observed in males at the highest dose. In females at the intermediate and highest doses, exophthalmus, tremors, abdominogenital staining, reddish brown staining of the cage-pan liner, chromorhinorrhoea, chromodacryorrhoea, decreased faeces, dehydration, diarrhoea and unthriftiness were observed. In the FOB, males at the highest dose (2000 mg/kg) showed decrease in foot splay, hindlimb grip strength and urine pools. In females at the highest dose, localized spasms/twitching or tremors, exophthalmos, walking on toes, and slightly impaired gait were observed. A reduction in motor activity was observed in females at the two higher doses at weeks 4 and 13 respectively. No treatment-related effects on necropsy and neuropathological examination were found. Measurements of AChE activity in brain, erythrocyte or plasma were not included in this study. On the basis of the clinical signs, observations in the FOB, and effects on body weight, body-weight gain and food consumption in animals at the two higher doses, the NOAEL was 20 mg/kg, equal to 1.2 mg/kg bw per day (Freeman, 1995)." (Evaluated in JMPR, 2004.)

### *Developmental neurotoxicity*

In a peer-reviewed, published developmental neurotoxicity study (not GLP; Klimisch score 2) pregnant female Wistar rats (six per dose level) were orally (gavage) administered carbosulfan (purity 92%) at 0, 0.5, 1, 2 or 4 mg/kg bw per day over GDs 1 to 15. The dams were allowed to deliver, and two days after delivery, each litter was culled to eight pups per dam to improve lactation efficiency. The pups were weaned on PND 24. Pups were selected from each of the six litters and examined. After the final day of behavioural testing; postnatal day (PND) 32 animals were terminated and their brains removed for biochemical and histopathological examination as described below.

Pup brain homogenate AChE activity on PND 32 was significantly reduced ($p < 0.05$) by 50% or more at all carbosulfan dose levels. Reductions in pup body weight (from ca 20% at PND 1 to less than 10% at PND 35 compared to controls) occurred following maternal dosing at 2 mg/kg bw per day and above. Maternal dosing at 2 mg/kg bw per day and above resulted in significantly increased ($p < 0.05$) latency to surface righting in the pups on PNDs 5 and 6. Over PNDs 7 to 9 pups from all carbosulfan-treated mothers displayed significantly ($p < 0.05$) increased latency to surface righting (three-fold or greater compared to controls). Dose-related and significantly delayed ($p < 0.05$) negative geotaxis re-orientation occurred on PNDs 13–17 in pups derived from mothers dosed at 1 mg/kg bw per day or more. A dose-related and significantly reduced ($p < 0.05$) incidence of mid-air righting occurred on PNDs 13–15; in detail, 2–3/6 pups were capable of mid-air righting in the 2 mg/kg bw per day group and 1/6 pups were capable of mid-air righting in the 4 mg/kg bw per day group. On PNDs 16 and 17 significantly reduced incidence of mid-air righting occurred at 1 mg/kg bw per day and above. Significantly ($p < 0.05$) reduced (ca 8 seconds or more) latency to fall in the hanging wire test occurred in all pups derived from carbosulfan-treated mothers on PNDs 13–19. Significant dose-related reductions ($p < 0.05$) in mean number of open-arm entries and time spent in open arms in the elevated plus maze also occurred in all pups derived from carbosulfan-treated mothers on PND 30. Pups derived from mothers dosed at 2 mg/kg bw per day and above displayed significantly delayed light–dark habituation time on PND 30. Irrespective of dose, maternal treatment with carbosulfan resulted in evidence of oxidative effects in pup brains manifesting as significant increases ($p < 0.05$) in tissue malondialdehyde and protein carbonyls, and significantly reduced ($p < 0.05$) tissue glutathione and glutathione peroxidase levels. Pups derived from mothers treated with carbosulfan displayed morphological changes in the cerebellar Purkinje cell layer at all dose levels (Banji et al., 2014). Critically, the determination that there was a 50% or greater reduction in brain AChE (sampled at PND 32) in the weaned offspring of dams dosed with carbofuran between GDs 1 and 15 is not consistent with the known toxicokinetic and toxicological properties of carbosulfan, based on the following weight of evidence:

- The finding that 50% inhibition of brain AChE compared to controls occurs in offspring at PND 32, following the oral dosing of their mothers at 0.5 mg/kg bw per day is not consistent with all other data evaluated in this monograph.
- The final dose of carbosulfan administered to the dams was on GD 15. Elimination of carbosulfan and its metabolites in rats is largely complete within 48 hours following dosing, with most elimination occurring within the first 24 hours. Accordingly, essentially no toxicologically meaningful amounts of carbosulfan or its associated residues could have been transferred to nursing pups over the lactational period (PNDs 0–21). Even if the rat pups received toxicologically significant exposures via the transmammary route, elimination would have been effectively complete by the time of brain sampling for AChE determination which occurred after behavioural testing on PND 32.
- While age-related changes in rat brain AChE sensitivity to inhibition by some anticholinesterases have been detected (Kousba, Poet & Timchalk, 2007), the most sensitive postnatal age is five days. By PND 17 the sensitivity to AChE inhibition by anticholinesterases is approximately five-fold less pronounced compared with PND 5. There is no evidence for an intrinsically age-related sensitivity of rat brain cholinesterases to anticholinesterases at PND 32.
- The blood–brain barrier in rats is functionally mature at birth (Schmitt et al. 2017). Accordingly, there is no brain dispositional explanation as to why carbosulfan would induce such high levels of brain AChE inhibition in 32-day-old rats.
- From the limited histopathology provided there is insufficient change in cell numbers to account for a 50% decrease in enzyme activity.
- There is a discrepancy in the study description, in that pup weights are reported up to PND 35, yet it is stated that the animals were terminated on PND 32.

Accordingly, because of its limitations and the incongruence of the results with the known kinetics of carbosulfan in rats, the known dose responses of brain AChE to orally administered carbosulfan in the rat, the unverifiability of the dosimetry used and study findings in a short published scientific paper (thus raw data not available for review) plus the overall weight of evidence presented in this monograph,

Banji et al., 2014 is not considered a suitable basis for the determination of health-based guidance values (HBGVs) for carbosulfan.

***Delayed neurotoxicity***

Carbosulfan administered orally at 500 mg/kg bw (that is a $LD_{70}$ dose) following atropine sulfate pretreatment induced no observable neurobehavioural signs and no microscopic anatomical pathology evidence of delayed neurotoxicity in the hen model. Neurotarget esterase measurements were not performed (Gephart et al., 1979).

**(b) Immunotoxicity**

No studies were submitted.

**(c) Studies on metabolites**

For evaluation of metabolites, refer to the previous monograph on carbofuran.

## 3. Microbiological aspects

There was no information available in the public domain and no experimental data were submitted that addressed the possible impact of carbosulfan residues on the human intestinal microbiome.

## 4. Observations in humans

Biomonitoring data on 64 (2017) or 70 (2018) workers involved in manufacturing/formulating carbosulfan suggested no inhibition of blood cholinesterase.

# Comments

## Biochemical aspects

Following oral dosing, carbosulfan undergoes substantial presystemic degradation resulting in a lower acute oral toxicity compared with carbofuran (Umetsu & Fukuto, 1982). Oral uptake is rapid. Based on the elimination fraction of carbosulfan-derived radiolabel in mass balance studies, estimated oral absorption in rats was up to 91.4% of administered dose (AD). Radioactivity derived from carbosulfan was widely distributed in rats. In rats carbosulfan was rapidly metabolized and excreted, predominantly in urine (up to 91.4% of AD). Excretion is largely complete by 48 hours following dosing. In rats the rate of excretion is increased following repeated dosing (Fang & El Naggar, 1995; Liss et al. 1981; Marsden, Ruwano & Fukuto, 1982; Selim, 1982; Wu, 1982a, b).

Two hepatic metabolic pathways were identified: the sulfur oxidation (detoxification) pathway, and the carbofuran (toxication) pathway. In vitro, human biomaterials detoxified carbosulfan more extensively than with comparable rodent biomaterials. Enzymes in the CYP3A subfamily were the major in vitro catalysts of carbosulfan metabolism (Abass et al., 2009, 2010).

In rats the major urinary metabolites were 3-hydroxy-carbofuran, 3-keto-7-phenol-carbofuran, 3-hydroxy-7-phenol-carbofuran, 7-phenol-carbofuran and dibutylamine. The major faecal metabolites were 3-hydroxy-carbofuran and carbofuran (Fang & El Naggar, 1995; Marsden, Ruwano & Fukuto, 1982).

## Toxicological data

Values for the acute oral median lethal dose ($LD_{50}$) of carbosulfan in rats ranged from 7.9 to 300 mg/kg bw (Costa Vieira, 2004a; Durando, 2007; Lowe, 2014a, b, c, 2015; Madurapathi, 2014a). The dermal $LD_{50}$ was greater than 2000 mg/kg bw. The inhalation median lethal concentration ($LC_{50}$) of carbosulfan (four hour, nose only) ranged from 0.05 to 0.38 mg/L (Penteado, 2005a; Subashini, 2014). Stabilized carbosulfan was not irritating to rabbits' skin (Maduropathi, 2014d; Verdolin Milo Collor, 2004b). Carbosulfan was slightly to mildly irritating to the eyes of rabbits (Lowe, 2007d; Verdolin Milo Collor, 2004a) and was not skin sensitizing in a Guinea pig maximization test (Penteado, 2005b).

In general, in short-term and long-term studies of toxicity, the most sensitive effect of oral administration of carbosulfan was the inhibition of acetylcholinesterase (AChE) activity, accompanied at the same or higher doses by clinical signs indicative of a cholinergic toxidrome.

In a 90-day oral toxicity study in rats (not GLP), carbosulfan was administered at dietary concentrations of 0, 10, 20 or 500 ppm (equal to 0, 0.25, 0.50 and 13.6 mg/kg bw per day for males, 0, 0.19, 0.38 and 9.8 mg/kg bw per day for females). The no-observed-adverse-effect level (NOAEL) was 20 ppm (equal to 0.38 mg/kg bw per day, due to inhibition of brain AChE at 500 ppm (equal to 9.8 mg/kg bw per day) (Marshall et al., 1979).

In a 24-month combined chronic oral toxicity and carcinogenicity study in mice, carbosulfan was administered at dietary concentrations of 0, 10, 20, 500 or 2500 ppm (equal to 0, 1.3, 2.5, 62 and 320 mg/kg bw per day for males, 0, 1.5, 3.1, 72 and 337 mg/kg bw per day for females). The NOAEL for toxicity was 20 ppm (equal to 2.5 mg/kg bw per day), due to reduced brain AChE activity at 500 ppm (equal to 62 mg/kg bw per day). The NOAEL for carcinogenicity was 2500 ppm (equal to 320 mg/kg bw per day), the highest dose tested (DeProspo, Norvell & Fletcher, 1982a).

In a 104-week combined chronic oral toxicity and carcinogenicity study in rats, carbosulfan was administered at dietary concentrations of 0, 10, 20, 500 or 2500 ppm (equal to 0, 0.5, 1.0, 26.8 and 152.8 mg/kg bw per day for males, 0, 0.6, 1.2, 34.7 and 213.3 mg/kg bw per day for females). The NOAEL for toxicity was 20 ppm (equal to 1.0 mg/kg bw per day) due to reduced brain AChE activity, increased incidence of focal iris atrophy and decreased body weight, at 500 ppm (equal to 26.8 mg/kg bw per day). The NOAEL for carcinogenicity was 2500 ppm (equal to 152.8 mg/kg bw per day), the highest dose tested (DeProspo, Norvell & Fletcher, 1982b).

Overall, the Meeting concluded that carbosulfan is not carcinogenic in mice or rats.

Carbosulfan was evaluated for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found.

The Meeting concluded that carbosulfan is unlikely to be genotoxic.

Given the lack of genotoxicity and the absence of carcinogenicity in mice and rats, the Meeting concluded that carbosulfan is unlikely to pose a carcinogenic risk to humans.

In a three-generation reproductive toxicity study in rats, carbosulfan was administered at dietary concentrations of 0, 10, 20 or 250 ppm (equivalent to 0, 0.67, 1.3 and 16.7 mg/kg bw per day). Cholinesterase activities were not determined in this study. The NOAEL for parental effects was 20 ppm (equivalent to 1.3 mg/kg bw per day), based on decreased body weight at 250 ppm (equivalent to 16.7 mg/kg bw per day). The NOAEL for reproductive toxicity was 20 ppm (equivalent to 1.3 mg/kg bw per day) due to effects on litter size at the next highest dietary concentration of 250 ppm (equivalent to 16.7 mg/kg bw per day). The NOAEL for offspring toxicity was 20 ppm (equivalent to 1.3 mg/kg bw per day) based on reductions in pup weight and pup weight gain at 250 ppm (equivalent to 16.7 mg/kg bw per day) (Kehoe & MacKenzie, 1982).

In a developmental toxicity study in rats, carbosulfan was administered by gavage at dose levels of 0, 2, 10 or 20 mg/kg bw per day. Cholinesterase activities were not measured in this study. The maternal NOAEL was 2 mg/kg bw per day based on an increased incidence of oral discharge (consistent with cholinergic toxicity) at 10 mg/kg bw per day. The embryo/fetal NOAEL was 2 mg/kg bw per day based on a reduction in fetal weight at 10 mg/kg bw per day (Janes, Rodwell & Blair, 1980a).

In a developmental toxicity study in rabbits, carbosulfan was administered by gavage at dose levels of 0, 2, 5 or 10 mg/kg bw per day. The maternal and embryo/fetal NOAEL was 10 mg/kg bw per day, the highest dose tested. Cholinesterase activities were not measured in this study (Janes, Rodwell & Blair, 1980b).

Overall, the Meeting concluded that carbosulfan is not teratogenic.

In an acute neurotoxicity study in rats, carbosulfan was administered by gavage at doses of 0, 0.5, 5 or 30 mg/kg bw. The NOAEL was 0.5 mg/kg bw, based on decreased AChE activity in the brain and erythrocytes at 5 mg/kg bw (Knapp, 1996).

In a 13-week neurotoxicity study in rats, carbosulfan was administered at dietary concentrations

of 0, 20, 1000 or 2000 ppm (equal to 0, 1.2, 65 and 131 mg/kg bw per day for males, 0, 1.4, 79 and 152 mg/kg bw per day for females). Measurements of AChE activity were not included in this study. The NOAEL was 20 ppm (equal to 1.2 mg/kg bw per day), based on tremors, reduced motor activity, decreased food consumption and decreased body weight and body weight gain at 1000 ppm (equal to 65 mg/kg bw per day) (Freeman, 1995).

In a delayed neurotoxicity study in hens, carbosulfan was administered orally as a single 500 mg/kg bw dose. Neuropathy target esterase measurements were not performed. No neurobehavioural signs, nor any microscopic anatomical pathology evidence of delayed neurotoxicity were observed (Gephart et al., 1979).

The Meeting concluded that carbosulfan is neurotoxic but does not induce delayed neurotoxicity.

No immunotoxicity studies were submitted, but no evidence of immunotoxicity was observed in repeat-dose studies of toxicity.

Based on the available data the Meeting concluded that carbosulfan is unlikely to be immunotoxic.

### Toxicological data on metabolites and/or degradates

The conversion of carbosulfan to carbofuran involves the formation of dibutylamine. The major metabolites present in carbosulfan dietary residues, except for dibutylamine, are derived from the metabolism of carbofuran (see previous monograph on carbofuran).

While dibutylamine is a major metabolite of carbosulfan occurring in rats, it is unlikely to have any anticholinesterase effects. There is insufficient data to derive a potency factor. The Meeting concluded that dibutylamine is toxicologically relevant, but can be covered by the health-based guidance values (HBGVs) for carbosulfan.

## Microbiological aspects

There was no information available in the public domain and no experimental data were submitted that addressed the possible impact of carbosulfan residues on the human intestinal microbiome.

## Human data

In reports on manufacturing plant personnel for 2017 and 2018 no adverse health effects were noted.

The Meeting concluded that the existing database on carbosulfan was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

# Toxicological evaluation

The Meeting re-affirmed an acceptable daily intake (ADI) of 0–0.01 mg/kg bw, based on a combined point of departure of 1.3 mg/kg bw per day derived from the NOAEL values of 1.2 mg/kg bw per day in the 13-week neurotoxicity study in rats, 1 mg/kg bw per day for toxicity in the 104-week study of toxicity and carcinogenicity in rats, and 1.3 mg/kg bw per day for parental and offspring toxicity in the three-generation reproductive toxicity study in rats. The NOAEL of 0.38 mg/kg bw per day in the 90-day rat study was not considered appropriate to derive the ADI because of the wide dose spacing, with the least-observed-adverse-effect level (LOAEL) of 9.8 mg/kg bw per day. A safety factor of 100 was used.

The Meeting re-affirmed an acute reference dose (ARfD) for the general population of 0.02 mg/kg bw, based on the NOAEL of 0.5 mg/kg bw in the acute neurotoxicity study in rats. The Meeting noted the wide dose range between the NOAEL of 0.5 mg/kg bw and the LOAEL of 5 mg/kg bw, and what is likely to be the higher $C_{max}$ associated with gavage dosing compared to dietary exposure. For these reasons the NOAEL for the acute neurotoxicity study was not used as the point of departure for the ADI. A safety factor of 25 was considered appropriate because the acute toxic effects of carbofuran are dependent on $C_{max}$ rather than the area under the concentration–time curve (AUC). Also, data indicated that the sensitivities of humans and laboratory animals (rats, dogs) to inhibition of AChE activity by carbofuran were similar. A full justification for the use of a total uncertainty factor of 25 was provided at JMPR, 2008 (JMPR, 2010)

***Levels relevant to risk assessment of carbosulfan***

| Species | Study | Effect | NOAEL | LOAEL |
|---|---|---|---|---|
| Mouse | 24-Month combined study of toxicity and carcinogenicity[a] | Toxicity | 20 ppm, equal to 2.5 mg/kg bw per day | 500 ppm, equal to 62 mg/kg bw per day |
| | | Carcinogenicity | 2500 ppm, equal to 320 mg/kg bw per day[c] | - |
| Rat | Acute neurotoxicity study[b] | Neurotoxicity | 0.5 mg/kg bw[c] | 5 mg/kg bw |
| | 13-Week study of neurotoxicity[a] | Neurotoxicity | 20 ppm, equal to 1.2 mg/kg bw per day | 1000 ppm, equal to 65 mg/kg bw per day |
| | 104-Week study of toxicity and carcinogenicity[a] | Toxicity | 20 ppm, equal to 1.0 mg/kg bw per day | 500 ppm, equal to 26.8 mg/kg bw per day |
| | | Carcinogenicity | 2500 ppm, equal to 152.8 mg/kg bw per day[c] | - |
| | Three-generation study of reproductive toxicity[a] | Reproductive toxicity | 20 ppm, equivalent to 1.3 mg/kg bw per day | 250 ppm, equivalent to 16.7 mg/kg bw per day |
| | | Parental toxicity | 20 ppm, equivalent to 1.3 mg/kg bw per day | 250 ppm, equivalent to 16.7 mg/kg bw per day |
| | | Offspring toxicity | 20 ppm, equivalent to 1.3 mg/kg bw per day | 250 ppm, equivalent to 16.7 mg/kg bw per day |
| | Developmental toxicity study[b] | Maternal toxicity | 2 mg/kg bw per day | 10 mg/kg bw per day |
| | | Embryo/fetal toxicity | 2 mg/kg bw per day | 10 mg/kg bw per day |
| Rabbit | Developmental toxicity study[b] | Maternal toxicity | 10 mg/kg bw per day[c] | - |
| | | Embryo/fetal toxicity | 10 mg/kg bw per day[c] | - |

[a] Dietary administration

[b] Gavage administration

[c] Highest dose tested

*Acceptable daily intake (ADI) applies to carbosulfan**

0–0.01 mg/kg bw

*Acute reference dose (ARfD) applies to carbosulfan***

0.02 mg/kg bw

* Applies to carbosulfan and dibutylamine expressed as carbosulfan, plus 10 times carbofuran and metabolites (see carbofuran evaluation, p 96, in previous monograph)

** Applies to carbosulfan and dibutylamine expressed as carbosulfan, plus 20 times carbofuran and metabolites (see carbofuran evaluation, p 96, in previous monograph)

## Information that would be useful for the continued evaluation of the compound

Clarification of the level of carbofuran in the carbosulfan test articles used in the toxicology studies. Results from epidemiological, occupational health and other such observational studies of human exposure.

*Critical end-points for setting guidance values for exposure to carbosulfan*

| | |
|---|---|
| ***Absorption, distribution, excretion and metabolism in mammals*** | |
| Rate and extent of oral absorption | Rapid and extensive (up to 91%) |
| Dermal absorption | No data<br>Rabbit: reduction in brain cholinesterase activity at 50 mg/kg bw/ day<br>Rat: substantial conversion of carbosulfan to carbofuran occurred in rat skin in vitro |
| Distribution | Wide; highest concentrations in gastrointestinal tract, liver, kidney, fat, thymus and central nervous system |
| Potential for accumulation | Low |
| Rate and extent of excretion | Rapid; >70% dose eliminated within 24 hours and nearly complete within 120 hours;<br>Mainly via urine (65 to >90% dose) |
| Metabolism in animals | 3-Hydroxy-carbofuran, 3-keto-7-phenol-carbofuran, 3-hydroxy-7-phenol-carbofuran and 7-phenol-carbofuran |
| Toxicologically significant compounds in animals and plants | Carbosulfan, dibutylamine, carbofuran and its metabolites<br>(see carbofuran evaluation p 96, in previous monograph) |
| ***Acute toxicity*** | |
| Rat, $LD_{50}$, oral | ≤ 7.9 to < 300 mg/kg bw |
| Rat, $LD_{50}$, dermal | > 4000 mg/kg bw |
| Rat, $LC_{50}$, inhalation[a] | 0.05 to 0.38 mg/L (four-hour, nose-only exposure) |
| Rabbit, dermal irritation | Stabilized carbosulfan: not irritating |
| Rabbit, ocular irritation | Slightly irritating |
| Mouse, dermal sensitization | No data |
| Guinea pig dermal sensitization | Not a sensitizer (Magnusson & Kligman) |
| ***Short-term studies of toxicity*** | |
| Target/critical effect | Inhibition of cholinesterase activity (rat, rabbit) |
| Lowest relevant oral NOAEL | 1.2 mg/kg bw per day (rat) |
| Lowest relevant dermal NOAEL | 5 mg/kg bw per day (rabbit) |
| Lowest relevant inhalation NOAEC | 0.65 µg/L (rat) |
| ***Long-term studies of toxicity and carcinogenicity*** | |
| Target/critical effect | Inhibition of cholinesterase activity (mouse, rat),<br>Increased incidence of focal iris atrophy (rat),<br>Decreased bodyweight (rat) |
| Lowest relevant NOAEL | 1 mg/kg bw per day (rat) |
| Carcinogenicity | Not carcinogenic |
| ***Genotoxicity*** | Unlikely to be genotoxic |
| ***Reproductive toxicity*** | |
| Target/critical effect | Reduced fetal and maternal body weights |
| Lowest relevant parental NOAEL | 1.3 mg/kg bw per day (rat) |
| Lowest relevant offspring NOAEL | 1.3 mg/kg bw per day (rat) |
| Lowest relevant reproductive NOAEL | 1.3 mg/kg bw per day (rat) |
| ***Developmental toxicity*** | |
| Target/critical effect | Decreased maternal and embryo/fetal body weight (rat) |
| Lowest relevant maternal NOAEL | 2 mg/kg bw per day (rat) |
| Lowest relevant embryo/fetal NOAEL | 2 mg/kg bw per day (rat) |

| | |
|---|---|
| ***Neurotoxicity*** | |
| Acute neurotoxicity NOAEL | 0.5 mg/kg bw (rat) |
| Subchronic neurotoxicity NOAEL | 1.2 mg/kg bw per day, (rat, acetylcholinesterase activity not assessed) |
| Developmental neurotoxicity NOAEL | No data |
| Delayed neurotoxicity | Negative |
| ***Other toxicological studies*** | |
| Immunotoxicity | No specific data; unlikely to be immunotoxic based on the available data |

**Studies on toxicologically relevant metabolites**

| | |
|---|---|
| ***Dibutylamine; carbofuran and its metabolites*** | See carbofuran evaluation p 96, in the previous monograph |
| ***Microbiology data*** | No data submitted |
| ***Human data*** | In reports on manufacturing plant personnel, no adverse health effects were noted |

## Summary

| | Value | Study | Safety factor |
|---|---|---|---|
| ADI | 0–0.01 mg/kg bw[a] | 13-week neurotoxicity study, the 104-week study of toxicity and carcinogenicity, and the three-generation reproductive toxicity study (all rat) | 100 |
| ARfD | 0.02 mg/kg bw[b] | Acute neurotoxicity study (rat) | 25 |

[a] Applies to carbosulfan and dibutylamine expressed as carbosulfan, plus 10 times carbofuran and its metabolites (see carbofuran evaluation p 96, in previous monograph)

[b] Applies to carbosulfan and dibutylamine expressed as carbosulfan, plus 20 times carbofuran and its metabolites (see carbofuran evaluation p 96, in previous monograph)

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# Cyantraniliprole (addendum)

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## Explanation

Cyantraniliprole is the ISO-approved common name for 3-bromo-1-(3-chloro-2-pyridyl)-4′-cyano-2′-methyl-6′-(methylcarbamoyl)pyrazole-5-carboxanilide (IUPAC), with the Chemical Abstracts Service number 736994-63-1. It is a ryanoid insecticide that upregulates ryanodine receptors in the endoplasmic/sarcoplasmic reticulum membrane, increasing calcium-induced calcium release, depleting calcium stores and thus leading to muscular paralysis and ultimately death.

Cyantraniliprole was previously evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 2013 (JMPR, 2014a) when an acceptable daily intake (ADI) of 0–0.03 mg/kg body weight (bw) per day was established based on adverse liver effects in dogs. An acute reference dose (ARfD) was not considered necessary. At the time of the previous evaluation some studies were available and evaluated for the metabolites IN-JSE76, IN-F6L99 and IN-N5M09. Additional studies performed with the metabolites IN-M2G89, IN-K5A78, IN-K5A79, IN-F6L99, IN-N5M09 and IN-JSE76 were reviewed by the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR). There was no other new information on the toxicology of cyantraniliprole.

All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with current test guidelines, unless otherwise specified. No additional information from a literature search was identified that complemented the toxicological information submitted for the current assessment.

# Evaluation for acceptable daily intake

## 1. Biochemical aspects

### 1.2 Biotransformation

According to the previous evaluation of cyantraniliprole (see the 2013 monograph; JMPR, 2014b), none of the metabolites IN-M2G98, IN-K5A78, IN-K5A79, IN-F6L99. IN-N5M09 and IN-JSE76 were detected in urine, faeces or bile, and they did not appear as part of the proposed metabolic pathway in rats (Gannon, 2009a, b).

## 2. Toxicological studies

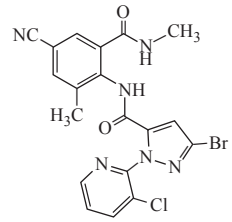
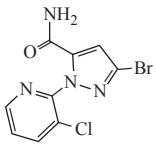
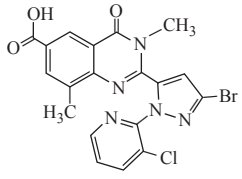
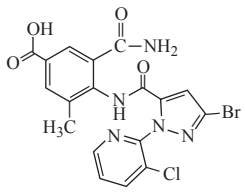
### 2.1 Special studies

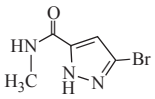
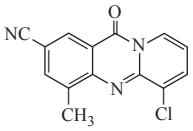
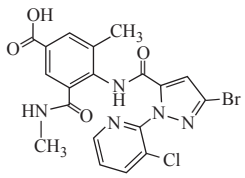
#### (a) Studies on metabolites

***Summary***

A summary of the studies available for the cyantraniliprole metabolites IN-M2G98, IN-K5A78, IN-K5A79, IN-F6L99. IN-N5M09 and IN-JSE76 is provided in Table 1 below.

***Table 1. Summary of cyantraniliprole metabolites for which experimental studies have been submitted; parent compound mentioned for comparison purposes***

| Compound, codes and structure | Detection in rat ADME | Genotoxicity studies | General toxicity studies |
|---|---|---|---|
| **Cyantraniliprole (parent)**  | Yes<br>ca 5% AD in urine | Ames test: negative<br>In vitro chrom. abb.: negative<br>In vitro cell mutation: negative<br>In vivo micronucleus: negative<br>In vivo micronucleus:vnegative | Full data set, robust reference values derived<br>Rat $LD_{50}$, oral: > 5000 mg/kg bw<br>Rat 28-day repeat dose, oral: NOAEL: 53 mg/kg bw per day (liver hypertrophy and thyroid follicular cell hypertrophy at 175 mg/kg bw per day)<br>ADI: 0–0.03 mg/kg bw per day<br>ARfD unnecessary |
| IN-M2G98  | No | Ames: negative<br>In vitro micronucleus: negative<br>In vitro chrom. abb.: negative<br>In vitro cell mutation: negative | Rat $LD_{50}$, oral: 175 mg/kg bw<br>Rat 28-day repeat dose, oral: No NOAEL (reduced body weight, damage to olfactory epithelium at the lowest dose tested,12 mg/kg bw per day) |
| IN-K5A78  | No | Ames: negative<br>In vitro micronucleus: negative<br>In vitro cell mutation: negative | No data |
| IN-K5A79  | No | Ames: negative<br>In vitro micronucleus: negative<br>In vitro chrom. abb.: negative<br>In vitro cell mutation: negative | Rat $LD_{50}$, oral: >5 000 mg/kg bw<br>Rat 28-day repeat dose, oral: NOAEL 1 912 mg/kg bw per day (no effects at highest dose, 1 912 mg/kg bw per day) |

| Compound, codes and structure | Detection in rat ADME | Genotoxicity studies | General toxicity studies |
|---|---|---|---|
| IN-F6L99  | Yes | Ames: negative[a]<br>Ames: negative<br>In vitro micronucleus: negative<br>In vitro chromosomal aberration: negative | Mouse $LD_{50}$, oral: > 2000 mg/kg bw[a] |
| IN-N5M09  | Yes | Ames: negative[a]<br>Ames: negative<br>In vitro micronucleus: negative<br>In vitro chromosomal aberration: negative | Mouse $LD_{50}$, oral: > 5000 mg/kg bw[a] |
| IN-JSE76  | Yes | Ames: negative[a]<br>In vitro cell mutation: negative[a]<br>In vitro chromosomal aberration: negative[a]<br>In vitro micronucleus: negative | Rat $LD_{50}$, oral: > 5000 mg/kg bw[a]<br>Rat 28-day repeat dose, oral: NOAEL 1454 mg/kg bw per day, the highest dose tested[a] |

ADME: Absorption, distribution, metabolism and excretion; AD: Administered dose; $LD_{50}$: Median lethal dose
NOAEL: No-observed-adverse-effect level; ADI: Acceptable daily intake; ARfD: Acute reference dose;
[a] Previously evaluated by the Meeting at JMPR 2013 (JMPR, 2014b)

### *Metabolite IN-M2G98*

An acute oral toxicity study using the "up-and-down" procedure was performed with female Crl:CD(SD) rats. In total, six animals were used; they were 10 weeks old at the start of dosing. The test substance IN-M2G98 (95.9% purity, batch C1365-031) was suspended in 0.1% Tween 80 (v/v) in aqueous 0.5% methylcellulose, and administered to rats by oral gavage, one at 55 mg/kg bw, three at 175 mg/kg bw and two at 550 mg/kg bw, at a minimum of 48 hour intervals. The total dosing volume was 20 mL/kg bw.

Animals were monitored daily for mortality and clinical signs. Rats were euthanized by isoflurane anaesthesia and exsanguination, then subjected to gross necropsy.

Two of two rats dosed at 550 mg/kg bw died, as did one of three rats at 175 mg/kg bw. No biologically relevant loss in body weight was reported. Clinical signs were reported in rats prior to death, namely abnormal gait, circling movement, splayed hindlimbs, ataxia, coldness to touch, dehydration, red nasal discharge, hypoactivity, moribundity, low posture ruffled fur and tremors. The rats that died were also examined post mortem. Wet ventral skin and a red-stained nose were reported in the 175 mg/kg bw group rat that died. No further gross lesions were observed.

In the surviving rats, abnormal gait, circling movement, splayed hindlimbs, ataxia, hypoactivity, ptosis, hunched posture, low posture and ruffled fur were also reported at 175 mg/kg bw. The clinical signs were gone in surviving animals by day 5. No clinical signs were reported at 55 mg/kg bw. No gross lesions were reported at any dose level.

The oral median lethal dose ($LD_{50}$) for this study was 175 mg/kg bw, with a very wide 95% confidence interval of 42.89–1040 mg/kg bw: the comparable value for the parent, cyantraniliprole, was greater than 5000 mg/kg bw (Fallers 2016a).

A 28-day repeat dose oral toxicity study was performed in rats according to OECD TG 407 (2008). Groups of 10 male and 10 female Crl:CD (SD) rats per dose were fed diets for 28 days containing IN-M2G98 (purity 95.9%, batch C1365-031) at concentrations of 0, 150 or 350 ppm (equal to 0, 12 and 22 mg/kg bw per day for males, 0, 12 and 21 mg/kg bw per day for females). Two further groups were fed IN-M2G98 at 750 or 1500 ppm but were terminated without further investigation on day 8 due to severe body weight loss and clinical signs including dehydration, hunched posture, hypoactivity, high

posture, ruffled fur, orbital tightening, and/or nose bulging (the latter two were considered signs of pain/distress).

Animals were monitored throughout the study for clinical signs, weighed regularly and their food consumption was measured. Animals were fasted for 15 hours prior to termination. At scheduled termination blood samples were taken from the vena cava under isoflurane anaesthesia. All required haematological parameters were determined, as were all clinical chemistry parameters in addition to β-oxidation activity (using $^{14}$C-labelled palmitoyl CoA), total cytochrome P450 content, and hepatic microsomal uridine diphosphate glucuronosyltransferase (UDP-GT) activity, using 4-nitrophenol. Urinalysis was also performed. The rats were killed and subjected to a full gross examination. All required organs were weighed, and a representative selection of organs and tissues taken for histopathology.

All remaining rats (that is, those dosed at 0, 150 and 350 ppm) survived until scheduled termination. Body weight compared to controls was 6% and 20% lower in males and 6% and 17% lower in females at 150 and 350 ppm respectively (Table 2). Reductions in body weight gain between day 1 and day 4, compared to controls, were statistically significant: −57.1% and −121% in males, −98.2 and −284% in females at 150 and 350 ppm respectively (Table 3). Although body weight gain did recover slightly thereafter, it remained significantly lower throughout the study. Food consumption was also significantly reduced, by 6.2% and 30.3% compared to controls in males, and by 14.3% and 40.4% in females at 150 and 350 ppm respectively. No further treatment-related clinical signs were reported.

***Table 2. Mean body weight in grams (standard deviation) and percentage difference compared to controls***

| | Time point | | | | | |
|---|---|---|---|---|---|---|
| | **Day 1** | **Day 4** | **Day 8** | **Day 15** | **Day 22** | **Day 29** |
| **Dose (ppm)** | **Males** | | | | | |
| 0 | 241.8 (14.3)<br>- | 265.4 (14.2)<br>- | 301.6 (14.8)<br>- | 351.0 (18.4)<br>- | 394.0 (22.0)<br>- | 425.4 (23.5)<br>- |
| 150 | 238.6 (12.1)<br>−1.3% | 248.7* (13.4)<br>−6.3% | 287.5 (18.1)<br>−4.7% | 335.0 (21.1)<br>−4.6% | 374.8 (22.0)<br>−4.9% | 399.1* (21.7)<br>−6.2% |
| 350 | 245.1 (13.6)<br>1.3% | 240.1* (16.2)<br>−9.5% | 262.4* (19.2)<br>−13.0% | 291.1* (21.7)<br>−17.1% | 323.6* (29.7)<br>−17.9% | 340.4* (29.0)<br>−20% |
| 750 | 240.9 (11.2)<br>−0.4% | 214.5* (12.4)<br>−19.2% | 198.7 (13.6)<br>−34.1% | - | - | - |
| 1500 | 236.8 (16.3)<br>−2.1% | 203.4* (15.7)<br>−23.4% | 170.6* (14.9)<br>−43.4% | - | - | - |
| | **Females** | | | | | |
| 0 | 167.9 (10.6)<br>- | 173.0 (9.6)<br>- | 184.6 (11.9)<br>- | 209.7 (10.8)<br>- | 229.8 (14.6)<br>- | 239.5 (16.2)<br>- |
| 150 | 167.4 (8.5)<br>−0.3% | 167.5 (7.1)<br>−3.2% | 181.2 (11.4)<br>−1.8% | 199.3 (6.3)<br>−5.0% | 219.3 (10.9)<br>−4.6% | 225.5 (12.6)<br>−5.9% |
| 350 | 168.9 (13.0)<br>0.6% | 159.5* (8.3)<br>−7.8% | 168.6* (13.6)<br>−8.7% | 180.3* (13.3<br>−14.0% | 192.6* (15.6)<br>−16.2% | 198.5* (13.2)<br>−17.1% |
| 750 | 165.8 (13.3)<br>−1.2% | 149.2* (10.6)<br>−13.7% | 139.2* (11.4)<br>−24.6% | - | - | - |
| 1500 | 169.1 (7.4)<br>0.7% | 142.5* (6.8)<br>−17.6% | 119.3* (10.0)<br>−35.4% | - | - | - |

* Statistically significant according to a Dunnett's two-sided or Dunnett's non-parametric t-test at $p < 0.05$

***Table 3. Mean body weight gain in grams (standard deviation) and percentage difference compared to controls***

| Dose (ppm) | Time point | | | | | |
|---|---|---|---|---|---|---|
| | Day 1-4 | Day 4-8 | Day 1-8 | Day 8-15 | Day 15-22 | Day 1-29 |
| | | | **Males** | | | |
| 0 | 23.6 (1.9)<br>- | 36.2 (3.6)<br>- | 59.8 (3.0)<br>- | 49.5 (6.1)<br>- | 43.0 (6.5)<br>- | 183.6 (13.7)<br>- |
| 150 | 10.1* (4.5)<br>−57.1% | 38.8 (6.7)<br>7.1% | 48.9* (8.7)<br>−18.2% | 47.6 (4.1)<br>−3.8% | 39.7 (6.1)<br>−7.6% | 160.5* (13.4)<br>−12.6% |
| 350 | −5.0* (8.4)<br>−121% | 22.3* (7.9)<br>−38.3% | 17.4* (8.4)<br>−70.9% | 28.7* (7.7)<br>−42.0% | 32.5* (9.2)<br>−24.5% | 95.4* (17.6)<br>−48.1% |
| 750 | −26.4* (4.2)<br>−212% | −15.9* (4.3)<br>−144% | −42.2* (7.3)<br>−171% | - | - | - |
| 1500 | −33.5* (4.3)<br>−242% | 32.8* (4.0)<br>−191% | −66.2* (6.5)<br>−211% | - | - | - |
| | | | **Females** | | | |
| 0 | 5.1 (6.1)<br>- | 11.7 (5.5)<br>- | 16.8 (8.8)<br>- | 25.1 (6.1)<br>- | 20.1 (6.1)<br>- | 71.6 (13.9)<br>- |
| 150 | 0.1 (3.4)<br>−98.2% | 13.7 (6.1)<br>17.6% | 13.8 (6.7)<br>−17.7% | 18.1* (6.8)<br>−27.8% | 20.0 (7.0)<br>−0.6% | 58.0* (9.6)<br>−19.0% |
| 350 | −9.4* (5.7)<br>−284% | 9.0 (5.9)<br>−22.8% | −0.4* (2.8)<br>−102% | 11.7* (4.4)<br>−53.3% | 12.4* (6.6)<br>−38.5% | 29.5* (7.3)<br>−58.8% |
| 750 | −16.6* (7.4)<br>−425% | −10.0* (2.8)<br>−185% | 26.6* (6.0)<br>−258% | - | - | - |
| 1500 | −26.6* (5.1)<br>−620% | −23.2* (6.8)<br>−299% | −49.8* (8.3)<br>−397% | - | - | - |

* Statistically significant according to a Dunnett's two-sided or Dunnett's non-parametric t-test at $p < 0.05$

Increased liver weight relative to body weight (by 17.3% for males and 8.4% for females, compared to controls) and minimal centrilobular hepatocellular hypertrophy, the latter only in males, was reported in the groups receiving 350 ppm. These effects were concordant with increased cholesterol (by 25% in males and 71% in females), total hepatic microsomal cytochrome P450 enzyme content (87% and 91% in males and females respectively) and increased UDP-GT activity (by 100% and 124%, in male and females respectively, compared to controls), all seen at a dose of 350 ppm. Increased UDP-GT activity (by 38% in males and 28% in females) was also reported at a dose level of 150 ppm. Whilst they were treatment-related, these effects were considered adaptive by the report author, and therefore not adverse. However, in the view of the Meeting, the magnitude of the liver weight changes and correlated effects on cholesterol indicate that the effects on the liver were indeed adverse.

Degeneration of olfactory epithelium in the nose and, less frequently, focal erosion or ulceration of the olfactory epithelium, of minimal severity, were reported in males (7/10 and 10/10) and in females (9/10 and 10/10) at 150 and 350 ppm respectively. These effects were treatment-related. Autophagic vacuoles in acinar cells of the pancreas of males (4/10) and females (4/10) were reported at 350 ppm. Autophagic vacuolation of acinar cells was interpreted as a homeostasis mechanism that plays a major role in catabolism of lysosomal digestion of organelles or cytoplasmic contents (Nolte et al, 2016). Consequently, this change was considered to be a secondary response to reduced body weight, and not a direct effect of the test article.

No NOAEL could be derived from this study due to the adverse effects on body weight, body weight gain and the olfactory epithelium reported at the lowest dose of 150 ppm (equal to 12 mg/kg bw per day) (Murkerji 2016).

Metabolite IN-M2G98 was evaluated in four in vitro genotoxicity studies. These studies are summarized in Table 4, and additional details are given below.

*Table 4. Summary of genotoxicity studies with IN-M2G98*

| Type of study | Test system | Concentration/ dose range tested | Metabolic activation | Batch/ Purity | Result | Reference |
|---|---|---|---|---|---|---|
| Reverse gene mutation test in bacteria (Ames test) | *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2 *uvr*A | 1.5–5000 µg/plate | ± S9 | E117295-44 92.7% | Negative | Wagner, 2013 |
| Mammalian cell micronucleus | Human peripheral lymphocytes | 137.5–2000 µg/mL | ± S9 | C1365-031 95.9% | Negative | Gilby, 2022a |
| Mammalian cell chromosomal aberration | Human peripheral lymphocytes | 250–3020 µg/mL | ± S9 | E117295-44 92.7% | Negative | Glover, 2013 |
| Mammalian cell forward gene mutation (HPRT) | Chinese hamster ovary (CHO) | 500–2320 µg/mL | ± S9 | E117295-44 92.7% | Negative | Clarke, 2013 |

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*;

HPRT: Hypoxanthine–guanine phosphoribosyl transferase

No evidence of mutagenicity was reported in the bacterial reverse gene mutation (Ames) test in *Salmonella typhimurium* and *Escherichia coli* tester strains, following OECD TG 471 (1997). No increase in mutation frequency was reported in any of the strains at any concentration tested, neither with, nor without metabolic activation, in the initial or in the confirmatory tests. No precipitation was reported at any concentration tested. All positive controls caused distinct increases in mutation frequency. The positive and negative controls were within the range of the historical control data (HCD) (Wagner, 2013).

No evidence of clastogenicity, nor of aneugenicity was reported in the in vitro micronucleus test using human lymphocytes, following OECD TG 487 (2016). No statistically significant increases in micronucleus formation were reported after treatment with IN-M2G98 at any of the concentrations tested, neither using three hours exposure with S9 (rat liver supernatant fraction obtained by centrifuging at 9000 g), nor without metabolic activation using a three hours or 24 hours exposure. No precipitation was reported. Significant cytotoxicity was only reported at 550 µg/mL (20 hours without S9). No statistical significance was reported from any of the trend tests. Micronucleus formation that occurred following treatment with the test substance was within the range of the negative HCD values. The positive controls caused distinct increases in micronucleus formation. All positive and negative controls were within the range of the HCD (Gilby, 2022a).

No evidence of clastogenicity was reported in the in vitro chromosomal aberration test using human lymphocytes, which broadly followed OECD TG 473 (1997). However only 100 cells were counted instead of the prescribed 300. No statistically significant increases in chromosomal aberrations were reported following treatment with IN-M2G98 at any of the concentrations tested, neither using four hours exposure with S9 activation, nor without metabolic activation using four hours or 22 hours exposure. Significant cytotoxicity was reported at 2000 µg/mL and above (four hours with S9), 2750 µg/mL, (four hours without S9), and at 1000 µg/mL (22 hours without S9). Precipitation was reported at 2250 µg/mL and above. No statistical significance was revealed from results of any of the trend tests. Chromosomal aberrations following treatment with the test substance were within the range of the negative HCD values. The positive controls caused distinct increases in chromosomal aberrations. The positive and negative controls were within the range of HCD values (Glover, 2013).

No evidence of mutagenicity was reported in the in vitro hypoxanthine–guanine phosphoribosyl transferase (HPRT) forward gene mutation test using Chinese hamster ovary cells that followed OECD TG 476 (1997). No significant increases in mutation frequencies were reported following treatment with IN-M2G98 at any of the concentrations tested, neither with, nor without metabolic activation. No significant cytotoxicity was reported and relative cloning efficiency was reduced to around 57% at the highest dose. No precipitation was reported at any concentration tested. Mutation frequencies following treatment with the test substance were within the range of the negative HCD values. The positive controls caused distinct increases in mutation frequencies. The positive and negative controls were within the range of the HCD values (Clarke 2013).

***Metabolite IN-K5A78***

Neither acute toxicity nor repeated dose toxicity studies were submitted for this metabolite.

Metabolite IN-K5A78 was evaluated in three in vitro genotoxicity studies. These studies are summarized in Table 5, and additional details are given below.

***Table 5. Summary of genotoxicity studies with IN-K5A78***

| Type of study | Test system | Concentration/ dose range tested | Metabolic activation | Batch/ purity | Result | Reference |
|---|---|---|---|---|---|---|
| Reverse gene mutation test in bacteria (Ames test) | *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2*uvr*A | 33.3–5000 µg/plate | ± S9 | E119780-79 96.4% | Negative | Myhre, 2015 |
| Mammalian cell micronucleus | Human peripheral lymphocytes | 250–1000 µg/mL | ± S9 | E119780-79 96.4% | Negative | Gilby, 2022b |
| Mammalian cell forward gene mutation (HPRT) | Chinese hamster ovary (CHO) | 15.6–2000 µg/mL | ± S9 | E119780-79 96.4% | Negative | Roy, 2015 |

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*;

HPRT: Hypoxanthine–guanine phosphoribosyl transferase

No evidence of mutagenicity was reported in the bacterial reverse gene mutation (Ames) test in *Salmonella typhimurium* and *Escherichia coli* tester strains that followed OECD TG 471 (1997). No increase in mutation frequency was reported in any of the strains at any concentration tested, neither with, nor without metabolic activation, in the initial or in the confirmatory test. Precipitation was reported at 3333 µg/mL and above. All positive controls caused distinct increases in mutation frequency. The positive and negative controls were within the range of the HCD values (Myhre, 2015).

No evidence of clastogenicity, nor of aneugenicity was reported in an in vitro micronucleus test using human lymphocytes, following OECD TG 473 (2016). No statistically significant increases in micronucleus formation were reported following treatment with IN-K5A78 at any of the concentrations tested, neither using three hours exposure with S9, nor three or 20 hours without metabolic activation. Precipitation was reported at 1000 µg/mL. Significant cytotoxicity was reported at 350 µg/mL and above in the 20-hour exposure without S9 activation. No statistical significance was apparent from any of the trend tests. Micronucleus formation following treatment with the test substance was within the range of the negative HCD values. The positive controls caused distinct increases in micronucleus formation. All other positive and negative controls were within the range of the HCD values (Gilby, 2022b).

No evidence of mutagenicity was reported in the in vitro HPRT forward gene mutation test using CHO cells following OECD TG 476 (1997). A statistically significant increase in the mutation frequency was reported at 125 µg/mL with S9 activation, however this result was isolated, not part of a trend and well within the HCD range for the vehicle control. Moreover, a repeat of the S9 activated

test was negative at all concentrations. No statistically significant increases in mutation frequency were reported following treatment with IN-K5A78 at any of the concentrations tested, neither with, nor without metabolic activation. No significant cytotoxicity was reported and relative survival was reduced to ca 44% at the highest dose without S9 activation. Precipitation was reported at 250 µg/mL and above. No statistical significance was apparent from any of the trend tests conducted. Mutation frequencies following treatment with the test substance were within the range of the negative HCD values. The positive controls caused distinct increases in mutation frequencies. The positive and negative controls were within the range of HCD values (Roy 2015).

***Metabolite IN-K5A79***

An acute oral toxicity study using the "up-and-down" procedure was performed with female Crl:CD (SD) rats. In total, three animals were used; they were 10 weeks old at the start of dosing. The test substance, IN-K5A79 (92.9% purity, batch C1365-061), was suspended in 0.1% Tween-80 (v/v) in 0.5% aqueous methylcellulose and administered to three rats by oral gavage at 5000 mg/kg bw. The total dosing volume of 20 mL/kg bw.

Animals were monitored daily for mortality and clinical signs. Rats were euthanized by isoflurane anaesthesia and exsanguination, then subjected to gross necropsy.

None of the rats died. One of the three rats did not gain body weight between day 8 and day 15, however this does not affect the result. No clinical signs were reported. No gross lesions were reported.

Based on this study, the oral $LD_{50}$ was greater than 5000 mg/kg bw, similar to that of the parent, cyantraniliprole (Fallers, 2016b).

A 28-day repeat dose oral toxicity study was performed in rats following OECD TG 407 (2008). Groups of 10 male and 10 female Crl:CD (SD) rats per dose were fed diets for 28 days containing IN-K5A79 (purity 92.9%, batch C1365-061) at concentrations of 0, 100, 400, 3000 or 20 000 ppm (equal to 0, 8.5, 33.7, 262 and 1800 mg/kg bw per day for males, 0, 8.9, 35.6, 278 and 1912 mg/kg bw per day for females). Animals were monitored throughout the study for clinical signs, weighed regularly and food consumption was measured. Animals were fasted for 15 hours prior to termination. At scheduled termination, blood samples were taken from the vena cava under isoflurane anaesthesia. All required haematological parameters were determined, as were all clinical chemistry parameters in addition to β-oxidation activity (using $^{14}$C-labelled palmitoyl CoA), total cytochrome P450 content, and hepatic microsomal UDP-GT activity (using 4-nitrophenol). Urinalysis was also performed. The rats were killed and subjected to a full gross examination. All required organs were weighed, and a representative selection of organs and tissues was taken for histopathology.

All rats survived until scheduled termination. Statistically significant increases in body weight, body weight gain, food intake and food efficiency were seen in all male groups compared to the control group, however there was no clear dose–response relationship, and these weight changes were not considered treatment-related. Irregular, statistically significant differences were also seen in the same parameters for females, but again, they were not regarded as treatment-related. No treatment-related clinical findings were reported.

No treatment-related effects were seen in the haematology and clinical chemistry measurements, nor from urinalysis. Hepatic cytochrome P450 activity was significantly lower (0.72-fold that of controls) in males at 3000 ppm, but not significantly different in males or females at 20 000 ppm. No statistically significant effects on hepatic peroxisomal β-oxidation, nor on UDP-GT activity were reported at any dose in either sex.

No gross findings in any group were attributed to treatment. Statistically significant increases in absolute liver weight were seen (by 10.2% and 12.9% respectively) in males and females treated at 20 000 ppm, however changes in liver weight relative to body weight (7.5% and 6.6% respectively) were not significantly different at any dose level, and did not correlate with histopathological findings. A similar finding was reported for absolute adrenal weight (increased by 23.3%) in females in the 20 000 ppm group, but once again, the increase in adrenal weight relative to body weight (by 16.5%) was not statistically significant and there were no histopathological changes. No other statistically significant changes in organ weight were considered to be treatment-related and no treatment-related

histopathological changes were reported. Although some changes were substantial, both in absolute and relative terms, these changes were not consistent between sexes, there was no apparent dose–response relationship, differences from the control group were rarely statistically significant and there were no histopathological, nor clinical chemistry correlates. Consequently none of the observed changes were considered treatment-related.

Thyroid hormone measurements were presented in a second report based on the same 28-day repeat dose oral toxicity study in rats, as per OECD TG 407 (2006). At scheduled termination, blood samples were taken from the vena cava under isoflurane anaesthesia, and the thyroid hormones triiodothyronine (T3), thyroxine (T4) and thyroid-stimulating hormone (TSH) were measured. No treatment-related changes in T3, T4 or TSH were evident.

The NOAEL in this 28-day repeat toxicity study was 20 000 ppm (equal to 1912 mg/kg bw per day), the highest dose tested, based on a lack of adverse effects at 20 000 ppm (Sayers 2016a, b).

Metabolite IN-K5A79 was evaluated in four in vitro genotoxicity studies. These studies are summarized in Table 6, and additional details are given below.

***Table 6. Summary of genotoxicity studies with IN-K5A79***

| Type of study | Test system | Concentration/ dose range tested | Metabolic activation | Batch/ purity | Result | Reference |
|---|---|---|---|---|---|---|
| Reverse gene mutation test in bacteria (Ames test) | *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2 *uvr*A | 33.3–5000 µg/plate | ± S9 | 103533-68 98.5% | Negative | Myhre, 2017 |
| Mammalian cell micronucleus | Human peripheral lymphocytes | 500–2000 µg/mL | ± S9 | DA 7090-378MWall 95.6% | Negative | Gilby, 2022c |
| Mammalian cell chromosomal aberration | Human peripheral lymphocytes | 25–2000 µg/mL | ± S9 | 103533-68 98.5% | Negative | Kellum, 2015 |
| Mammalian cell forward gene mutation (HPRT) | Chinese hamster ovary (CHO) | 299–4787 µg/mL | ± S9 | 103533-68 98.5% | Negative | Dutta, 2015 |

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*;

HPRT: Hypoxanthine–guanine phosphoribosyl transferase

No evidence of mutagenicity was reported in the bacterial reverse gene mutation (Ames) test in *Salmonella typhimurium* and *Escherichia coli* tester strains conducted to OECD TG 471 (1997). No increase in mutation frequency as reported in any of the strains at any of the concentrations tested, neither with, nor without metabolic activation, in the initial or in the confirmatory test. No precipitation was reported at any of the concentrations used. All positive controls caused distinct increases in mutation frequency. The positive and negative controls were within the range of the HCD values (Myhre, 2017).

No evidence of clastogenicity, nor aneugenicity was reported from the in vitro micronucleus test using human lymphocytes following OECD TG 487 (2016). No statistically significant increases in micronucleus formation were reported following treatment with IN-K5A79 at any of the concentrations used, neither following three hours exposure with S9, nor three hours and 20 hours exposures without metabolic activation. No precipitation or significant cytotoxicity were reported at any of the concentrations employed. No statistical significance was revealed from any of the trend tests on the results of three hours or 20 hours exposures without S9 metabolic activation. From results of the

three hour treatment in the presence of S9, the initial trend test (that is, using micronuclei per 1000 cells) was significantly significant (see Table 7), however a subsequent trend test of micronuclei per 2000 cells (as required by the OECD guideline), was no longer significant (see Table 8). In the case of all treatments the extent of micronucleus formation after exposure to the test substance was within the range of the negative HCD values, however HCD for micronuclei per 2000 cells were not reported. The positive controls caused distinct increases in micronucleus formation. All other positive and negative controls were within the range of the HCD values.

***Table 7. Three-hour treatment with IN-K5A79 in the presence of S9 metabolic activation, (micronucleus count per 1000 cells examined)***

| Treatment group | | Micronuclei (per 1000 cells) | *p*-value | *p*-value trend test | Mean cytostasis (%) | Historical control data |
|---|---|---|---|---|---|---|
| Vehicle (negative control) | | 6 | | | 0.0 | Mean 6.2 |
| | | 7 | | | | 95% C.I. |
| | | 5 | | | | 1.7–10.8 |
| | | 7 | | | | |
| | Mean | 6.3 | | | | |
| IN-K5A79; 500 µg/mL | | 5 | 1.000 | | 0.0 | |
| | | 4 | | | | |
| | Mean | 4.5 | | | | |
| IN-K5A79; 1000 µg/mL | | 7 | 0.440 | 0.369 | 0.0 | |
| | | 7 | | | | |
| | Mean | 7.0 | | | | |
| IN-K5A79; 2000 µg/mL | | 7 | 0.090 | 0.020 | 3.4 | |
| | | 9 | | | | |
| | Mean | 8.0 | | | | |
| CPA (positive control); 10 µg/mL | | 18 | < 0.001 | | 51.0 | Mean 21.1 |
| | | 20 | | | | 95% C.I. |
| | Mean | 19.0 | | | | 10.2–32.1 |

C.I.: Confidence interval; CPA; Cyclophosphamide; Source: Gilby 2022c

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*

***Table 8. Three-hour treatment with IN-K5A79 in the presence of S9 metabolic activation, (micronucleus count per 2000 cells examined)***

| Treatment group | | Micronuclei (per 2000 cells) | *p*-value | *p*-value trend test | Mean cytostasis (%) | Historical control data |
|---|---|---|---|---|---|---|
| Vehicle (negative control) | | 14 | | | 0.0 | No data |
| | | 16 | | | | |
| | | 12 | | | | |
| | | 12 | | | | |
| | Mean | 13.5 | | | | |
| IN-K5A79; 500 µg/mL | | 10 | 1.000 | | 0.0 | |
| | | 11 | | | | |
| | Mean | 10.5 | | | | |

| Treatment group | | Micronuclei (per 2000 cells) | *p*-value | *p*-value trend test | Mean cytostasis (%) | Historical control data |
|---|---|---|---|---|---|---|
| IN-K5A79; 1000 µg/mL | | 11 | 0.634 | 0.532 | 0.0 | |
| | | 20 | | | | |
| | Mean | 15.5 | | | | |
| IN-K5A79; 2000 µg/mL | | 14 | 0.597 | 0.233 | 3.4 | |
| | | 17 | | | | |
| | Mean | 15.5 | | | | |
| CPA (positive control); 10 µg/mL | | 35 | < 0.001 | | 51.0 | No data |
| | | 42 | | | | |
| | Mean | 38.5 | | | | |

C.I.: Confidence interval; CPA; Cyclophosphamide; Source: Gilby, 2022c

No evidence of clastogenicity was reported in the in vitro chromosomal aberration test using human lymphocytes, which followed OECD TG 473 (2014). No statistically significant increases in chromosomal aberrations were reported following treatment with IN-K5A79 at any concentration tested, neither following four hours exposure with S9, nor four hours and 22 hours exposures without metabolic activation. Significant cytotoxicity was reported at 250 µg/mL and above (four hours with S9) and 500 µg/mL and above (22 hours without S9). No precipitation was reported at any concentration tested. No statistical significance was apparent from any of the trend tests employed. Chromosomal aberrations following treatment with the test substance were within the range of the negative-control HCD values. The positive controls caused distinct increases in chromosomal aberrations. The positive and negative controls were within the range of HCD values (Kellum, 2015).

No evidence of mutagenicity was reported in the in vitro HPRT forward gene mutation test using Chinese hamster ovary cells, following OECD TG 476 (1997). No statistically significant increases in mutation frequencies were reported following treatment with IN-K5A79 at any concentration tested, neither with, nor without metabolic activation. No significant cytotoxicity was reported and relative survival was reduced to ca 43% at the highest dose. Precipitation was reported at 2394 µg/mL and above. No statistical significance was apparent from any of the trend tests employed. Mutation frequencies following treatment with the test substance were within the range of the negative-control HCD values. Positive controls caused distinct increases in mutation frequencies. The positive and negative controls were within the range of HCD values (Dutta, 2015).

***Metabolite IN-F6L99***

A mouse acute oral $LD_{50}$ of greater than 2000 mg/kg bw had previously been determined (JMPR, 2014b; Finlay, 2006).

Metabolite IN-F6L99 was previously evaluated in a negative Ames test (Myhre, 2006) and is included in the table below for the sake of completeness. Metabolite IN-F6L99 was further evaluated by the current Meeting in the light of two new in vitro genotoxicity studies. These studies are summarized in Table 9, and additional details are given below.

***Table 9. Summary of genotoxicity studies with IN-F6L99***

| Type of study | Test system | Concentration/ dose range tested | Metabolic activation | Batch/purity | Result | Reference |
|---|---|---|---|---|---|---|
| Reverse gene mutation test in bacteria (Ames test) | *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2 *uvr*A | 33.3–5000 µg/plate | ± S9 | 4004042 98.6% | Negative | Myhre, 2006[a] |

| Type of study | Test system | Concentration/ dose range tested | Metabolic activation | Batch/purity | Result | Reference |
|---|---|---|---|---|---|---|
| Mammalian cell micronucleus | Human peripheral lymphocytes | 35–2000 µg/mL | ± S9 | 200007-352L 99.8% | Negative | Gilby, 2020a |
| Mammalian cell chromosomal aberration | Human peripheral lymphocytes | 50–2000 µg/mL | ± S9 | 200007-352L 99.8% | Negative | Kellum, 2018a |

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*;

[a] Previously evaluated by JMPR 2013 (JMPR, 2014b)

No evidence of clastogenicity, nor aneugenicity was reported in an in vitro micronucleus test using human lymphocytes, following OECD TG 487 (2016). No statistically significant increases in micronucleus formation were reported following treatment with IN-F6L99 at any concentration tested, neither after three hours exposure with S9, nor three hours or 20 hours without metabolic activation. Noteworthy however, was the frequency of micronuclei at 2000 µg/mL, which were above the HCD range in the initial three hour exposure in the absence of S9. Consequently this experiment was repeated, and the frequency of micronuclei found this time to be within the HCD range, and clearly indicating a negative result. The initial 20 hour treatment in the absence of S9 did not achieve significant cytostasis at up to 1000 µg/mL. An additional study resulted in significant cytostasis at 700 µg/mL and above. No statistical significance was apparent from any of the trend tests employed. Micronucleus formation following treatment with the test substance was within the range of the negative HCD values. The positive controls caused distinct increases in micronuclei formation. The positive and negative controls were within the range of HCD values (Gilby, 2020a).

No evidence of clastogenicity was reported in the in vitro chromosomal aberration test using human lymphocytes following OECD TG 473 (2016). No statistically significant increases in chromosomal aberrations were reported following treatment with IN-F6L99 at any concentration tested, neither following four hours exposure with S9, nor four hours or 22 hours without metabolic activation. Significant cytotoxicity was reported at 2000 µg/mL (four hours without S9) and 300 µg/mL (22 hours without S9). No precipitation was reported at any concentrations tested. No statistical significance was apparent from any of the trend tests employed. Chromosomal aberrations following treatment with the test substance were within the range of the negative HCD values. The positive controls caused distinct increases in chromosomal aberrations. The positive and negative controls were within the range of HCD values (Kellum, 2018a).

### *Metabolite IN-N5M09*

A rat acute oral $LD_{50}$ of greater than 5000 mg/kg bw had previously been determined (JMPR, 2014a; Carpenter, 2010).

Metabolite IN-N5M09 was previously evaluated in a negative Ames test (Wagner & VanDyke, 2009a) and is included in the table below for the sake of completeness. Metabolite IN-N5M09 was further evaluated by the current Meeting in the light of three new in vitro genotoxicity studies. These studies are summarized in Table 10, and additional details are given below.

***Table 10. Summary of genotoxicity studies with IN-N5M09***

| Type of study | Test system | Concentration/ dose range tested | Metabolic activation | Batch/purity | Result | Reference |
|---|---|---|---|---|---|---|
| Reverse gene mutation test in bacteria (Ames test) | *Salmonella typhimurium*; *Escherichia coli* | 1.5–5000 µg/plate | ± S9 | N5M09-003 99.9% | Negative | Wagner & VanDyke, 2009a[a] |

| Type of study | Test system | Concentration/ dose range tested | Metabolic activation | Batch/purity | Result | Reference |
|---|---|---|---|---|---|---|
| Reverse gene mutation test in bacteria (Ames test) | *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2 *uvr*A | 1.5–5000 µg/plate | ± S9 | D100855-058 99.9% | Negative | Wagner & VanDyke, 2015 |
| Mammalian cell micronucleus | Human peripheral lymphocytes | 6.25–50 µg/mL | ± S9 | G4907-147 99.7% | Negative | Gilby, 2020b |
| Mammalian cell chromosomal aberration | Human peripheral lymphocytes | 0.5–500 µg/mL | ± S9 | D100855-058 99.9% | Negative | Kellum, 2018b |

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*;

[a] Previously evaluated by JMPR 2013 (JMPR, 2014a)

No evidence of mutagenicity was reported in the bacterial reverse gene mutation (Ames) test in *Salmonella typhimurium* and *Escherichia coli* tester strains, carried out according to OECD TG 471 (1997). No increase in mutation frequency was reported in any of the strains at any concentrations tested, neither with, nor without metabolic activation, in the initial or the confirmatory tests. Precipitation was reported at concentrations of 150 µg/plate and above. All positive controls caused distinct increases in mutation frequency. The positive and negative controls were within the range of HCD values (Wagner & VanDyke, 2015).

No evidence of clastogenicity, nor aneugenicity was reported from the in vitro micronucleus test using human lymphocytes, following OECD TG 487 (2016). No statistically significant increases in micronucleus formation were reported following treatment with IN-N5M09 at any concentration tested, neither using three hours exposure with S9, nor three hours or 20 hours exposure without metabolic activation. There was no evidence of significant cytotoxicity. No statistical significance was apparent from any of the trend tests employed. Micronucleus formation following treatment with the test substance was within the range of negative-control HCD values. The positive controls caused distinct increases in micronuclei formation. The positive and negative controls were within the range of HCD values (Gilby, 2020b).

No evidence of clastogenicity was reported from the in vitro chromosomal aberration test using human lymphocytes following OECD TG 473 (2016). No statistically significant increases in chromosomal aberrations were reported following treatment with IN-N5M09 at any concentration tested, neither using four hours exposure with S9, nor four hours or 22 hours exposure without metabolic activation. There was no evidence of significant cytotoxicity. Precipitation was reported at concentrations of 10–25 µg/mL and above. No statistical significance was apparent from any of the trend tests employed. Chromosomal aberrations following treatment with the test substance were within the range of negative-control HCD values. The positive controls caused distinct increases in chromosomal aberrations. The positive and negative controls were within the range of HCD values (Kellum, 2018b).

***Metabolite IN-JSE76***

A rat acute oral $LD_{50}$ of greater than 5000 mg/kg bw had previously been determined (JMPR, 2014a; Oley, 2009). The NOAEL from a 28-day oral rat study, also evaluated at that time, was 1445 mg/kg bw per day, the highest dose tested (JMPR, 2014a; Anand, 2010; Mawn, 2011).

Metabolite IN-JSE76 was previously evaluated in an Ames test (Wagner & VanDyke, 2009b), an in vitro chromosomal aberration test (Gudi & Rao, 2010) and an in vitro HPRT forward gene mutation test (Clarke, 2009), and these are included in the table below for the sake of completeness. All results

were negative. Metabolite IN-JSE76 was further evaluated by the current Meeting in the light of one in vitro micronucleus test. This study is summarized in Table 11, and additional details are given below.

***Table 11. Summary of genotoxicity studies with IN-JSE76***

| Type of study | Test system | Concentration/ dose range tested | Metabolic activation | Batch/purity | Result | Reference |
|---|---|---|---|---|---|---|
| Reverse gene mutation test in bacteria (Ames test) | *Salmonella typhimurium* and *Escherichia coli* | 50–5000 µg/plate | ± S9 | JSE76-005 93.8% | Negative | Wagner & VanDyke, 2009b[a] |
| Mammalian cell mutation | Human peripheral lymphocytes | 100–1500 µg/mL | ± S9 | JSE76-005 93.8% | Negative | Clarke, 2009[a] |
| Mammalian cell chromosomal aberration | Human peripheral lymphocytes | 156–2000 µg/mL | ± S9 | JSE76-005 93.8% | Negative | Gudi & Rao, 2010[a] |
| Mammalian cell micronucleus | Human peripheral lymphocytes | 500–2000 µg/mL | ± S9 | D100082-299 96.7% | Negative | Gilby, 2022d |

[a] Previously evaluated by JMPR 2013 (JMPR, 2014a)

In an in vitro micronucleus test using human lymphocytes, largely following OECD TG 487 (2016), a statistically significant increase in micronuclei was initially reported at 2000 µg/mL following exposure for 20 hours without S9 activation (Table 12), and a trend test also indicated the values were statistically significant, however the number of micronuclei counted was within the 95% confidence interval of the HCD range. The initial analysis was based on a count of 1000 cells (Table 12). A subsequent analysis, counting 2000 cells in total (as required by OECD TG 487) reinforced these statistical analyses (data not shown). According to the criteria in TG 487, this second result was neither clearly positive, nor clearly negative. Consequently, an additional repeat of the 20-hour exposure without S9 was undertaken, the result of which was no longer statistically significant (Table 13). The result was therefore taken as clearly negative for the 20-hour exposure without S9 activation.

***Table 12. Twenty hour treatment with IN-JSE76 in the absence of S9; 1000 cells examined[a]***

| Treatment group | | Micronuclei (per 1000 cells) | *p*-value | *p*-value trend test | Mean cytostasis (%) | Historical control data |
|---|---|---|---|---|---|---|
| Vehicle (negative control) | | 4 | | | 0.0 | Mean 5.9 95% C.I. 1.4 – 10.4 |
| | | 3 | | | | |
| | | 5 | | | | |
| | | 3 | | | | |
| | Mean | 3.8 | | | | |
| IN-JSE76; 500 µg/mL | | 3 | 1.000 | | 0.0 | |
| | | 2 | | | | |
| | Mean | 2.5 | | | | |
| IN-JSE76; 1000 µg/mL | | 6 | 0.096 | 0.084 | 0.0 | |
| | | 5 | | | | |
| | Mean | 5.5 | | | | |
| IN-JSE76; 2000 µg/mL | | 6 | 0.021* | 0.005** | 31.6 | |
| | | 7 | | | | |
| | Mean | 6.5 | | | | |

| Treatment group | | Micronuclei (per 1000 cells) | *p*-value | *p*-value trend test | Mean cytostasis (%) | Historical control data |
|---|---|---|---|---|---|---|
| MMC; 0.1 µg/mL (positive control) | | 21 | < 0.001 | | 9.1 | Mean 22.8 95% C.I. 14.2–35.9 |
| | | 16 | | | | |
| | Mean | 18.5 | | | | |
| COL; 0.02 µg/mL (positive control) | | 13 | < 0.001 | | 22.4 | Mean 16.3 95% C.I. 8.3–24.2 |
| | | 15 | | | | |
| | Mean | 14.0 | | | | |

C.I.: Confidence interval; MMC; Methyl methanesulfonate; COL: Colchicine; Source: Gilby 2022d
S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*

***Table 13. Additional 20 hour treatment with IN-JSE76 in the absence of S9; 1000 cells examined*** [a]

| Treatment group | | Micronuclei (per 1000 cells) | *p*-value | *p*-value trend test | Mean cytostasis (%) | Historical control data |
|---|---|---|---|---|---|---|
| Vehicle | | 4 | | | 0.0 | Mean 5.9 95% C.I. 1.4–10.4 |
| | | 9 | | | | |
| | | 6 | | | | |
| | | 9 | | | | |
| | | 7.0 | | | | |
| IN-JSE76; 1000 µg/mL | | 6 | 1.000 | | 5.1 | |
| | | 10 | | | | |
| | Mean | 8.0 | | | | |
| IN-JSE76; 1500 µg/mL | | 7 | 1.000 | 0.465 | 7.9 | |
| | | 4 | | | | |
| | Mean | 5.5 | | | | |
| IN-JSE76; 2000 µg/mL | | 6 | 0.791 | 0.938 | 13.2 | |
| | | 10 | | | | |
| | Mean | 8.0 | | | | |
| MMC; 0.1 µg/mL | | 19 | < 0.001 | | 23.9 | Mean 22.8 95% C.I. 14.2–35.9 |
| | | 23 | | | | |
| | Mean | 21.0 | | | | |
| COL; 0.02 µg/mL | | 20 | < 0.001 | | 22.5 | Mean 16.3 95% C.I. 8.3–24.2 |
| | | 19 | | | | |
| | Mean | 19.5 | | | | |

C.I.: Confidence interval; MMC; Methyl methanesulfonate; COL: Colchicine; Source: Gilby 2022d
S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*

No other statistically significant increases in micronuclei formation were reported following treatment with IN-JSE76 at any concentrations tested, for three hours exposure, with or without metabolic activation. No precipitation was reported. Significant cytotoxicity was not reported. No statistical significance was apparent from any of the trend tests employed. Micronucleus formation following treatment with the test substance was otherwise within the range of the negative-control HCD values. The positive controls caused distinct increases in micronuclei formation. All other positive and negative controls were within the range of HCD values. Overall, no clear evidence of clastogenicity, nor of aneugenicity was provided by the in vitro micronucleus assays using human lymphocytes (Gilby, 2022d).

# Comments

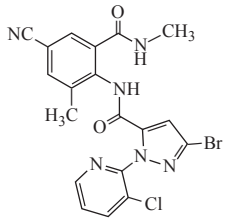
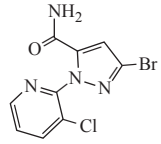
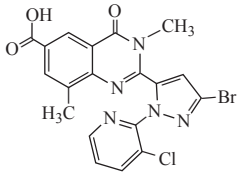
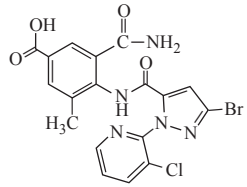
## Biochemical aspects

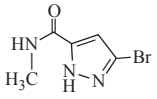
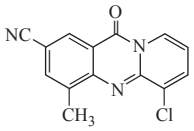
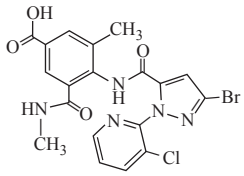
None of the metabolites were detected in urine, faeces or bile, nor were they part of the metabolic pathways proposed for rats.

## Toxicological data on metabolites and/or degradates

A toxicological evaluation was performed for the metabolites shown in Table 1, which shows an overall summary of the toxicological characterization of the metabolites requested for consideration in the residue definition.

***Table 14. Summary of the toxicological characterization of plant/livestock metabolites of cyantraniliprole***

| **Compound, codes and structure** | **Major rat metabolite** (>10% AD) | **Genotoxicity assessment** (data, QSAR, RA) | **General toxicity** | **Toxicological reference values** |
|---|---|---|---|---|
| **Cyantraniliprole**<br> | Parent | Not genotoxic (in vitro and in vivo data) | Full data set | ADI: 0.03 mg/kg bw<br>ARfD: not required |
| **IN-M2G89**<br>3-Bromo-1-(3-chloropyridin-2-yl)-*1H*-pyrazole-5-carboxamide<br> | No | Not genotoxic (in vitro data) | Rat $LD_{50}$, oral: 175 mg/kg bw<br>Rat 28-day repeat dose, oral: No NOAEL. Reduced body weight, bw gain; damage to olfactory epithelium at 12 mg/kg bw/day (ldc) | ADI: 10 times more toxic than parent<br>ARfD: 0.1 mg/kg bw per day |
| **IN-K5A78**<br>2-[3-Bromo-1-(3-chloropyridin-2-yl)-*1H*-pyrazol-5-yl]-3,8-dimethyl-4-oxo-3,4-dihydroquin**a**zoline-6-carboxylic acid<br> | No | Not genotoxic (in vitro data) | No information | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **IN-K5A79**<br>4-({[3-Bromo-1-(3-chloropyridin-2-yl)-*1H*-pyrazol-5-yl] carbonyl} amino)-3-carbamoyl-5-methylbenzoic acid<br> | No | Not genotoxic (in vitro data) | Rat $LD_{50}$, oral: >5000 mg/kg bw<br>Rat 28-day repeat dose, oral NOAEL: 1912 mg/kg bw per day (highest dose tested) | Parent ADI |

| Compound, codes and structure | Major rat metabolite (>10% AD) | Genotoxicity assessment (data, QSAR, RA) | General toxicity | Toxicological reference values |
|---|---|---|---|---|
| **IN-F6L99**<br>3-Bromo-*N*-methyl-*1H*-pyrazole-5-carboxamide<br> | No | Not genotoxic (in vitro data) | Mouse $LD_{50}$, oral: >2000 mg/kg bw [a] | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **IN-N5M09**<br>6-chloro-4-methyl-11-oxo-11Hpyrido[2,1-b]quinazoline-2-carbonitrile<br> | No | Not genotoxic (in vitro data) | Mouse $LD_{50}$, oral: >5000 mg/kg bw [a] | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **IN-JSE76**<br>4-[[[3-Bromo-1-(3-chloro-2-pyridinyl)-*1H*-pyrazol-5-yl] carbonyl]amino]-3-methyl-5-[(methylamino)-carbonyl]benzoic acid<br> | No | Not genotoxic (in vitro data) | Rat $LD_{50}$, oral: >5000 mg/kg bw [a]<br>Rat 28-day repeat-dose, oral NOAEL: 1454 mg/kg bw per day (highest dose tested)[a] | Parent ADI |

ldc: Lowest dose tested; ADI: Acceptable daily intake; ARfD: Acute reference dose;

TTC: Threshold of toxicological concern; RA: Read across;

[a] Previously evaluated by JMPR 2013 (see resulting monograph from this meeting), but included here for completeness

The Meeting concluded that IN-M2G89 is toxicologically relevant and estimated to be 10-fold more potent than the parent based on the 28-day rat studies. The Meeting concluded that IN-K5A78, IN-K5A79, IN-F6L99, IN-N5M09 and IN-JSE76 are not toxicologically relevant.

## Toxicological evaluation (addendum)

The Meeting re-affirmed the ADI of 0–0.03 mg/kg bw for cyantraniliprole, established by the JMPR 2013 Meeting. The current Meeting concluded that the parent ADI applies also to metabolites IN-K5A79 and IN-JSE76, and to 10 times IN-M2G98.

The Cramer class III value of 1.5 µg/kg bw per day applies to IN-K5A78, IN-F6L99 and IN-N5M09.

The current Meeting re-affirmed the conclusion of the 2013 Meeting that it was not necessary to establish an ARfD for cyantraniliprole. The current Meeting established an ARfD of 0.1 mg/kg bw for metabolite IN-M2G98 based on a LOAEL of 12 mg/kg bw per day in the 28-day oral study in rats, and applying a safety factor of 100. Given the lack of toxicity at 55 mg/kg bw in the acute toxicity study, an additional safety factor on the LOAEL was not judged necessary.

***Critical end-points for setting guidance values for exposure to cyantraniliprole (addendum)***

| | |
|---|---|
| **Studies on toxicologically relevant metabolites** | |
| IN-M2G98 | Acute oral $LD_{50}$: ca 175 mg/kg bw (rat)<br>28-day LOAEL: 12 mg/kg bw per day (rat)<br>Not genotoxic (Ames, in vitro micronucleus, in vitro chromosomal aberration, in vitro cell mutation) |
| IN-K5A78 | Not genotoxic<br>(Ames, in vitro micronucleus, in vitro cell mutation) |
| IN-K5A79 | Acute oral $LD_{50}$: > 5000 mg/kg bw (rat)<br>28-day NOAEL: 1912 mg/kg bw per day (rat)<br>Not genotoxic (Ames, in vitro micronucleus, in vitro chromosome aberration assay, in vitro cell mutation) |
| IN-F6L99 | Acute oral $LD_{50}$: > 2000 mg/kg bw (mouse)[a]<br>Not genotoxic (Ames, in vitro micronucleus, in vitro chromosome aberration assay) |
| IN-N5M09 | Acute oral $LD_{50}$: >5000 mg/kg bw (mouse)[a]<br>Not genotoxic (Ames, in vitro micronucleus, in vitro chromosome aberration assay) |
| IN-JSE76 | Acute oral $LD_{50}$: >5000 mg/kg (rat)[a]<br>28-day NOAEL: 1454 mg/kg bw per day (rat)[a]<br>Not genotoxic (Ames test[a], in vitro micronucleus assay, in vitro chromosome aberration assay[a], in vitro cell mutation[a]) |

[a] Previously evaluation by JMPR in 2013

*Acceptable daily intake (ADI) applies to cyantraniliprole, IN-K5A79, IN-JSE76, and 10 × IN-M2G98*

0 – 0.03 mg/kg bw

*Acute reference dose (ARfD) applies to IN-M2G98*

0.1 mg/kg bw

*Acute reference dose (ARfD) unnecessary for cyantraniliprole, IN-K5A79 and IN-JSE76*

## Summary

| | Value | Study | Safety factor |
|---|---|---|---|
| ADI [a] | 0–0.03 mg/kg bw | Ninety-day and one-year toxicity studies (dog) | 100 |
| ARfD for IN-M2G98 | 0.1 mg/kg bw | 28-day study (rat) | 100 |
| ARfD for parent, *IN-K5A79 and IN-JSE76* | Unnecessary | | |

[a] Applies to parent and metabolites IN-K5A79 and IN-JSE76, and 10 × IN-M2G98

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All unpublished references were submitted to WHO by Exponent International Ltd, Harrogate, United Kingdom on behalf of Syngenta Crop Protection AG, 4058 Basel, Switzerland.

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# Emamectin benzoate (addendum)

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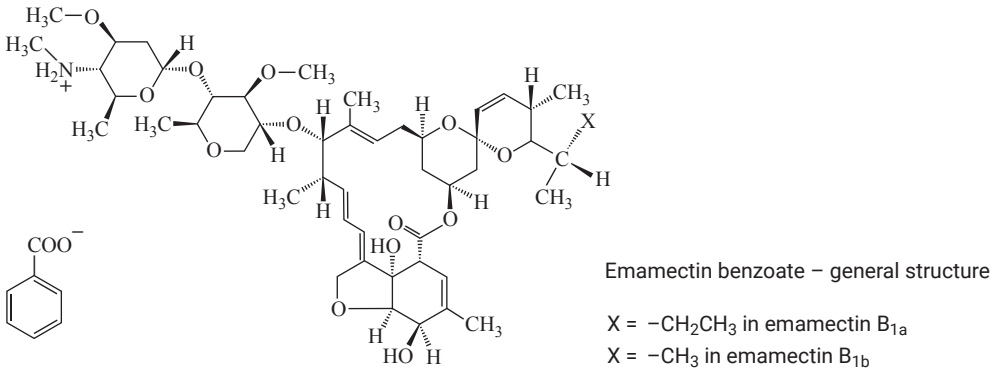
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## Explanation

Emamectin benzoate is the International Organization for Standardization (ISO)-approved name for (4″*R*)-4″-deoxy-4″-(methylamino)avermectin B1 benzoate, with the Chemical Abstracts Service number 155569-91-8. It is a macrocyclic lactone insecticide belonging to the avermectin group. It is a mixture of at least 90% (4″*R*)-4″-deoxy-4″-(methylamino)avermectin B1a benzoate, and at most 10% (4″*R*)-4″-deoxy-4″-(methylamino)avermectin B1b benzoate, both as their salts. Emamectin is structurally similar to abamectin and ivermectin (see Fig.1 below). Emamectin was originally developed as the hydrochloride salt MK 243 (L-656, 748-010V), but the commercial product was subsequently changed to the benzoate salt MK 244 (L-656, 748-038W) and benzoate hydrate (L-656, 748-052S) because of their improved storage and handling characteristics.

Emamectin was previously evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 2011 when an acceptable daily intake (ADI) for emamectin benzoate of 0–0.000 5 mg/kg body weight (bw) and an acute reference dose (ARfD) of 0.03 mg/kg bw were established. Emamectin benzoate was evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2013. The Committee confirmed the health based guidance values (HBGVs) established by JMPR in 2011. The 2014 JMPR Meeting withdrew the ARfD of 0.03 mg/kg bw and established a new ARfD of 0.02 mg/kg bw. Emamectin was evaluated by the present Meeting within the programme of follow-up evaluations after a spontaneous submission of data on the parent compound and its photodegradation metabolites, due to a request for additional information on analytical methodology, storage stability and MRLs. The present Meeting evaluated all newly provided studies. The results of these studies did not affect the previously established ADI or ARfD for emamectin benzoate, thus the details are presented here, but were not included in the meeting report.

***Figure 1. Chemical structure of emamectin benzoate***




Studies were performed with emamectin benzoate, unless stated otherwise. All studies were conducted according to internationally recognized guidelines (generally those of the Organisation for Economic Co-operation and Development, OECD), and followed good laboratory practice (GLP) or were otherwise quality audited; if this was not the case it is indicated.

# Evaluation for acceptable daily intake

## 1. Biochemical aspects

No new data were submitted on this topic.

## 2. Toxicological studies

### 2.1 Acute toxicity

#### (a) Lethal doses

In an oral study with emamectin benzoate technical (purity 96.6%) in female CRL:(NMRI) BC mice, clinical signs of toxicity were observed at both dose levels tested. At the higher dose, clinical signs (decreased activity, hunched back, inco-ordination, tremors) were observed one to three hours after dosing. Emamectin benzoate technical caused mortalities at 175 mg/kg bw (3/3) and 55 mg/kg bw (1/3). The median lethal dose ($LD_{50}$) for emamectin benzoate technical was calculated as 55 mg/kg bw in female mice (Tavaszi, 2010).

### 2.2 Short-term studies of toxicity

#### (a) Oral administration

***Rat***

In a 28-day dose range-finding oral toxicity study, groups of eight male and eight female HsdRccHan:WIST rats were fed diets containing emamectin benzoate (purity 96.2%, batch MK 244G) at mean dietary

concentrations of 0, 25, 50 or 100 ppm (equal to average doses of 0, 2.6, 5.3 and 8.9 mg/kg bw per day for males, 0, 2.6, 5.2 and 9.8 mg/kg bw per day for females). For comparative purposes, additional groups of eight males and eight females were fed diets containing emamectin benzoate from an alternative source (purity 95.6%, batch MK 244) at 50 ppm (equal to 5.2 and 5.3 mg/kg bw per day for males and females respectively). Animals were subject to detailed observations for clinical signs, and their body weights were recorded daily until day 8 of the study and then on days 11, 15, 18, 22, 25, 29 or at termination. Cage-side observations were recorded twice daily for mortality and clinical signs. Food consumption was determined as a weekly mean.

Treatment-related clinical signs of toxicity were observed at 100 ppm (with MK 244G) on days 5–16. Signs comprised tremors in all males and females, decreased activity (8/8 males, 2/8 females), piloerection (5/8 males, 2/8 females); vocalization (5/8 females); shaking (2/8 females); increased response to touch (2/8 females) and staining around the nose (1/8 males). Due to the increased severity of these signs, animals at 100 ppm were terminated on day 16. Tremors were observed in one of eight males at 50 ppm on day 15 only. There were no changes in the clinical condition of animals administered MK 244G at 25 ppm. No treatment-related clinical signs of toxicity were observed in animals administered MK 244 at 50 ppm.

In animals fed emamectin at 100 ppm, suppression of body weight compared to controls was statistically significant, with a time-related decrease compared to controls from day 6 to day 16 (termination), showing a maximum difference of up to 24% in males and 17% in females. Similarly, a statistically significant decrease in food consumption was observed at 100 ppm (MK 244G), in males during weeks 1 and 2 (21% and 39% lower respectively compared to controls) and females during week 2 (20% lower than controls). Significantly reduced food consumption was observed in males fed emamectin benzoate (MK 244G) at 25 and 50 ppm during week 1 (4% and 5% lower, respectively); in these animals food consumption was comparable to controls for the remainder of the study. There were no effects on food consumption in animals fed MK 244 at 50 ppm.

Under the experimental conditions the administration of emamectin benzoate at 100 ppm resulted in body weight and food consumption decrease and severe clinical signs of toxicity (tremors and decreased activity). Dietary concentrations of 25 and 50 ppm were tolerated well and there were minimal effects due to treatment. The two batches used at 5 mg/kg bw per day displayed similar effects on body weight, food consumption and clinical signs of toxicity (Townsley, 2007a).

In a 90-day oral toxicity study, groups of 12 male and 12 female HsdRccHan:WIST rats were fed diets containing emamectin benzoate (purity 96.2%, batch MK 244G) at concentrations targeted to give received doses of 0, 0.5, 2.5 or 5.0 mg/kg bw per day (actual received doses were 0, 0.5, 2.5 and 4.9 mg/kg bw per day for males, 0, 0.5, 2.5 and 5.1 mg/kg bw per day for females). Cage-side observations were made twice a day. Detailed clinical observations and body weights were recorded twice weekly throughout the study and at termination. Food consumption was determined as a weekly mean for each cage. Ophthalmology examination was conducted before treatment commenced and during the week prior to termination in controls and males at 2.5 mg/kg bw per day, and in females at 5 mg/kg bw per day. At week 13 animals were subject to a functional observation battery (FOB), consisting of assessment in the home cage, removal from cage, standard arena, handling, reflex tests and quantitative measurements; motor activity was also recorded. Urinalysis was conducted during the last week of treatment. At termination sampling were taken for a standard set of haematology and clinical chemistry analyses, and the following organs were weighed: adrenal glands, ovaries, brain, spleen, epididymides, testes, heart, thymus, kidneys, uterus (with cervix) and liver. All animals were subjected to a full macroscopic examination. A microscopic examination was conducted on a full set of tissues from male and female controls, from males at 2.5 mg/kg bw per day and females at 5 mg/kg bw per day, and from any animal killed intercurrently (males at 5 mg/kg bw per day). In addition, sciatic nerve samples were examined from males at 0.5 mg/kg bw per day and females at 2.5 mg/kg bw per day. Sciatic nerve samples from females receiving 0.5 mg/kg bw per day were not examined due to the findings in female of the 2.5 mg/kg bw per day group being comparable to the concurrent control.

Treatment-related clinical observations were noted in males at 5 mg/kg bw per day during weeks 5–14. These were piloerection (12/12 males, 10/12 females), shaking (11/12 males, 9/12 females) and thinness (7/12 males, 5/12 females). Other treatment-related effects in these animals that occurred less frequently and in fewer individuals included tremors (2/12 males), sides pinched

(1/12 male) and hunched (1/12 male), absence of pinna reflex (7/12 females), reduced splay reflex (4/12 females) and ungroomed (4/12 females). In males at 5 mg/kg bw per day (actual 4.9 mg/kg bw per day), two animals were killed due to clinical signs in weeks 5 and 9, and the remaining animals were terminated for humane reasons on day 76 due to the severity of the effects and loss of body weight. At 2.5 mg/kg bw per day, reduced splay reflex was seen in 2/12 females and in 1/12 male during weeks 13–14. No treatment-related effects in clinical condition were seen in animals dosed at 0.5 mg/kg bw per day.

At 5 mg/kg bw per day (actual 4.9 mg/kg bw per day), decreases in the body weight of males (adjusted to initial body weight) were statistically significant from week 6 onward, by up to 18% compared to controls. At week 11 these animals showed absolute body weight loss and were therefore terminated early. Females receiving an actual dose of 5.1 mg/kg bw per day displayed a significant body weight decrease from week 9 but the effect was less severe than in males, at 7% compared to controls. There were no treatment-related effects on body weight in animals at 0.5 or 2.5 mg/kg bw per day. Food consumption was significantly reduced in males at 5 mg/kg bw per day (actual 4.9 mg/kg bw per day) from week 5, and food utilization decreased in weeks 5–8. Females in the 5 mg/kg bw per day group showed reduced food utilization during weeks 5–8, weeks 9–13, and over the whole study length.

No treatment-related effects were observed as a result of ophthalmoscopic examination.

There were several findings from the FOB (clinical assessments) which were treatment-related. In females receiving an actual dose of 5.1 mg/kg bw per day, an absence of pinna reflex was noted in 7/12 rats, reduced splay reflex in 4/12 rats and "thin" was reported for 4/12 rats. At 2.5mg/kg bw per day, a reduced splay reflex was observed in 1/12 male and 2/12 female rats. Changes in fore- and hind-limb grip strength compared to controls were statistically significant (reduced by 53% and 23%, respectively) in females receiving an actual dose of 5.1 mg/kg bw per day. No effects on grip strength were seen in either sex at 0.5 or 2.5 mg/kg bw per day. There were no treatment-related effects on motor activity at any dose levels in either sex.

A statistically significant reduction in urine volume was reported for females at 2.5 and 5 mg/kg bw per day (by 34% and 50% respectively) when compared to controls. Urine specific gravity was higher in females at 2.5 and 5 mg/kg bw per day, achieving statistical significance (1%) at the high dose, probably reflecting the lower urine volume. It was noted that urine volume in control animals was greater than the historical control range, whilst urine volume and specific gravity values for animals dosed at 2.5 and 5 mg/kg bw per day fell within historical control ranges. In the absence of correlating pathological findings these changes were considered of doubtful relation to treatment, and of no toxicological significance.

There were no treatment-related or toxicologically relevant effects on haematology parameters. A marginal but statistically significant increase in haemoglobin (Hb) was reported in females at 5 mg/kg bw per day (by 4%) and in males at 2.5 mg/kg bw per day (by 3%), and mean cell Hb concentrations were minimally higher (by 2% compared with controls, but statistically significant) in males at 2.5 mg/kg bw per day. In the absence of any effects on other red blood cell (RBC) parameters in males or females at the higher dose, these differences were considered neither related to treatment nor of biological relevance.

Clinical chemistry analysis showed a statistically significant lowering of cholesterol (by 16%) and creatinine (by 13%) levels in females at 5 mg/kg bw per day compared to controls. For creatinine, it was noted that there was one particularly high outlier value in the control group results.

In females receiving 5 mg/kg bw per day, absolute and adjusted (for body weight) spleen weights were lower (with statistical significance at 17% and 14%, respectively), and brain and kidney weights similarly adjusted were higher compared to controls, reaching statistically significance higher at 5% and 7%, respectively. The higher brain and kidney weights were considered to probably reflect lower body weights rather than a direct effect on these organs. Adrenal weights were higher (with statistical significance) before and after adjustment for body weight (by 13% at 0.059 g) in males at 2.5 mg/kg bw per day when compared to controls (0.052 g). However, it was noted that the control group mean weight was unusually low, and the 2.5 mg/kg bw per day group mean adrenal weight was within the range of HC means (0.054–0.059 g). There were no treatment-related histopathology findings corresponding to the organ weight changes described.

There were no treatment-related findings at gross pathology examination. Histopathology

examination revealed an increased incidence of focus inflammatory cell infiltration/necrosis in the liver of females at 5 mg/kg bw per day (4/12) when compared to controls (1/12). At 5 mg/kg bw per day, in both sexes, there was an increased incidence and severity of nerve fibre degeneration/demyelination in the sciatic nerve. This finding was recorded in the control group in 2/12 males and 2/11 females, all at minimal severity. In contrast, all 24 animals in the 5 mg/kg bw per day dose group were affected, and in the majority of these the severity of the lesion was greater than that seen in the controls. Moving from 0.5 to 2.5 mg/kg bw per day in males there appeared an increased incidence in sciatic degeneration (4/12). However, as the severity was the same as in concurrent controls and the incidence was within the laboratory HC range, the incidence of degeneration/demyelination in the sciatic nerve in the mid dose males was not considered biologically relevant and of doubtful relation to treatment.

***Table 1. Key findings of the 90-day dietary toxicity study in rat***

| | Dose level (mg/kg bw per day) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Males | | | | | Females | | | | |
| | 0 | 0.5 | 2.5 | 4.9 | HCD (min–max) | 0 | 0.5 | 2.5 | 5.1 | HCD (min–max) |
| **Liver – histopathology** | | | | | | | | | | |
| Number examined | 12 | 0 | 12 | 12 | | 12 | 0 | 0 | 12 | |
| Focus inflammatory cell infiltration/necrosis | | | | | | | | | | |
| All | 2 | 0 | 2 | 3 | | 1 | 0 | 0 | 4 | |
| minimal | 2 | 0 | 2 | 3 | | 1 | 0 | 0 | 4 | |
| **Sciatic nerve – histopathology** | | | | | | | | | | |
| Number examined | 12 | 12 | 12 | 12 | | 12 | 0 | 12 | 12 | |
| Degeneration/demyelination | | | | | | | | | | |
| All | 2 | 3 | 4 | 12 | 0–6 | 2 | 0 | 2 | 12 | 1–4 |
| minimal | 2 | 3 | 4 | 1 | | 2 | 0 | 2 | 4 | |
| slight | | | | 8 | | | | | 8 | |
| moderate | | | | 3 | | | | | – | |

HCD: Historical control data from five studies conducted between 2005 and 2006 Source: Townsley, 2007b

The NOAEL was 0.5 mg/kg bw per day based on increased incidence of reduced splay reflex in males and females at 2.5 mg/kg bw per day (Townsley, 2007b).

### *Dog*

A study with emamectin benzoate (purity 92.8% B1a and 4.1% B1b, batch L-656, 748) was performed in dogs, two of each sex per dose. Initially the test item was administered in the diet at doses corresponding to 0, 0.5, 2.5, 12.5 and 25 mg/kg bw per day. Due to poor food consumption, probably due to palatability issues, administration was stopped at day 4 and the route of administration changed to oral gavage at doses of 0, 0.5, 2.5, 5 and 7.5 mg/kg bw per day on day 8 or day 9. The intention was to dose with 11 administrations.

Mydriasis and ataxia were observed in one high-dose male on day 5, that is one day after the termination of the dietary administration phase.

Mydriasis, ataxia, tremors and lateral recumbency were observed at 2.5, 5 and 7.5 mg/kg bw per day after dosing by oral gavage. The severity was comparable between groups; the onset of clinical signs was dose-dependent. Ataxia was first observed in the 7.5, 5 and 2.5 mg/kg bw per day groups on days 8, 9 and 12, respectively. On day 10 dosing was terminated for all animals in the 7.5 mg/kg bw per day group and for one female in the 5 mg/kg bw per day group due to severe ataxia/lateral recumbency. After termination of dosing these dogs were observed until the end of the study. Decreased food and water consumption and decreases in body weight were recorded for all animals dosed with the three highest dose levels. Slight effects on haematological and clinical chemistry parameters were observed in animals dosed with 2.5 mg/kg bw per day, probably related to stress associated with the poor physical condition

of the animals. No treatment-related changes in electrocardiograms were observed. No treatment-related effects were observed at the 0.5 mg/kg per day dose level (Lankas, 1992a)

**(b) Dermal application**

A four-week dermal toxicity study was performed according to OECD test guideline 410 in New Zealand White rabbits. Groups of five male and five female rabbits were treated for four weeks, on five days each week via semi-occluded application to shaven skin for six hours for each treatment. Emamectin benzoate (purity 97.9%) was applied in solid form at concentrations of 0, 100, 300 and 1000 mg/kg bw on each of these days. There were no effects due to treatment on body weight, body weight gain or food consumption. No local clinical signs were observed that could be ascribed to test item application. No changes that could be considered treatment-related were observed in haematology or clinical chemistry parameters. At necropsy no macroscopic findings or changes in organ weights were recorded. The results of the histopathology evaluation showed no treatment-related effects. The no-observed-adverse effect level (NOAEL) was considered to be 1000 mg/kg bw per day. According to the Draize classification criteria, emamectin benzoate is thus considered to be 'not irritant' to rabbit skin (Zelenak, 2017).

**(c) Exposure by inhalation**

***Mouse***

A five-day preliminary inhalation study was performed in CF-1 (BR) mice by nose-only exposure. Groups of two male and two female mice were treated for five days for six hours per day with target concentrations of 0, 1, 5, 10, 25 or 125 µg/L of emamectin benzoate technical (purity 95.7%) delivered as particulates. Treatment with 132 and 25.1 µg/L resulted in adverse clinical signs, body weight loss and reduced food consumption, and treatment was stopped after one and two exposure periods, respectively. At 10.9 µg/L lower growth was observed and reduced splay reflex between days 3 and 5. At 5.04 and 1.05 µg/L no clinical signs indicative of systemic toxicity were observed, but slightly lower growth was observed at 5.04 µg/L in both sexes, and at 1 µg/L in males only. Food consumption for animals exposed to 1.05, 5.04 or 10.9 µg/L was not affected. A no-effect concentration for histopathological changes could not be determined since degeneration of olfactory epithelium in the dorsal medial meatus was observed at all exposure levels, with gradually increasing incidence and severity as concentrations increased. Exposure concentrations no higher than 4 µg/L were recommended for the subsequent 30-day study (Pinto, 2007).

***Rat***

A five-day preliminary inhalation study was performed in Crl:CD (SD) rats by nose-only exposure. Groups of five male and five female rats were treated for six hours per day on five consecutive days using target concentrations of 0, 1, 3, 10 or 30 µg/L of emamectin benzoate technical (purity 96.2%) in aerosol. Piloerection and hunched body posture was observed at an achieved concentration of 28.3 µg/L. After five exposures, hunched posture, rolling gate and intermittent or continuous tremors were observed up to two hours post-dose at 28,3 µg/L. Body weight loss was observed at 28.3 µg/L, correlating with lower food consumption. Landing foot splay values at 28.3 µg/L were lower (statistically significant) for males on days 3 and 5, and for females on day 5, possibly because control values were higher at these time points. No differences in the lung or brain weights or macroscopic findings were observed. Microscopic examination of the sciatic nerve indicated no treatment-related changes at the highest concentration.

Microscopic findings at achieved concentrations of 28.3 µg/L were squamous metaplasia of the respiratory epithelium in the nasal cavity, pharynx and larynx, olfactory epithelial atrophy in the nasal cavity and larynx, and epithelial degeneration in the carina of the trachea. These findings were graded as minimal or mild. Microscopic findings at an achieved concentration of 9.1 µg/L were limited to minimal or mild squamous metaplasia and/or inflammatory cell infiltration in the larynx and nasal cavity. These microscopic findings are considered an adaptive response. The no-observed-adverse-effect concentration, which was primarily based on systemic effects, was 9.1 µg/L (Bain, 2014).

## 2.3 Genotoxicity

An updated package of genotoxicity studies on emamectin benzoate was submitted from the current manufacturing source. In addition, some older studies were provided. The results of all studies were negative for genotoxicity.

### (a) In vitro studies

***Bacterial reverse mutation assays***

This study was performed according to OECD Guideline TG 471 (2020). The potential of emamectin benzoate (90.2% B1a, 1.64% B1b) to induce gene mutations in a plate incorporation test (Experiment I) and a pre-incubation test (Experiment II) using the *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100, and the *Escherichia coli* strains WP2 *uvrA* (pKM101) and WP2 (pKM101) was studied. The test item was assayed at concentrations between 1 and 5000 µg/plate.

Cytotoxicity, as indicated by thinning of the background lawn and/or reduction in revertant numbers, was observed at the higher dose levels with all tester strains.

No relevant increase in the number of revertant colonies was observed following treatment with emamectin benzoate at any concentration level up to 5000 µg/plate with any of the test strains, in the presence or absence of metabolic activation with a rat liver supernatant fraction obtained by centrifuging at 9000 *g* (S9 mix). Precipitation was observed with all strains at the highest concentrations tested, both with and without S9. Appropriate reference mutagens were used as positive controls and an increase in induced revertant colonies was observed, demonstrating the sensitivity of the test system and the efficacy of the S9 mix.

Emamectin benzoate was considered not to be mutagenic in the *S. typhimurium* and *E. coli* reverse mutation assays (Chang, 2021)

An older study had been performed according to OECD Guideline TG 471 (1997). The potential of emamectin benzoate technical (purity 96.6%, batch SNA6A016) to induce gene mutations in the plate incorporation test (Experiment I) and the pre-incubation test (Experiment II) using the *S. typhimurium* strains TA1535, TA1537, TA98 and TA100, and the *E. coli* strains WP2 uvrA (pKM101) and WP2 (pKM101) was studied. The test substance was assayed at a concentration range 3–5000 µg/plate in Experiment I with all tester strains and in Experiment II with *E. coli* strains. In this latter experiment a concentration range of 1–2500 µg/plate was used with *S. typhimurium* tester strains.

Cytotoxic effects, as indicated by thinning of the background lawn and/or reduction in revertant numbers, were observed at higher concentrations with all strains used in both experiments, both with and without metabolic activation (S9 mix).

No substantial increase in revertant colony numbers was observed with any of the six tester strains following treatment with emamectin benzoate technical at any dose level up to 5000 µg/plate, in the presence or absence of metabolic activation. Precipitation was observed in all strains with and without S9 at the highest concentrations tested. Positive control chemicals showed appropriate responses for the relevant strains.

Emamectin benzoate technical was considered not to be mutagenic in the *S. typhimurium* and *E. coli* reverse mutation assays (Sokolowski , 2010)

***Gene mutation in mammalian cells***

A study was performed according to OECD Guideline TG 476 (1997). The potential of emamectin benzoate technical (MK244 technical purity 96.2%, batch SNA6B019) to induce gene mutation or clastogenic effects in mammalian cells was studied using L5178Y $TK^{+/-}$ mouse lymphoma cells, both in the presence and absence of S9 mix. Large and small mutant colonies were scored for all cultures in each experiment. Mutant frequencies were assessed in three independent experiments by cell growth in the presence of trifluorothymidine after a 48-hour expression period.

Emamectin benzoate technical was tested up to concentrations of 25 µg/mL and 20 µg/mL in the presence and absence, respectively, of S9 mix. Observed minimum acceptable relative total growth

(RTG) levels, compared to the solvent control cultures, were: 16 % (treated at 20 µg/mL in the presence of S9 mix) and 14 % (treated at 16 µg/mL in the absence of S9 mix). No significant increases in mutant frequency were observed. The positive controls induced appropriate increases in mutant frequency in all mutation experiments thus demonstrating the efficacy of the S9 mix, and that the assay was performing satisfactorily and capable of detecting known mutagens.

Emamectin benzoate technical was not mutagenic in L5178YTK$^{+/-}$ cells following in vitro treatment in either the presence or absence of S9 mix (Clay, 2006).

A study in Chinese hamster V79 cells according to OECD Guideline TG 476 (2016) was performed. Emamectin benzoate (purity 90.22% B1a, 1.64% B1b), was assessed for its potential to induce gene mutations at the *HPRT* locus using Chinese hamster V79 cells**.** Replicate cultures (four) were used for the solvent control and duplicate cultures for the test substance and positive controls. The treatment period was four hours both with and without metabolic activation.

Test substance concentrations between 8.5 µg/mL and 1090.0 µg/mL were used in the pre-experiment, with and without metabolic activation following a four-hour treatment. The highest concentration was limited by the solubility properties of the test substance. Precipitation occurred at 136.3 µg/mL and above, whether metabolic activation was used or not.

A relevant cytotoxic effect, indicated by a relative cloning efficiency of 50% or less, was observed at concentrations of 8.5 µg/mL and above without metabolic activation, and at 68.1 µg/mL and above in the presence of metabolic activation.

Based on the results of the pre-experiment the following concentrations were applied in the main experiment without metabolic activation: 0.31, 0.63, 1.25, 2.5, 5.0, 10.0, 15.0 and 20.0 µg/mL. Relevant cytotoxic effects indicated by a relative survival of under 50% (mean value of both parallel cultures) were detected at concentrations of 10.0 µg/mL and above. Hence, 10.0 µg/mL was the maximum concentration evaluated for induction of mutations without activation, achieving a relative survival between 10% and 20%. In the main experiment with metabolic activation (S9 mix) concentrations of 3.75, 7.5, 15.0, 30.0, 45.0, 60.0, 80.0, and 100.0 µg/mL were tested. Relevant cytotoxic effects indicated by a relative survival of under 50% (mean value of both parallel cultures) were detected at 30.0 µg/mL and above. Hence 30.0 µg/mL was the maximum concentration evaluated with S9 mix for induction of mutations, achieving a relative survival between 10% and 20%. Three lower concentrations (15.0, 7.50 and 3.75 µg/mL) were also included.

The observed mean mutation frequencies (MFs) for the solvent control and all evaluated concentrations were within the 95% of the control limits seen in solvent historical control data.

The positive controls induced distinct increases in mutant frequencies, demonstrating the sensitivity of the test system and efficacy of the S9 mix.

Emamectin benzoate technical did not induce gene mutations at the *HPRT* locus in hamster V79 cells (Sokolowski, 2020).

***Chromosome aberration assays***

Emamectin benzoate technical (MK244G purity 96.2%) was evaluated for its clastogenic potential in an in vitro cytogenicity assay using human lymphocytes, according to OECD Guideline TG 473 (1997).

In Experiment 1, cultures were treated for a period of three hours both in the presence and absence of S9 mix. The highest concentrations tested were 20 µg/mL with metabolic activation, and 15 µg/mL in the absence of activation. In Experiment 2 cultures were treated for a period of three hours in the presence of S9 mix at concentrations up to 10 µg/mL, and for 20 hours in the absence of S9 mix at concentrations up to 1.5 µg/mL. All cultures were harvested 68 hours after culture initiation.

The highest concentrations selected for chromosome aberration analysis were based on reductions in mitotic activity. Concentration-related reductions in mitotic activity were observed in cultures from both experiments, thus demonstrating that emamectin benzoate technical (MK244G) is biologically active in this test system. No statistically or biologically significant increases in the percentage of aberrant cells, compared to solvent control values, were recorded in cultures from either experiment. The sensitivity of

the test system and the metabolic activity of the S9 mix used were demonstrated by the increases in the percentage of aberrant cells induced by the positive control agents, mitomycin C and cyclophosphamide. It was concluded that, under the conditions of this assay, emamectin benzoate technical (MK244G) is not clastogenic to cultured human lymphocytes treated in vitro, either in the presence or the absence of S9 mix (Fox, 2006).

Emamectin benzoate (purity 90.2% B1a and 1.64% B1b) was assessed for its potential to induce micronuclei in human lymphocytes in vitro in three independent experiments conducted according to OECD TG 487 (2016). In each experimental group, two parallel cultures were analyzed. At least 1000 binucleated cells per culture were evaluated for cytogenetic damage.

The highest applied test substance concentration in this study (1670 µg/mL) was chosen with regard to its solubility and with respect to the current OECD Guideline 487. A cytostasis-based evaluation of cytotoxicity was made, expressing the proportion of second-division cells in the treated population relative to the untreated controls (CBPI). In Experiment I CBPI values in the absence and presence of metabolic activation (S9 mix) of 19.5% and 17.5% respectively were observed at the highest evaluable concentration, 20.0 µg/mL. Due to strong cytotoxic effects the next higher tested concentration was not evaluable (almost no cells available). Since CBPI does not always accurately reflect cytotoxicity and a microscopic precheck may be used to provide further insight. In this case a microscopic precheck of the specimen showed clear evidence of a cytotoxic effects at the highest evaluated concentration in both parts of the experiment, with a sizeable decrease in cell numbers and increase in the fraction of mononucleated cells without cytoplasm. The requirement for testing to a cytotoxic limit was considered met at these concentrations. In Experiment II, in the absence and presence of S9 mix, clear cytotoxicity (56.7% and 55.0% cytostasis) was observed at the highest evaluated concentration.

In Experiment I in the absence of S9 mix and Experiment II in the absence and presence of S9 mix, no relevant increases in the number of micronucleated cells were observed after treatment with the test substance. All values were within the 95% control limit of the historical control data and no concentration dependency was observed. In Experiment I in the presence of S9 mix, however, a value of 0.75% micronucleated cells after treatment with 20.0 µg/mL of the test substance represented a statistically significant increase, and concentration-dependency as tested by a trend test was observed. All values were within the 95% control limit of the historical control data, 0.00%–1.03% micronucleated cells. As indicated in the OECD test guideline for the assay, an additional experiment was conducted, Experiment II. In Experiment II in the presence of S9 mix, none of the positive findings described above were confirmed, and the criteria for a clearly negative outcome were fulfilled. Hence, overall, in the presence of S9 mix a negative result was obtained.

Appropriate mutagens were used as positive controls, and they demonstrated the correct performance of the test system.

One significant deviation from the OECD TG 487 was observed: cytochalasin B was added after a 16–20 hour recovery period. This might have resulted in an underestimation in the frequency of micronucleation. Therefore, based on this study alone no conclusion on the clastogenic or aneugenic properties of emamectin benzoate could be made. (Naumann, 2021)

**(b) In vivo studies**

Emamectin benzoate technical (purity 96.2%, MK244G) has been evaluated for its ability to induce micronucleated polychromatic erythrocytes in the bone marrow of CD-1 mice according to OECD TG 474 (1997). A single oral dose was given to groups of male mice at dose levels of 0, 50, 25 and 12.5 mg/kg bw. The highest dose level represented the maximum tolerated dose (MTD) based on patterns of clinical signs and lethalities over a four-day observation period. Bone marrow samples were taken 48 hours after dosing for the highest dose level only, and 24 hours after dosing for all other dose levels employed.

No statistically or biologically significant increases over the vehicle control values in the incidence of micronucleated polychromatic erythrocytes, were seen at either of the sampling times investigated. Comparison of the percentage of polychromatic erythrocytes showed no statistically or biologically important differences at either sampling time between the vehicle control animals and those treated with emamectin benzoate.

Cyclophosphamide, the test system positive control, induced statistically significant and biologically meaningful increases in micronucleated polychromatic erythrocytes compared to the vehicle controls, thus demonstrating the sensitivity of the test system to a known clastogen.

Under the conditions of test, emamectin benzoate technical is not clastogenic or aneugenic in the mouse bone marrow micronucleus test (Booth, 2006)

## 2.4 Reproductive and developmental toxicity

### (a) Multigeneration studies

***Rat***

In a rat reproductive toxicity study, four groups of 30 animals of each sex per group were administered emamectin benzoate (purity greater than 95%) through the diet at adjusted concentrations to achieve dose levels of 0, 0.1, 0.6 and 1.8 mg/kg bw per day for two generations (F0 and F1). The F0 animals were approximately seven weeks old at study initiation and received the test item for at least 70 consecutive days prior to mating and onward throughout the in-life phase of the study. Offspring selected to become the F1 generation received the test diet following weaning on postnatal day (PND) 22. The F0 and F1 males continued to receive the test article throughout mating and onward until the day of euthanasia. The F0 and F1 females continued to receive the test article throughout mating, gestation and lactation until euthanasia. For both generations (F1 and F2), eight pups per litter (four of each sex when possible) were selected on PND 4 to reduce the variability among the litters. Offspring (30 of each sex per group when possible) from the pairing of the F0 animals were selected on PND 21 to constitute the F1 generation. F0 males and females were exposed for 140–143 consecutive days, and F1 males and females were exposed for 145–163 and 146–163 consecutive days, respectively. All animals were observed twice daily for appearance and behaviour. Clinical observations, body weights and food consumption were recorded at appropriate intervals for males throughout the study and for females prior to mating and during gestation and lactation. Vaginal lavages were performed daily for determination of estrous cycles beginning 21 days prior to pairing. All F0 and F1 females were allowed to deliver and rear their pups until weaning on lactation day (LD) 21. Clinical observations, body weights and sexes for F1 and F2 pups were recorded at appropriate points. Developmental landmarks (balanopreputial separation and vaginal patency) were evaluated for the selected F1 rats. Open field observations and hindlimb and forelimb grip strengths were recorded for 20 F1 pups of each sex per group (a maximum of one pup of each sex per litter) on PNDs 22 and 49. On PND 21, the F1 and F2 pups selected for organ weights were necropsied and the selected organs weighed and retained. In addition, the brain, spleen and thymus gland from one pup of each sex per litter in each group from these F1 and F2 pups were examined microscopically. Anogenital distance was recorded for all F2 pups on PND 1. The remaining F1 pups that were not selected for organ weights or to be the F1 adult generation were maintained until necropsy on PND 28. Each surviving F0 and F1 animal received a complete detailed gross necropsy following the completion of weaning of the F1 and F2 pups, respectively, and selected organs were weighed. Spermatogenic end-points (sperm motility, morphology and numbers) were recorded for all F0 and F1 males, and ovarian primordial follicle counts were recorded for all F1 females in the control and high-exposure groups. Microscopic evaluations were performed on an adequate set of tissues from all F0 and F1 parental animals from the control and high-dose groups, and for all adult animals found dead and euthanized in extremis. The reproductive organs for the low- and mid-dose group F0 and F1 adults suspected of reduced fertility (those that failed to mate, conceive, sire or deliver healthy offspring) were also examined.

The homogeneity, stability and concentration of emamectin in test diets were acceptable.

Overall, during the course of the dosing of the F0 and F1 animals, the actual test article consumptions were close to the target doses (93%–103% and 92%–110%, respectively), except during lactation when substantial overdosing occurred. During lactation (when the medicated diets were not adjusted weekly), the mean compound intakes in the low-, mid- and high-dose groups were 0.24, 1.31 and 3.45 mg/kg bw per day for F0 animals and 0.23, 1.40 and 3.62 mg/kg bw per day forF1 animals.

*F0 generation parental animals*

There were no treatment-related mortalities or clinical signs of toxicity. No treatment-related effects on body weight or body weight gain were observed in F0 parental animals. Occasional statistically significant higher body weight gain was reported in F0 males at low and high dose in weeks 11–12, and higher body weight in F0 females at high dose (by 6%) in week 20. As no consistent dose- or time-dependent trend was observed, these isolated higher body weight values were considered unrelated to treatment. A statistically significant increase in body weight compared to control was reported in F0 females at high dose (by 6%) on LD 21. This significant difference was considered a consequence of body weight loss (an 11 g loss) in control females. Treatment-related reduced food consumption was observed in F0 females at 1.8 mg/kg bw per day during the periods LD 7–14 and 14–21 ($p < 0.01$; evaluated as g/animal per day and g/kg bw per day) resulting in a statistically significant lower food consumption for the entire lactation period (days 1–21). This lower food consumption at 1.8 mg/kg bw per day occurred when the exposure was at the highest level during the F0 generation (3.45 mg/kg bw per day), when the dietary concentrations were not adjusted weekly. In these animals, body weight during LDs 7–14 and 14–21 were similar to controls. Food efficiency was lower (not statistically significant) during LDs 7–14. Other occasional statistically significant food consumption or food efficiency differences from control were reported in males and/or females. Since these changes were not consistent in their direction, lacked a dose–response relationship, and did not appear as a time-dependent trend they were considered unrelated to treatment.

Reproductive performance, estrous cyclicity, gestation length, sperm motility, concentration and morphology were all unaffected by treatment. No treatment-related findings were observed at macroscopic examination or from inspection of implantation sites.

Relative weights (to body weight) of adrenal and brain were significantly lower ($p < 0.05$ or $p < 0.01$) than those of the control group (by 13% and 7% respectively) for females fed 1.8 mg/kg bw per day. Since the final body weight of these animals was significantly ($p < 0.05$) higher than that of the control groups, these lower mean relative organ weights were due to the observed body weight difference, and not related to treatment. There were no treatment-related histopathology findings in the corresponding organs. In males at 1.8 mg/kg bw per day, absolute seminal vesicle/coagulation glands (with accessory fluids) weights (by 11%) and left testis (but not right) weight (by 7%) were significantly ($p < 0.01$) higher than those of the control group. There were no treatment-related histopathology findings in these or other organs.

*F1 Litter*

There were no treatment-related effects on litter parameters; pups born, live litter size, percentage of males per litter at birth, or postnatal survival between birth and PND 21. Treatment-related whole-body tremors were observed in nearly all pups at 1.8 mg/kg bw per day on PND 14 and/or PND 21. It is noted that these findings occurred in the period of the highest maternal compound intake (3.8 mg/kg bw per day). Some whole-body tremors were also observed in two male and one female F1 pups at 0.6 mg/kg bw per day group only on PND 14 (all three pups were from the same litter). None of the F1 pups in the 0.6 mg/kg bw per day group exhibited this finding on PND 21. Also, in contrast to the F1 group at 1.8 mg/kg bw per day, where tremors were observed during the anonymized PND 22 open field evaluation, no tremors were seen at 0.6 mg/kg bw per day during the PND 22 open field evaluation. Therefore, the limited occurrence of transient tremors in three F1 pups from one litter at 0.6 mg/kg bw per day, without progression, on lactation day 21, was considered of doubtful relation to treatment. No tremors were noted in the F2 groups at 0.1 or 0.6 mg/kg bw per day. No other test article-related clinical findings were noted in the F1 pups.

Pup deaths were noted in all groups, including the controls. The number of F1 pups (F1 litters) that were found dead or were euthanized in extremis were: 22 (11), 36 (11), 30 (9) and 21(12) in the control, 0.1, 0.6 and 1.8 mg/kg bw per day groups, respectively. In the same respective F1 groups 3 (2), 19 (6), 7 (5) and 4 (4) pups (litters) were missing and presumed to have been cannibalized. In addition there were 13 pups (two litters) from the 1.8 mg/kg bw per day group where pups had drowned.

Compared to controls the decrease in body weight of pups from the 1.8 mg/kg bw per day group was statistically significant on PNDs 14 and 21, by approximately 15% and 30%, respectively in both sexes. For these animals body weight gain was also significantly suppressed by approximately

30% in the period PND 7–14 and approximately 63% at PNDs 14–21, in both sexes. The decreased body weights were considered secondary to the treatment-related tremors that impaired the ability of pups to nurse, and possibly increased the caloric demand on the animal, resulting in slower/reduced growth in the 1.8 mg/kg bw per day group pups. There were no effects on the body weight of pups at the low or mid dose.

In weanlings (PND 21) dosed at 1.8 mg/kg bw per day, a significant decrease ($p < 0.01$) in absolute brain weight compared to controls was reported in males (by 11%) and females (by 7%). In these animals relative (to body weight) brain weight was increased, with statistical significance, by 38% and 30% in males and females respectively. Absolute and relative spleen weights were significantly ($p < 0.01$) decreased in males (absolute by 53%, relative by 26%), and in females (absolute by 48%, relative by 26%), and absolute thymus weights were significantly decreased ($p < 0.01$) in males (by 36%) and females (by 29%) at 1.8 mg/kg bw per day. The slight decreases seen in absolute brain weight, moderate increases in relative brain weight, and moderate decreases in absolute thymus weight were considered secondary to a significant decrease seen in the body weights of male pups (by 28%) and female pups (by 37%).

Microscopic examination revealed a significantly ($p < 0.05$) increased incidence of minimal neuronal cytoplasmic alteration in the brain of male (13/21) and female (12/22) pups at 1.8 mg/kg bw per day when compared to control males (2/22) and females (0/22). This cytoplasmic alteration was noted in the brainstem, in the neurons, of the dorsolateral to ventrolateral pontine nuclei, predominantly in the spinal nuclei of the trigeminal nerve. The cytoplasmic alteration was a very subtle lesion characterized as: peripherally condensed Nissl substance, a peripheral nucleus that was slightly condensed and a pale central cytoplasmic compartment that appeared very finely granular. In the spleen, there was a significantly increased ($p < 0.05$) incidence and severity of decreased extramedullary haemopoiesis in males (6/21; two minimal and four mild) at 1.8 mg/kg bw per day when compared to controls (0/22). This finding was also observed in females (2/22; both minimal) at the high dose, compared to controls (0/22), but did not achieve statistical significance. The mild lesions noted in four males correlate well with the previously described decreased spleen weights noted in this group. There were no other treatment-related histopathology findings.

In open field observations and grip strength evaluation on PDN 22, several treatment-related effects were observed in male and female offspring at 1.8 mg/kg bw per day. These were whole-body tremors, slight (1.5 mm) tremors, impairment of mobility (slight or moderate), gait (hunched or dragging or splayed hindlimbs), gait score (slight or considerable impairment), grip strength (reduced forelimb and hindlimb) and rearing (fewer counts). A slightly longer time to first step was also observed in females. Differences from the control group were significant ($p < 0.05$ or $< 0.01$). On PND 49 the test article-related changes observed were lower mean hindlimb grip strength and rearing counts for the 1.8 mg/kg bw per day group males and females when compared to control group values; the differences were generally significant at $p < 0.05$ or $< 0.01$. Tremors were not observed in these animals, and no effects on forelimb grip strength, mobility, gait or gait score were noted. No treatment-related effects on open field parameters or grip strength were observed at 0.1 or 0.6 mg/kg bw per day on PND 22 or PND 49.

The mean age of attainment of balanopreputial separation in 1.8 mg/kg bw per day males (48.9 days) was significantly ($p < 0.01$) later than in the concurrent control group (43.7 days). Males' body weight at balanopreputial attainment at 1.8 mg/kg bw per day (208.5 g) was lower by 5.3% ($p < 0.01$) than for the control group males (220.1 g). Similarly, the mean age of attainment of vaginal patency for 1.8 mg/kg bw day females (37.0 days) was significantly later ($p < 0.01$) than that of the concurrent control females (32.9 days). The mean body weight on the day of attainment of vaginal patency (98.1 g) was lower by 12.1% ($p < 0.01$) than that of the concurrent control group females (111.6 g). Days of attainment of these developmental landmarks were at the upper bound of the historical control data (49 days for balanopreputial separation; 37 days for vaginal patency). The Meeting considered the relationship between the reduced body weight and these developmental landmarks to be uncertain. No effects on these developmental landmarks were seen in the groups receiving 0.1 or 0.6 mg/kg bw day.

*F1 generation parental animals*

At clinical observations hair loss on various body surfaces was increased in F1 females at 1.8 mg/kg bw per day (17/336 animals) when compared to control (5/54 animals).

During the post-weaning period (weeks 17–39) significantly lower ($p < 0.05$ or $< 0.01$) mean body weights, ranging from 37.8% and 40.3% lower (in study week 17) to 7.1% and 7.6% lower (in study week 25 for males or week 26 for females), were observed at 1.8 mg/kg bw per day. In these animals, mean body weight gain during weeks 17–18 was significantly lower ($p < 0.01$). Thereafter (weeks 18–39) mean body weight gain was generally similar to that in the control group. Occasionally significant ($p < 0.05$ or $< 0.01$) differences in mean body weight gain were noted for males in the 1.8 mg/kg bw per day group. However, when the entire premating period was evaluated, mean body weight gains in these males and females were similar to the control group. Body weights were not affected by treatment during the remaining life of this generation of males and females (including the gestation and lactation periods) at the high dose, or in animals in the low- and mid-dose groups. Significantly lower food consumption ($p < 0.05$ or $< 0.01$) was reported during the first weeks of the postweaning period (week 17–20) in animals at 1.8 mg/kg bw per day. However, because the mean body weights were lower and food efficiency was similar to the control group, mean weekly food consumption when evaluated relative to body weight was generally higher than for controls throughout the generation, often with statistical significance at $p < 0.05$ or $< 0.01$. Therefore, the decreased absolute food consumption during the early postweaning period was considered secondary to, or a residual effect of the reduced body weight noted at weaning for these animals. Food consumption and efficiency were unaffected by treatment during the reminder of the generation in males or females (including gestation) at the high dose, or in animals of the low- and mid-dose groups. During lactation significantly lower ($p < 0.05$ or $< 0.01$) food consumption was observed in females in the 1.8 mg/kg bw per day group on LDs 14–21. It is of note that in this lactation period an overdosing occurred (actual consumption 3.6 mg/kg bw per day) which also corresponded to the onset of tremors noted in the F2 pups in this group and markedly lower mean pup body weight gain (see below). The lower parental female food consumption during the last week of lactation was considered secondary to the reduced lactational demand because the whole-body tremors impaired the ability of the pups to nurse. There were no effects on food efficiency at the high dose or food consumption and efficiency at the low and mid doses.

Assessment of reproductive performance showed that at 0.6 and 1.8 mg/kg bw per day, the mating (83.3%–82.7%) and fertility (70.0%–75.9%) indices of males were lower when compared to control mating (93.3%) and fertility (86.7%) values. These differences were not statistically significant. Since the number of infertile males and females were similar to those of the F0 concurrent control these differences were not considered treatment-related. The precoital interval was longer in 1.8 mg/kg bw per day animals, but this was primarily due to five females in this group that did not mate with the first male used for breeding, compared to just one of the concurrent controls. Therefore, this longer precoital interval was not considered treatment-related. Gestational length and parturition were not affected by treatment.

Estrous cyclicity, gestation length, sperm motility, concentration and morphology, and ovarian follicle counts were unaffected by treatment. No treatment-related findings were observed at macroscopic examination or on implantation sites.

A slight but statistically significant decrease in absolute brain weight was reported in rats receiving 1.8 mg/kg bw per day, lower by 5% in males and 6% in females compared to controls: similarly the brains of females at 0.6 and at 0.1 mg/kg bw per day, by 3% and 4%, respectively compared to controls. Decreased absolute brain weights at the low and mid dose were considered unrelated to treatment as the values were within the historical control range and virtually identical to the F0 parental female control value. The brain weight of animals at 1.8 mg/kg bw per day were below the lower end of the historical control ranges. The lower absolute brain weight in these animals was considered to be a residual effect of the lower brain weight observed in F1 pups at weaning (by 7% in males; 11% in females) as the majority of brain mass in adults is reached during the lactation period (PNDs 20–21). Therefore, lower absolute brain weights in the F1 animals resulted from a decrease in organ weight around weaning, which in turn was secondary to the decreased body weights in this group at weaning. In addition, there were no histopathological correlates to explain the decreased brain weight, nor treatment-related microscopic findings in other organs and tissues.

*F2 Litter*

There were no treatment-related effects on litter parameters; number of pups born, live litter size, percentage of males per litter at birth or postnatal survival between birth and PND 21.

Treatment-related whole-body tremors were observed in nearly all pups at 1.8 mg/kg bw per day on PND 14 and/or PND 21. It was noted that these findings occurred in the period of the highest maternal compound intake (3.6 mg/kg bw per day).

A slight statistically significant increased anogenital distance (AGD) relative to cubic root of body weight compared to controls, was reported in male pups at 0.6 and 1.8 mg/kg bw per day, by 7% and 6% respectively. No significant difference was observed in the absolute AGD. Given that the reported AGD changes were slight, with no dose–response relationship, and that virtually all individual AGD litter mean values at the high dose fell within the range of the concurrent controls, these changes were not considered of biological significance and of doubtful relation to treatment. There were no significant effects on AGD (absolute or relative to cubic root of body weight) in females. Additionally, considering the endocrine mediated parameters in the two-generation reproductive toxicity study and endocrine mechanistic studies, there is no indication that emamectin interferes with androgen action. The weak positive result from the in vitro steroidogenesis assay (estrogen decrease) was not concordant with an AGD increase in males.

Treatment-related reduced body weight was noted in animals at 1.8 mg/kg bw per day. At birth the decrease was by 8% in males and 10% in females compared to controls, the latter achieving statistical significance. Although not achieving statistical significance, the lower body weight (up to 10%) persisted through PNDs 4, 7 and 11. In the period PND 14–21 body weights were significantly lower by up to approximately 25% in males and females. Body weight gain in these animals was significantly lower ($p < 0.05$ or $< 0.01$) during PND 7–21. Body weights of animals at the low and mid dose were similar to controls.

The were no treatment-related necropsy findings.

As in F1, the F2 offspring at 1.8 mg/kg bw per day, showed a significant ($p < 0.01$) decrease in absolute brain weight compared to controls, by 5% in males and 7% in females. In these animals brain weight relative to body weight was increased to a statistically significant extent by 25% and 24% in males and females respectively. Absolute and relative spleen weight were significantly decreased ($p < 0.01$) in males (absolute by 42%, relative by 44%) and females (absolute by 24%, relative by 24%), and absolute thymus weights were significantly decreased ($p < 0.01$) in males (by 28%) and females (by 27%) at 1.8 mg/kg bw per day. The slight decrease in absolute brain weights and moderate increase in relative brain weights, and the moderate decrease in absolute thymus weight were considered secondary to a significant decrease in pup body weights, by 23% in males and 26% in females.

Microscopic evaluation of F2 pups revealed a significantly ($p < 0,05$) increased incidence of minimal neuronal cytoplasmic alteration in the brain of male (12/21) and female (12/22) pups at 1.8 mg/kg bw per day when compared to control male (0/22) and female (1/22) pups. This is the same cytoplasmic alteration in the brainstem that was previously discussed for the F1 weanlings. Looking at the spleen, decreased extramedullary haematopoiesis was noted in one pup per sex from the 1.8 mg/kg bw per day group.

The NOAEL for parental toxicity was 0.6 mg/kg bw per day, based on decreased body weights in F1 males and females, and increased hair loss on various body surfaces in F1 females at 1.8 mg/kg bw per day.

The NOAEL for reproductive toxicity was 1.8 mg/kg bw per day, the highest dose tested.

The NOAEL for offspring toxicity was 0.6 mg/kg bw per day, based on reduced body weights, clinical signs of neurotoxicity, neural cytoplasmic alteration in the brain, decreased spleen weights, reduced extramedullary haemopoiesis in both sexes of the F1 and F2 generations, and delayed developmental landmarks in both sexes of the F1 generation seen at 1.8 mg/kg bw per day (Stump, 2009).

**(b) Developmental toxicity**

***Rat***

In a dose ranging study (not guideline compliant), 10 presumed-pregnant female SD rats per group were given emamectin benzoate (MK-0243) by gavage, in 5 mL/kg deionized water, at dose levels of 0, 1.25, 2.5, 5.0 and 10.0 mg/kg bw per day from GD 6 to GD 17. Body weights were recorded at regular intervals during the course of the study. On GD 14, approximately 24 hours after the eighth dose all females, except those in the 10 mg/kg bw per day group, were bled from the orbital sinus for haematological and serum biochemical analyses. All animals were observed for physical signs once daily, prior to dosing, on day 0, between GDs 6 and 20, and 1–5 hours post dosing (GDs 6–17). Animals in the 0, 1.25, 2.5 and 5.0 mg/kg/day groups were euthanized on GD 20 and the uterus of each animal examined to determine reproductive status. Implants were counted and each classified as a live fetus, dead fetus or resorption. All fetuses were weighed and examined externally.

There were no treatment-related mortalities, but the all high-dose rats had to be terminated prematurely. All females at 10 mg/kg bw per day were euthanized early on GDs 12–14 due to adverse physical signs and body weight loss. Clinical signs consisted of tremors (8/10), unkempt coat (5/10) and nasal discharge (2/10). Tremors were first observed on GDs 10, 11 or 12, and in all cases persisted until termination. Nasal discharge and unkempt coat were seen one or two days after the tremors appeared. There were no treatment-related clinical signs of toxicity at lower doses.

At 10 mg/kg bw per day half of the females lost body weight between GD 10 and GD 12. Females at 2.5 and 5 mg/kg bw per day displayed a slightly higher body weight (by 4%–8%) compared to controls from GD 10 to GD 20.

There were no treatment-related effects on haematological, clinical chemistry, or pregnancy parameters, embryo survival, fetal weights or from external fetal examination. Based on the results of the study the 10 mg/kg bw per day dose was not tolerated by pregnant female rats (Manson, 1992a).

***Rabbit***

In a dose ranging study (not guideline compliant), six non-pregnant female New Zealand White rabbits per group were given emamectin benzoate (MK-0243) by gavage in 4 mL/kg of deionized water at dose levels of 0, 1.37, 2.74, 5.48 and 10.96 mg/kg bw per day for 13 consecutive days. Body weights were recorded at regular intervals during the course of the study. All females were observed daily for physical signs prior to dosing and at 1–5 hours after dosing. Samples were taken 24 hours after the last dose from all animals for haematology and clinical chemistry analyses. No postmortem examination was performed.

There were no deaths during the study. All females given 10.96 mg/kg bw per day were euthanized early due to adverse physical signs and body weight losses. One female showed decreased food consumption following the second dose and was laterally recumbent and lethargic with tremors and laboured breathing on the morning of day 6. This animal was euthanized and discarded without further examination on day 6. Another female in this group showed decreased food consumption and decreased activity from the morning of day 6. Tremors were first observed in this animal after the sixth dose and were seen both before and after dosing thereafter until day 8. Mydriasis was seen after the seventh dose and approximately 24 hours later the animal was laterally recumbent with laboured breathing so the animal was not dosed. Three other animals in this group displayed tremors following the seventh dose and two of these also showed mydriasis. Prior to the eighth dose all four remaining animals exhibited tremors, decreased food intake and, in three animals, decreased water intake. After the eighth dose, one animal was ataxic. All animals in this high-dose group showed a body weight loss between days 1 and 5, or on day 7 ranging from 61 g to 370 g.

At doses of 5.48 mg/kg per day and lower there were no treatment-related effects on mean body weight gain, haematology parameters, or serum biochemical parameters. Based on the results, a dose of 10.96 mg/kg bw per day was not tolerated by non-pregnant female rabbits (Manson, 1992b).

In a dose-ranging study (not guideline compliant), 10 artificially inseminated female New Zealand White rabbits per group were given emamectin benzoate (MK-0243) by gavage in 4 mL/kg of deionized water at dose levels of 0, 2, 4, 6 and 8 mg/kg bw per day from GD 6 to GD 18. Food

consumption was measured every third day on all females over a 24 hour period beginning on GD 0. On GD 19, approximately 24 hours after the last dose, all females were bled from the medial auricular artery for haematological and serum biochemical analyses. All animals were observed daily for physical signs prior to dosing, as well as 1–5 hours post dosing (GDs 6 to 18). Reproductive status and uterus were evaluated in all animals at GD 28. Implants were counted and each classified as a live fetus, dead fetus or resorption. All fetuses were weighed and examined externally. In addition, the heads of two fetuses from the high-dose group that were seen to be misshapen were examined in free-hand coronal sections.

There were no deaths or abortions during the study. Treatment-related physical signs were noted at 8 mg/kg bw per day and consisted of five animals with soft and/or small faeces at the end of or following the treatment period. One female also produced no urine. Another animal which had small or soft faeces after the treatment period also had tremors beginning on GD 19 which persisted until termination. The remaining physical signs that were observed (alopecia, pulling fur, blood in pan, diarrhoea, mucoid/gelatinous/tarry faeces, sediment in pan, sore mouth and scab in ear) were not considered treatment-related since they appeared in all groups, or were observed in only one or two animals per group.

From GD 14 to GD 19 there were significant ($p < 0,05$), treatment-related average maternal body weight losses in groups dosed at 6 and 8 mg/kg bw per day. The maternal body weight losses resulted in a significant ($p < 0,05$) decrease in body weight gain during the overall dosing period (GD 6–19) in the 6 mg/kg bw per day group and an average body weight loss in the 8 mg/kg bw per day group. During the post-treatment period (GD 19–28) the body weight of these animals tended to recover. Body weights of females at 2 and 4 mg/kg bw per day were comparable to those of the controls. There were dose- and treatment-related decreases in average maternal food consumption in the 6 and 8 mg/kg bw per day groups on GD 16 and 19. Food consumption was also decreased on GD 22 in the 8 mg/kg bw per day group. The decreases on GDs 16 and 22 for rabbits in the 8 mg/kg bw per day group were statistically significant ($p < 0,05$) compared to the controls. There were no treatment-related effects on food consumption at 2 and 4 mg/kg bw per day.

There were no treatment-related effects on haematology parameters. Slight increases in mean serum glucose (by 9%–14%), moderate increases in mean serum cholesterol (by 8%–56%), slight decreases in mean serum potassium (by 12%–17%) and moderate decreases in mean serum triglycerides (by 23%–33%) were seen in all treated groups. However, these changes were not dose-related and all individual values were either within the range of the concurrent control group (cholesterol and triglycerides) or within the range of historical control values for this laboratory (historical median and range for glucose,124 mg/dL, 83–199 mg/dL; for potassium, 3.8 mEquiv./L, 3.1–4.4 mEquiv./L), except for one potassium value in the 2 mg/kg bw per day group, at 3.0 mEquiv./L. Four control animals had potassium values above the range of the historical controls.

At caesarean section, no treatment-related effects on embryo survival were seen in the groups given 8 mg/kg bw per day or less, based on the average number of implants and live fetuses per pregnant female and the percentage of resorptions plus dead fetuses/implants. One litter in each of the 2 and 8 mg/kg bw per day groups had a large number of resorptions; six and four respectively. The scale of these resorptions was not considered treatment-related since only single litters were affected and there were no similar effects at 4 and 6 mg/kg bw per day. There was a slight fetal weight decrease (by 6%; not significant as $p > 0.05$) at 8 mg/kg bw per day. This effect was largely due to a single fetus.

One fetus (fetal incidence, 1.59%) in the 8 mg/kg bw per day group displayed cleft palate and hydrocephaly and another fetus from a different litter showed only hydrocephaly. The fetuses were from the two dams that lost the most body weight during the dosing period. The former dam also developed tremors after the dosing period. The relationship to treatment of these findings is uncertain since there were only two affected fetuses and cleft palate is occasionally seen in control rabbit fetuses from this laboratory, where records showed an overall fetal incidence of 0.04%, and a highest fetal incidence of 1.85%. Hydrocephaly was not recorded in this laboratory because coronal sectioning had not been a standard procedure for fetal rabbits. Based on the marked maternal toxicity (body weight loss and clinical signs) observed at 8 mg/kg bw per day, 6 mg/kg bw per day was selected as the high dose level for the main rabbit developmental toxicity study (Manson, 1992c).

## 2.5 Special studies

### (a) Immunotoxicity

In an immunotoxicity study, emamectin benzoate (purity 96.6%) was administered in the diet to female CD-1 mice (10 per group) at concentrations of 0, 10, 30, or 60 ppm (equal to 0, 1.7, 4.8, or 10.6 mg/kg bw per day expressed as free base) for at least 28 days. A positive control group of 10 females received 10 mg/kg bw per day of cyclophosphamide (CPA) by gavage for the same study period. On day 25 all test animals received a single intravenous injection of the antigen, sheep red blood cells (sRBCs), using 0.25 mL per animal containing $2 \times 10^8$ cells per animal. The animals were monitored regularly for viability and clinical signs of toxicity. Body weights and food consumption were measured. Blood samples were collected on day 29 for a specific anti-sRBC IgM response by enzyme-linked immunosorbent assay (ELISA). After the completion of treatment all animals were sacrificed and liver (with gall bladder), spleen and thymus weighed then preserved.

No mice died during the study, and no treatment-related clinical signs were observed. A definitive effect of treatment on body weight gain could not be determined, however, a slight (but not statistically significant) decrease in body weight gain was observed for the 60 ppm treatment group over the 28-day treatment period. The body weight changes at 10, 30 and 60 ppm compared to controls over the 28-day treatment period were by +6.8%, −4.5% and −20.5% respectively. No treatment-related changes in food or water consumption were observed.

There was no evidence of a immunosuppressant effect on the humoral system. The CPA-treated group showed a lower (with statistical significance) antibody-specific IgM response compared to controls, confirming the reliability of the study since CPA is a known immunosuppressant.

The NOAEL for immunotoxicity was 60 ppm (equal to 10.6 mg/kg bw per day), the highest dose tested (Donald & Smith, 2012).

### (b) Endocrine disruption

A number of studies were evaluated to assess the potential endocrine effect of emamectin benzoate. These consisted of an in vitro aromatase inhibition assay, an H295R steroidogenesis assay, an androgen receptor in vitro transactivation assay, and a Hershberger assay.

In a H295R steroidogenesis in vitro assay, emamectin benzoate (purity 97.0%) was tested for 48 hours at seven concentrations up to 125 µM together with the positive control inducer, forskolin and positive control inhibitor, prochloraz. Dimethylsulfoxide (DMSO) was used as the vehicle and the concentration of vehicle in the incubations was kept constant at 0.1% (v/v). Cell viability was determined using the 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The concentration of estradiol and testosterone in the exposure medium was determined by liquid chromatography with tandem mass spectrometry (LC-MS/MS). Three independent experiments were conducted. In the first experiment, emamectin benzoate was tested at a final concentration of 125 µM, the highest soluble concentration, however, the top two concentrations (12.5 and 125 µM) were toxic to the cells. In the second experiment, the highest two concentrations (1.25 and 3.956 µM) were toxic to the cells. In the third experiment emamectin benzoate was not cytotoxic at any concentration tested; the highest concentration tested was 1.25 µM.

The assay acceptability criteria were all met in the above assays.

#### *H295R steroidogenesis assay*

In a first experiment, emamectin benzoate caused statistically significant ($p < 0.05$) decreases in estradiol release at all five noncytotoxic concentrations tested, ranging from 125 pM to 1.25 µM, with resulting estradiol release relative to the solvent control ranging from 0.67 to 0.90. In the second experiment, emamectin benzoate caused statistically significant ($p < 0.05$) decreases in estradiol at the two highest non-cytotoxic concentrations tested (125 and 395.6 nM) with changes relative to the solvent control of 0.86 and 1.01, respectively. In the third experiment, statistically significant ($p < 0.001$) decreases in estradiol were observed at the two highest noncytotoxic concentrations tested (395.6 nM and 1.25 µM) with resulting estradiol release relative to the solvent control of 0.65 and 0.84, respectively.

In the first experiment, emamectin benzoate caused a statistically significant decrease ($p < 0.001$) in testosterone release at the highest non-cytotoxic concentration of 1.25 µM, with a fold change relative to the solvent control was 0.79. In the second experiment, emamectin benzoate caused statistically significant ($p < 0.05$) decreases in testosterone at non-cytotoxic concentrations of 3.956 nM, 12.5 nM, 125 nM and 395.6 nM). The average fold change relative to the solvent control ranged from 0.88 to 0.97 . In the third experiment, emamectin benzoate caused a statistically significant decrease ($p < 0.001$) in testosterone release at the highest non-cytotoxic concentration of 1.25 µM. The average fold change relative to the solvent control was 0.78.

The results of the above study were assessed according to the criteria of OECD TG 456 (updated July 2023), and the decision matrix applied. For estradiol, a dose-related decrease was observed in at least two adjacent concentrations in all three experiments. In the first two experiments the maximum fold changes were above the threshold of 66.7% of control, therefore they were judged equivocal. The third experiment had the maximum fold change slightly below the threshold (65%) and was judged positive. Applying the decision matrix, emamectin benzoate is weakly positive for estradiol inhibition. For testosterone, the results of the first, second and third experiment are negative, equivocal, and negative, respectively. Applying the decision matrix emamectin benzoate is negative for testosterone inhibition (Kelsall, 2021).

***Aromatase assays***

In an in vitro aromatase inhibition assay, emamectin benzoate (purity 97.0%) was assessed for its potential inhibition on the catalytic activity of aromatase (CYP19). The assays included three valid independent runs. Within each run, the test item was tested at eight concentrations (in triplicate) ranging from 0.1 nM to 31.6 µM (the highest soluble concentration), together with a complete dose response curve for the positive control inhibitor 4-hydroxy-androstenedione (4-OH-ASDN, formestane). As substrate for the aromatase assay, a mix of unlabelled and tritiated 4-androstene-3,17-dione (ASDN) was used at a concentration of 100 nM in each incubation. Aromatase activity was assessed by measuring the release of tritiated water for 15 minutes. The vehicle was DMSO and the concentration of vehicle in the incubations was kept constant at 1% (v/v).

The acceptance and performance criteria of the assay were met. No notable inhibition of aromatase activity was observed. The maximum inhibition noted in the three runs with samples was 17.7% (82.3% of control enzyme activity remaining at 31.6 µM, in run 1). It was not possible to calculate the mean half-maximal inhibitory concentration ($IC_{50}$) for emamectin benzoate, as enzyme activity in the presence of emamectin benzoate ranged from 82.3% to 105.6% of the control value in individual assay runs and showed no relationship to concentration. It was concluded that emamectin benzoate had no effect on aromatase activity (Cheung, 2021).

***Androgen receptor transactivation assay***

In an in vitro assay, emamectin benzoate (purity 97.0%) was evaluated for androgen receptor (AR) agonist or antagonist activity using the stably transfected human androgen receptor transcriptional activation assay (AR-EcoScreen™). Two valid experiments for the AR agonist assay and three valid experiments for the AR antagonist assay were performed. Following a solubility analysis, the final concentrations ranged from 10 pg/mL to 1 µg/mL in the AR agonist assay, and from 10 pg/mL to 1 µg/mL or from 100 pg/mL to 316 ng/mL in the AR antagonist assay. Adequate positive controls for the agonist and antagonist assays were used.

All quality and acceptance criteria were met or accepted after evaluation. The maximum level of response induced by the test item compared to the response induced by 10 nM dihydrotestosterone (DHT; a response regarded as the $RPC_{max}$) was 0.1% in Experiment 1, and −0.4% in Experiment 2. Since the $RPC_{max}$ values were below 10% in both independent experiments, emamectin benzoate was considered to be negative in the AR agonist assay. In all three experiments, no log $IC_{30}$ (the concentration at which activity is inhibited to 30% of the positive control value) could be determined for the test item. As such, emamectin benzoate was also considered to be negative in the AR antagonist assay (Verkaart, 2021).

***Hershberger assay***

In a Hershberger assay, six peripubertal, orchido-epididymectomized male Crl:CD(SD) rats per group were administered emamectin benzoate (purity 97.0%) by gavage for 10 consecutive days at dose levels of 2.5, and 5 mg/kg bw per day for the androgen agonist analysis, and at 1, 2.5 and 5 mg/kg bw per day (co-administered with a subcutaneous injection of testosterone propionate at 0.2 mg/kg bw per day) for the androgen antagonist assay. Adequate positive controls for androgen agonism (testosterone propionate), antagonism (flutamide at 3 mg/kg bw per day), and a vehicle negative control 0.5% carboxymethyl cellulose (CMC) in de-ionized water were employed. Clinical observations (two hours after dosing) and body weights were recorded daily throughout the study. Adrenal glands, bulbourethral glands, glans penis, kidneys, levator ani plus bulbocavernosus (LABC) muscle group, liver, seminal vesicles with coagulating glands, and ventral prostate gland were examined and weighed.

All males survived to the scheduled necropsy. No emamectin benzoate-related clinical observations were noted. In one male given emamectin benzoate at 5 mg/kg bw per day and testosterone propionate at 0.2 mg/kg bw per day, opacity in the eye was observed. No clinical observations were noted in the testosterone propionate positive control group or in the flutamide/testosterone propionate positive control group. There were no treatment-related effects on body weight. The performance and acceptance criteria for control and high-dose treatment groups were met. No evidence of androgen agonism or antagonism was noted following administration of emamectin benzoate. Differences from the respective control groups were slight, not statistically significant, and/or not observed to occur in a dose-related manner. Mean weights of adrenal gland, kidney, and liver for all emamectin benzoate-treated groups were comparable to the respective controls. Testosterone propionate, the androgenic positive control substance, elicited the expected response.

Based on the results of the study there was no evidence of emamectin benzoate androgen agonism, antiandrogenic effect or 5α-reductase inhibition (Harrison, 2021).

## 2.6 Studies on metabolites and/or degradates

### (a) Metabolite 4″-epi-(*N*-formyl)-amino-4″-deoxy-avermectin B1 (L-657,831, FAB1a)

Metabolite 4″-epi-(*N*-formyl)-amino-4″-deoxy-avermectin B1 is a photodegradation product of the pesticide emamectin benzoate. An acute toxicity study in mice, a microbial mutagenesis assay and a 15-day dietary neurotoxicity study in mice have been performed

***Acute toxicity study***

No specific guideline was followed for this study.

Three groups of approximately seven-week-old, Crl:CD-1(ICR)BR male and female mice (five mice per sex) were given a single oral dose of L-657,831 (purity 81.5%; batch L-657,831-000S) in 0.5% aqueous methylcellulose at doses of 0, 50, 100 or 200 mg/kg bw in a dose volume of 10 mL/kg bw. Clinical observations were made daily, and body weights recorded pretest, and on days 7 and 14. On day 15 all surviving mice were killed with carbon dioxide, exsanguinated and examined macroscopically. Any gross lesions were examined histopathologically.

There were mortalities in 1/5 males and 0/5 females dosed of 50 mg/kg bw, and in all mice at doses of 100 and 200 mg/kg bw, occurring between approximately three hours and nine days after dosing. Physical signs noted in all groups included tremors, decreased activity, ptosis and bradypnea and lateral recumbency. These physical signs were generally seen within a few hours of dosing and lasted one to eight days. Surviving mice at 50 mg/kg bw were normal by day 5 with the exception of dermal irritation of the marginal pinnae, which was seen only in females at 50 and 100 mg/kg bw starting on day 3 and lasting until day 15. Irritation of the marginal pinnae was thought to be the result of a treatment-related photoreaction to light, as the females were housed on the top row of the caging and the males were housed below and would have received less exposure to the ambient room light.

On day 7, the two surviving female mice given 100 mg/kg bw had body weight losses of up to 32% compared with weights pretest. An 8% decrease in initial body weight was seen on day 7 in one male mouse given 50 mg/kg bw, but this weight loss was recovered by day 14. A single dose of the

metabolite did not appear to affect the body weight of female mice given 50 mg/kg bw.

No treatment-related gross or microscopic changes were noted in the postmortem examination.

In conclusion, the acute oral $LD_{50}$ of L-657,831 for male and female mice, sexes combined, was 66 mg/kg bw (range 57–76 mg/kg bw). For males the $LD_{50}$ was 62 mg/kg bw (range 47–81 mg/kg bw) and for females 71 mg/kg bw (range not given) (Lankas, 1995a).

In a non-guideline, but GLP-compliant study, metabolite 4″-epi-(*N*-formyl)-amino-4″-deoxy-avermectin B1 (L-657,831) was of similar acute toxicity in mice as the parent emamectin benzoate had been in a previous study (Tavaszi, 2010)

***Microbial mutagenesis assay***

Metabolite L-657,831 (purity 89.4%; batch L-657,831-000S008) was evaluated for mutagenic potential in a microbial mutagenesis test system using mutant strains of *Salmonella typhimurium* (TA1535, TA97a, TA98 and TA100) and *Escherichia coli* (WP2, WP2 uvrA, and WP2 uvrA pKM101). The study was performed in compliance with GLP; no specific guideline was stated but the study design was similar to OECD TG 471.

The strains were exposed for 48 hours to the test article as a solution in DMSO at concentrations of 100, 300, 1000, 3000 or 10 000 µg per plate in the presence and absence of mammalian metabolic activation.

Metabolite L-657,831 tested at the highest possible concentration did not produce any two-fold or greater increase in revertants relative to the solvent control in any of the tester strains, either with or without metabolic activation. Precipitate was seen on the plates at a test concentration of 3000 µg/plate but this did not interfere with scoring of the plates. Precipitate which interfered with scoring occurred on all plates at 10 000 µg/plate, the highest dose tested. No cytotoxicity was observed at any dose level.

The positive control compounds, 2-aminoanthracene and hydrazine sulfate, produced the expected increases in revertants.

Since metabolite 4″-epi-(*N*-formyl)-amino-4″-deoxy-avermectin B1 (L-657,831) did not induce sufficient revertant colonies compared with the solvent control in any tester strain at any concentration it was considered not mutagenic in the microbial mutagenesis assay (Sina, 1993a).

***Fifteen-day dietary neurotoxicity study in mice***

No specific guidelines were stated for this study, but it was GLP-compliant.

The mouse strain used in this study is deficient in the MDR1a P-glycoprotein and is therefore considered more sensitive to the effects of avermectins.

In a dietary neurotoxicity study, groups of ten male and ten female, 36-day-old, Crl:CF-1BR mice were continuously administered a diet containing metabolite L-657,831 (purity 89.4%; batch L-657,831–000S008) for 14 days, resulting in doses of 0, 0.04, 0.06, 0.07 and 0.23 mg/kg bw per day for males, 0, 0.04, 0.05, 0.08 and 0.24 mg/kg bw per day for females.

All animals were observed daily for mortality and clinical signs. Body weight was recorded before treatment and once a week during the study. Food consumption was measured weekly beginning during the prestudy week, based on a intake periods of 4–6 days. Food was withdrawn overnight prior to scheduled termination on day 15. All mice underwent necropsies limited to the central nervous system (brain and cervical, thoracic and lumbar segments of spinal cord) and sciatic nerve. Weights of brains were recorded. The tissues were fixed, stained and sections of the brain, spinal cord and peripheral nerve from all control and high-dose mice underwent histopathological examination.

No mortalities or treatment-related clinical signs were reported. Suppression of body weight gains compared to controls, by 17% and 43% for male and female mice, respectively, were observed in the top-dose group over the course of the study. The remaining treatment groups were unaffected. Average food consumption was comparable for all dose groups. No gross or microscopic changes related to treatment were observed. There were no treatment-related changes in brain weight.

In conclusion, the dietary administration of L-657,831 to CF-1 mice at dose levels of 0.04–0.24 mg/kg bw per day resulted in suppression of body weight gain compared to controls in high-dose male and female mice by 17% and 43%, respectively, over the course of the study. No clinical signs of toxicity (for example tremors) were observed at any dose level, nor were there treatment-related changes in brain weight or any gross or microscopic changes related to treatment.

In a non-guideline, GLP-compliant study, there was no evidence of neurotoxicity in CF-1 mice of 4″-*epi*-(*N*-formyl)-amino-4″-deoxy-avermectin B1 (L-657,831; Gerson, 1993a).

**(b) Metabolite 4″*epi*-amino-avermectin AB1a (L-653,649, NOA438309)**

Metabolite 4″ epi-amino-avermectin is a photodegradation and demethylated metabolite of emamectin and has been tested in a number of toxicity and genotoxicity studies.

***Acute oral toxicity in mice.***

The study was conducted to EPA OPPTS 870.1100 guidelines equivalent to OECD TG 401. The study was GLP-compliant

Metabolite L-653,649 (purity 93.1%, batch L-653,649-005S) was studied in an acute oral toxicity study in Crl:CD-1(ICR) BR mice. Groups of five mice per sex were given a single oral dose of L-653,649 in 0.5% aqueous methylcellulose at doses of 0, 50, 100 or 200 mg/kg body weight in a dose volume of 10 mL/kg bw. Individual animal observations for clinical signs were made daily. Body weights were recorded pretest and on days 7 and 14. On day 15 all surviving mice were killed with carbon dioxide, exsanguinated and underwent complete necropsies.

Mortality occurred on days 2 to 8 in one male, but no females, dosed at 50 mg/kg bw, in three males and one female at 100 mg/kg bw, and all males and females at 200 mg/kg bw. Physical signs noted in all groups included tremors, decreased activity, ptosis and bradypnea. These physical signs generally observed within a few hours of dosing or on day 2, and lasted 1–7 days. Lateral recumbency was seen at 100 and 200 mg/kg bw on days 1–6, 1–3 days prior to being found dead. Surviving mice at 50 and 100 mg/kg bw were normal by day 5. On day 7 the one surviving female mouse given 200 mg/kg bw showed a body weight loss of 25% compared to pretest weight, and was subsequently found dead on day 8. All four surviving female mice and one of two male mice given 100 mg/kg bw had body weight losses compared to pretest of 6%–13%; however, this weight loss was recovered by day 14. No apparent effect on body weight occurred in males and females given 50 mg/kg bw.

The results of gross and microscopic examinations (limited to one gross lesion) did not reveal any compound-related changes.

The acute oral $LD_{50}$ of L-653,649 for male and female mice, sexes combined, was 100 mg/kg bw (range 77–130 mg/kg bw); for males 81 mg/kg bw (range 52–126 mg/kg bw) and for females 123 mg/kg bw (range 93–162 mg/kg bw).

Metabolite 4″*epi*-amino avermectin AB1a (L-652, 649) was considered to have acute toxicity comparable to emamectin benzoate (MK-0244) (Lankas & Bagdon, 1995).

***Microbial mutagenesis assay***

Metabolite L-653,649 (purity 95.7%, batch L-653,649-005S003) was evaluated for mutagenic potential in a microbial mutagenesis test system using mutant strains of *Salmonella typhimurium* (TA1535, TA97a, TA98 and TA100) and *Escherichia coli* (WP2, WP2 uvrA, and WP2 uvrA pKM101), with and without a rat liver microsomal enzyme activation system (S9). The study was performed in compliance with GLP. No specific guideline was stated but the study design was similar to OECD TG 471.

The test article was dissolved in DMSO. In the first assay, the final concentrations tested were 100, 300, 1000, 3000 and 10 000 µg/plate with and without S9 metabolic activation.

The results indicated that L-653,649, at the highest testable concentrations, did not produce any two-fold or greater increases in revertants relative to the solvent control in any of the tester strains, either with or without metabolic activation. Precipitate was seen on the plates at test concentrations as

low as 1000 µg/plate in the *Salmonella* strains. Cytotoxicity, as indicated by thinning of the background lawn and/or inhibition of revertant numbers, was apparent in both *E. coli* and *S. typhimurium* strains at various doses. Inhibition of revertants was so extensive in the *Salmonella* strains that a repeat assay was performed over a lower range of concentrations for these strains.

Positive controls produced the expected increases in revertants in all tester strains.

In the repeat assay, L-653,649 did not produce any two-fold or greater increases in revertants relative to solvent control over the range of 1–300 µg/plate. No precipitate was seen on the plates in this test. Slight inhibition of the background lawn was seen with *S. typhimurium* TA97a without S9 at 300 µg/plate, and inhibition of revertant growth was seen in TA100 and TA97a at the highest concentration tested.

Positive controls produced the expected increases in revertants.

Metabolite 4″ *epi*-amino avermectin AB1a (L-652, 649) did not induce two-fold or greater dose-related increases in revertants relative to the solvent control in any of the test strains, and thus was not considered mutagenic in this microbial mutagenesis assay. (Sina, 1995)

***Exploratory comparative neurotoxicity study in dogs***

The study was explorative and was not conducted to specific guidelines; it was not GLP-compliant.

Metabolite L-653,649 (4″ *epi*-amino avermectin) along with emamectin hydrchloride, and a series of structurally related avermectins, L-682, 901 (4″*epi*-methylamino ivermectin), L-653, 648 (4″*epi*-acetyl avermectin), and L-655, 372 (4″ *epi*-dimethylamino avermectin) were tested. These were administered to beagle dogs in a two-week oral toxicity study to determine their comparative neurotoxic potential.

All compounds were administered once daily at a dose level of 1.5 mg/kg bw per day in propylene glycol:glycerol formal mixture at 60:40 (v/v) in a dose volume of 1 mL/kg bw. However, due to the shortage of L-682, 901, the dose of this compound was reduced to 0.5 mg/kg bw per day on day 11 and to 0.71 mg/kg bw per day on days 12 and 13. Each group contained two male and two female dogs. A control group also of two males and two females received vehicle only.

At study termination all dogs in the L-682, 901 group and all male dogs in the other treatment groups had received 13 daily doses of test compound. All control dogs and all female dogs in the treatment groups (with the exception of the L-682, 901 group) had received 14 daily doses.

All animals were observed daily for mortality and clinical signs. Additional examinations of the dogs' eyes for mydriasis were conducted approximately two hours after each dose. Body weights were obtained pretest and once in week 2, and food consumption was measured daily Wednesday to Friday in week 1 and Tuesday to Friday in week 2. Blood was collected in week 2 from all treated animals for possible analysis for plasma concentration of the test substances.

Selected tissues were taken at necropsy, fixed. Sections from the brain, cervical, thoracic and lumbar spinal cord, dorsal root ganglia, optic nerves and sciatic nerve were stained with haematoxylin and eosin and examined microscopically. Additionally, selected sections of midbrain and medulla oblongata were stained with a monoclonal antibody used for the detection of phosphorylated epitopes on neurofilaments.

All dogs survived to scheduled termination. Treatment-related physical signs consisted of mydriasis, tremors, drooling and lateral recumbency. Mydriasis was seen in dogs receiving L-653, 648 or L-653,649. Tremors occurred in dogs receiving emamectin hydrochloride, L-653,649 or L-655, 372. Additionally, one animal in the L-653,649 group was observed to be drooling during the latter part of week 2 and was laterally recumbent on the day of scheduled necropsy. Treatment-related decreases in food consumption occurred in animals receiving L-653, 648, L653, 649 and L-655, 372, most markedly in the latter two groups. Treatment-related reductions in average body weight were observed in all groups receiving the test compounds.

No treatment-related gross lesions were found at necropsy. Treatment-related microscopic changes consisted of neuronal degeneration in the brains of dogs receiving emamectin hydrochloride,

L-653,649 or L-655, 372. Affected neurons were most abundant in the midbrain (pontine nuclei) but were also found in the accessory cuneate nuclei, reticular formation and in cranial nerve nuclei located within the medulla of the brain. Sevier–Munger and SMI-31-stained sections of brain indicated that the perikarya of degenerative neurons contained abnormal accumulations of neurofilaments. Additional treatment-related microscopic findings consisted of degeneration of the white matter of the spinal cord and brain and peripheral nerve degeneration. Spinal cord degeneration was seen in dogs receiving emamectin, L-653,649 or L-655,372. A similar change was observed in the brain (cerebellar peduncles) of 1 dog receiving L-653,649 and one dog receiving L-655, 372. Very slight degenerative changes were noted in sections of spinal nerve roots or sciatic nerve obtained from dogs receiving emamectin, L-653,649 or L-655, 372, and were also noted in an optic nerve from one dog that received L-653,649.

Metabolites L-653,649 and L-655, 372 are respectively the unsubstituted and dimethylamino analogues of emamectin hydrochloride; they induced clinical signs and morphological lesions that are essentially identical to the changes induced by emamectin hydrochloride. However, L-682, 901 (4″*epi*-methlyamino-ivermectin) and L-653, 648 (4″*epi*-acetylamino-avermectin) did not produce the morphological changes found with equivalent dosage levels of emamectin hydrochloride. In addition, similar studies in rats and dogs at comparable or higher dosage levels with ivermectin and abamectin did not produce any morphological changes in the central or peripheral nervous system.

Although the mechanism of the morphological alterations is undetermined, the presence of phosphorylated epitopes on neurofilaments within the perikarya of neurons is an abnormal condition and suggests an effect on slow axonal transport systems. This morphological alteration is considered an unusual observation in dogs and was considered to be treatment-related (Lankas, 1992b).

### (c) Metabolite 8,9-*Z*-4″-*epi*-methylamino-avermectin B1a benzoate (L-695,638, 8,9-Z MAB1a, NOA438376)

Metabolite 8.9-*Z*-4″-*epi*-methylamino-avermectin B1a benzoate (L-695,638) is a photodegradate of emamectin benzoate. It was tested in a number of toxicity and genotoxicity studies.

#### *Acute oral toxicity study in mice*

The study was conducted according to OECD TG 401 (1981) and in compliance with GLP.

The acute oral toxicity of L-695,638 (purity 90.6%, batch L-695,638-001C) was studied in mice. Groups of Crl:CD-1(ICR) BR mice (five/sex per dose) were given L-695,638 in 0.5% aqueous methylcellulose as a single oral dose at 0, 50, 100 or 200 mg/kg bw in a volume of 10 mL/kg bw.

Individual animal observations for clinical signs were made daily. Body weights were recorded pretest and on days 7 and 14. On day 15 all surviving mice were killed with carbon dioxide, exsanguinated and underwent complete necropsies.

There were no deaths, physical signs or effects on body weight throughout the 15-day study. No lesions were seen at necropsy that were considered due to treatment.

In conclusion, the $LD_{50}$ of L-695,638 given as a single oral dose to male and female mice was greater than 200 mg/kg bw. Following oral dosing L-695,638 was less toxic than parent emamectin benzoate (Lankas, 1995b).

#### *Microbial mutagenesis assay*

The study was performed in compliance with GLP; no specific guideline was stated but the study design was similar to OECD TG 471.

Metabolite L-695, 638 (purity 92.9% , batch L-695,638-001C001), was evaluated for mutagenic potential in a microbial mutagenesis test system using mutant strains of *Salmonella typhimurium* (TA1535, TA97a, TA98 and TA100) and *Escherichia coli* (WP2, WP2 uvrA and WP2 uvrA pKM101). The strains were exposed to L-695,638 (purity 92.9%) as a solution in DMSO at concentrations of 3, 10, 30, 100, 300, 1000, 3000 and 10 000 µg/plate in the presence and absence of mammalian metabolic activation using a 48-hour exposure.

In the initial assay, L-695,638 was tested over a concentration range of 100 to 10 000 µg/plate

and did not produce any two-fold or greater increases in revertants relative to the solvent control in any of the tester strains, either with or without metabolic activation. Positive control compounds produced expected increases in revertants.

Precipitation, that did not interfere with the scoring, was seen at concentrations of 3000 and 10 000 µg/plate. Inhibition of bacterial lawn growth was noted at concentrations as low as 1000 µg/plate in *Salmonella* strains TA100, TA1535 and TA97a, and this interfered with the scoring of plates in many cases. Inhibition of revertant growth was also apparent in a number of strains, particularly with *Salmonella* strains. For this reason the experiment was repeated with *Salmonella* strains over a lower range of concentrations.

The repeat assay (TT #92-8072) tested L-695,638 (purity 92.9%) as a solution in DMSO at concentrations of 0, 3, 10, 30, 100 or 300 µg/plate against *Salmonella typhimurium* strains TA1535, TA97a, TA98 and TA100). The assay did not reveal any two-fold or greater increases in revertants relative to the solvent control for any of the tester strains with or without metabolic activation. No precipitate was present at 300 µg/plate, the highest dose tested. Slight thinning of background lawn was seen with TA97a with S9 mix at 300 µg/plate and some inhibition of revertant growth was seen with TA100 and TA97a. The positive control compounds, with and without S9 activation, produced the expected increases in revertants in all tester strains with the following exceptions: for TA97a the fold increase in revertant numbers was lower than acceptable after treatment with ICR-191 (an acridine mutagen) and methyl methanesulfonate (MMS); for TA98 results with ICR-191 were too high, while results with daunomycin were too low. The assay was, therefore, repeated for TA97a and TA98.

The second repeat assay (TT #92-8073) tested L-695,638 (purity 92.9%) as a solution in DMSO at concentrations of 0, 3, 10, 30, 100 or 300 µg/plate in *Salmonella typhimurium* strains TA97a and TA98. The experiment did not produce any two-fold or greater increases in revertants relative to the solvent control in any of the tester strains with or without metabolic activation. Slight cytotoxic effects were seen in TA97a tester strain. No precipitate was seen on any of the plates. All positive controls showed appropriate responses.

Metabolite 8.9-*Z*-4″-*epi*-methylamino-avermectin B1a benzoate (L-695,638) was not detectably mutagenic in this microbial mutagenesis assay (Sina, 1993b).

***Fifteen-day dietary neurotoxicity study in mice***

This study was not conducted according to regulatory guidelines but was performed according to GLP.

The mouse strain used in this study is deficient in MDR1a P-glycoprotein and is therefore considered to be more sensitive to the effects of avermectins.

In a dietary neurotoxicity study, groups of ten male and ten female, approximately 39-day-old, Crl:CF-1 BR mice were continuously administered diet containing L-695,638 (purity 93.7%, batch L-696, 638-001C001) at dose levels of 0, 0.05, 0.075, 0.10 or 0.30 mg/kg bw per day for 14 days. The actual, average consumed dose of L-695,638, corrected for measured dietary concentrations of the test article and mean dose group food consumption, was calculated on days 7 and 14. The mean L-695,638 consumption on day 7 was 0, 0.04, 0.07, 0.09 and 0.26 mg/kg bw per day for males, 0, 0.05, 0.08, 0.10 and 0.29 mg/kg bw per day for females. Mean consumption on day 14 was 0, 0.06, 0.08, 0.11 and 0.32 mg/kg bw per day for males, 0, 0.06, 0.08, 0.12 and 0.31 mg/kg bw per day for females. The study director stated that the dietary concentrations of the test substance fell below acceptable limits for females during week 2, so this study was repeated in female CF-1 mice.

All animals were observed daily for mortality and clinical signs. Body weight was recorded pretreatment and once a week during the study. Food consumption was measured weekly beginning during the pretest week. Food was withdrawn overnight prior to scheduled termination on day 15. All mice underwent necropsies limited to the central nervous system (brain and cervical, thoracic and lumbar segments of spinal cord) and sciatic nerve. Weights of brains were recorded. The tissues were fixed, stained and sections of the brain, spinal cord and peripheral nerve from all control and high-dose mice underwent histopathological examination.

No mortalities or treatment-related clinical signs were observed. There were no treatment-related effects on body weight or food consumption. No gross or microscopic changes were observed related to

treatment. No treatment-related changes in brain weights were observed.

In conclusion, there were no signs of neurotoxicity at any of the dose levels tested. Based on average daily food consumption and targeted dietary concentration, the average daily consumption of L-695,638 approximated to the target intake during week 1 (Gerson, 1993b).

Because dietary concentrations of the test substance fell below acceptable levels for females in week 2, the study was repeated in female mice as described immediately below.

***Fifteen day dietary neurotoxicity study in female mice***

This study was not conducted following any regulatory guidelines but was performed according to GLP.

The mouse strain used in this study is deficient in MDR1a P-glycoprotein and is therefore considered to be more sensitive to the effects of avermectins.

In a dietary neurotoxicity study, groups of ten female, 40-day-old, Crl:CF-1 BR mice were continuously administered a diet containing L-695,638 (purity 93.7%, batch L-596, 638-001C001), at dose levels of 0, 0.05, 0.075, 0.10 or 0.30 mg/kg bw per day for 14 days. This test article had been previously administered at the same dose levels to male and female CF-1 mice (TT #92-072-0) but due to low dietary concentrations of the test substance in the female diets during the week 2 (less than 15% of target dose), the study needed repeating in female mice. In the previous study, targeted dietary concentrations were achieved in male mice and no signs of toxicity observed.

All animals were observed daily for mortality and clinical signs. Body weight was recorded pretest and once a week during the study. Food consumption was measured weekly beginning at the pretest week, based on a six-day intake period.

The actual, average consumed dose of L-695,638, corrected for measured dietary concentrations of the test article and mean dose group food consumption, was calculated on days 7 and 14. The mean L-695,638 consumption was 0.041, 0.057, 0.086 or 0.243 mg/kg bw per day.

Food was withdrawn overnight prior to scheduled termination on day 15. All mice underwent necropsies limited to the central nervous system (brain and cervical, thoracic and lumbar segments of spinal cord) and sciatic nerve. Weights of brains were recorded. The tissues were fixed, stained and sections of the brain, spinal cord and peripheral nerve from all control and high-dose mice underwent histopathological examination.

There were no mortalities or treatment-related clinical signs. There were no treatment-related effects on body weight or food consumption. There were no gross or microscopic changes related to treatment. There were no treatment-related changes in brain weight.

It was concluded that 8.9-*Z*-4″-*epi*-methylamino-avermectin B1a benzoate (L-695,638) is not more toxic in female CF-1 mice than emamectin benzoate (Gerson, 1994).

**(d) Metabolite 4″*epi*-(*N*-formyl-*N*-methyl)-amino-4″-deoxy-avermectin B1 (L-660,599, MFB1a)**

Metabolite 4″*epi*-(*N*-formyl-*N*-methyl)-amino-4″-deoxy-avermectin B1 is a photodegradate of emamectin benzoate.

***Acute oral toxicity study in mice***

No specific guidelines were stated for this study, but it was performed according to GLP. The $LD_{50}$ of L-660,599, given as a single oral dose to male and female mice was investigated.

Groups of Crl:CD-1(ICR) BR mice (five per sex in each group) were given a single oral dose of L-660,599 (purity 96.6%, batch L-660,599-000N) in 0.5% aqueous methylcellulose at doses of 0, 25, 50, 100 or 200 mg/kg bw in a dose volume of 10 mL/kg bw. Individual animal observations for clinical signs were made daily. Body weights were recorded pretest and on days 7 and 14. On day 15 all surviving mice were killed with carbon dioxide, exsanguinated and underwent complete necropsies.

Mortality occurred in 2/5 males, but no females at 25 mg/kg bw, and all males and females at 50, 100 and 200 mg/kg bw ca 1.5 hours after dosing and day 5.

Physical signs noted in all groups included tremors, decreased activity, ptosis and bradypnea. Lateral recumbency was seen in all dose groups except 100 mg/kg bw. These physical signs were first seen within approximately one hour of dosing or on day 2, and lasted one to four days. Surviving mice that had shown clinical signs at 25 mg/kg bw, all recovered by day 6.

Body weight loss (by 13%) occurred in a single male given 25 mg/kg bw on day 7, however weight was recovered by day 14. No apparent effect on body weight occurred in the remaining animals in this dose group.

The gross examination did not reveal any compound-related changes.

The acute oral $LD_{50}$ for L-660,599 for male and female mice, sexes combined, was 31 mg/kg bw (range 26–37 mg/kg bw), for males 27 mg/kg bw (range 19–38 mg/kg bw) and for females 35 mg/kg bw (limits not given). It was concluded that 4″*epi*-(*N*-formyl-*N*-methyl)-amino-4″-deoxy-avermectin B1 (L-660,599) was more toxic after oral dosing than emamectin benzoate (Lankas, 1995c).

***Microbial mutagenesis assay***

Metabolite L-660,599 was evaluated for mutagenic potential in a microbial mutagenesis test system using mutant strains of *Salmonella typhimurium* (TA1535, TA97a, TA98 and TA100) and *Escherichia coli* (WP2, WP2 uvrA and WP2 uvrA pKM101). The study was performed in compliance with GLP; no specific guideline was stated but the study design was similar to OECD TG 471.

The strains were exposed to L-660,599 (purity 94.4%, batch L-660,599-000N004) as a solution in DMSO at concentrations of 100, 300, 1000, 3000 or 10 000 µg/plate in the presence and absence of mammalian metabolic activation.

Precipitation was seen on the plates at 10 000 µg/plate and this interfered with scoring of the plates at this level. No inhibition of bacterial lawn growth was noted at 10 000 µg/plate; no inhibition of revertant growth was apparent at any concentration. Metabolite L-660,599, tested at the highest scorable concentration of 3000 µg/plate, did not produce any two-fold or greater increases in revertants relative to the solvent control with any of the tester strains, with or without metabolic activation.

The positive control compounds, 2-aminoanthracene and hydrazine sulfate, produced the expected increases in revertants in all tester strains, in the presence of metabolic activation.

Metabolite 4″*epi*-(*N*-formyl-*N*-methyl)-amino-4″-deoxy-avermectin B1 (L-660,599) did not induce any two-fold or greater increase in revertant colonies compared to the solvent control in any tester strain at any concentration and was therefore not considered mutagenic in the microbial mutagenesis assay (Sina, 1993c).

***Fifteen day dietary neurotoxicity study in mice***

This study was not conducted in compliance with any specific regulatory guideline but was performed according to GLP.

The mouse strain used in this study is deficient in MDR1a P-glycoprotein and is therefore considered to be more sensitive to the effects of avermectins.

In a dietary neurotoxicity study, groups of approximately 39-day-old Crl:CF-1 BR mice, ten male and ten females, were continuously administered a diet containing L-660,599 (purity 98.1%, batch L-660,599-00N), at targeted dose levels of 0, 0.1, 0.3 or 0.9 mg/kg bw per day for 14 days. The actual, average consumed dose of L-660,599, corrected for measured dietary concentrations of the test article and mean dose group food consumption, was calculated on days 7 and 14. The mean L-660,599 consumption for day 7 was 0, 0.10, 0.31 or 1.01 mg/kg bw per day for males, 0, 0.11, 0.30 or 0.99 mg/kg bw per day females. The mean consumption for day 14 was 0, 0.11, 0.36 or 1.08 mg/kg bw per day for males, 0, 0.13, 0.44 or 1.20 mg/kg bw per day for females.

All mice underwent partial necropsy limited to brain, spinal cord and sciatic nerve. For all mice haemotoxylin/eosin stained sections of these tissues and gross findings from the partial necropsies were examined.

Three females and one male at 0.9 mg/kg bw per day, and three females and one male at 0.3 mg/kg bw per day were killed due to the severity of clinical findings on days 3–9 of the study. In addition, one 0.3 mg/kg bw per day female was found dead on day 12.

Tremors, starting on day 2, were seen in six mice at 0.9 mg/kg bw per day, followed by additional signs (lethargy, splayed limbs, shallow breathing and urine staining) in the more severely affected animals. At 0.3 mg/kg bw per day, tremors, starting on day 3, and/or additional clinical signs (lethargy, splayed limbs, hunched posture, piloerection, and ptosis) were observed in up to seven animals. These signs generally increased in severity with time. Clinical signs of toxicity were only seen in one male at 0.1 mg/kg bw per day, comprising slight tremors, hunched posture and piloerection on day 14. Four mid-dose and four high-dose animals were sacrificed from days 3 until 9 due to the severity of the clinical signs. One mid-dose female was found dead on day 12; the cause of death was also not evident at necropsy

Metabolite L-660,599 induced weight loss (pretest and terminal body weights compared) in the animals from the mid- and high-dose groups that had severe clinical signs. The range of weight loss was approximately 4–8 g, with decrements noted within the first three days. These changes in body weight were attributed to the severe, apparently continuous tremors experienced by these animals. With the exception of one 0.3 mg/kg bw per day male and one 0.1 mg/kg bw per day male, all animals with treatment-related changes in body weight were sacrificed prior to study termination due to the severity of their clinical condition.

No gross or microscopic lesions were found in either the early death/early sacrifice mice or in those that survived to scheduled necropsy. There was no effect of treatment on brain weights.

In conclusion, 4″*epi*-(*N*-formyl-*N*-methyl)-amino-4″-deoxy-avermectin B1 (L-660,599), the plant photometabolite/degradate of emamectin benzoate, caused clinical signs of toxicity (tremor, often associated with splayed limbs, decreased activity, hunched appearance, ptosis and weight loss) at all dose levels. These signs generally appeared on the second day following treatment in high-dose animals, the third day after exposure in mid-dose animals, and not until the final day of the study in one low-dose animal. While these findings were dose-related in terms of onset, there was no clear difference in severity between mid- and high-dose animals. The signs observed in this study were similar to those in a previous 14-day study in this strain of mouse when administered emamectin benzoate in the diet (Gerson, 1993c).

***Fifteen-day dietary neurotoxicity study in CF-1 mice***

This study was not conducted in compliance with any specific regulatory guideline but was performed according to GLP.

The mouse strain used in this study is deficient in MDR1a P-glycoprotein and is therefore considered to be more sensitive to the effects of avermectins.

In a dietary neurotoxicity study, groups of approximately 39-day-old Crl:CF-1 BR mice, ten male and ten females, were continuously administered diet containing L-660, 559 (purity 98.1%, batch L-660,599-000N), a plant photometabolite of emamectin benzoate, at target dose levels of 0, 0.05, 0.075, 0.10 and 0.30 mg/kg bw per day for 14 days.

All animals were observed daily for clinical signs. Body weight and food consumption were recorded pretest and once a week during the study. Doses of the test article, averages of six days, were calculated on days 7 and 14 of the study.

All mice underwent necropsies at scheduled termination on day 15, except animals in the 0.30 mg/kg bw per day group and one male in the 0.10 mg/kg bw per day group, which were terminated by study day 8 due to the severity of toxic signs.

Sections of brain (including cerebral cortex and subcortical white matter, cerebellum and pons) and spinal cord (cervical, thoracic and lumbar) from all mice in the control, 0.10 and 0.30 mg/kg bw per day groups, and sciatic nerve from all groups, were taken for histopathological examination.

The mean consumptions of L-660,599 for day 7 were 0, 0.06, 0.11, 0.14 and 0.42 mg/kg bw per day for males, 0, 0.07, 0.11, 0.14 and 0.37 mg/kg bw per day for females. The mean consumptions for day 94 were 0, 0.05, 0.07 and 0.09 mg/kg bw per day for males, 0, 0.05, 0.075 and 0.10 mg/kg bw per day for females.

At 0.30 mg/kg bw per day, 3/10 males and 5/10 females had tremors on days 2–4. Two to five days following the onset of the tremors additional treatment-related signs were seen: ptosis, decreased activity, hunched posture, splayed limbs, ataxia, laboured breathing, urine staining and/or lateral recumbency. Because of the severity of these signs, all eight of the animals were sacrificed on days 4–8. Decreased body weight and food consumption were seen in the mice with treatment-related clinical signs. The only treatment-related histopathological lesion was a very slight degeneration of the sciatic nerve in a 0.30 mg/kg bw per day male.

At 0.10 mg/kg bw per day, 3/10 male mice had tremors beginning on days 3 or 5. Tremors were subsequently accompanied in two of these animals by ptosis, decreased activity, and/or hunched posture. One of these animals was sacrificed on day 8 because of the severity of clinical signs. Decreased body weight and food consumption were seen in the mice with treatment-related clinical signs. The only treatment-related histopathological lesion was very slight degeneration of the sciatic nerve in the male that was terminated early. There were no treatment-related changes in brain weight.

No treatment-related clinical signs or effects on body weight or food consumption were seen in the groups dosed with 0.05 or 0.075 mg/kg bw per day.

Degeneration of the sciatic nerve was present in one control group female, four males receiving 0.1 mg/kg per day and one male receiving 0.3 mg/kg per day. No treatment-related histological tissue abnormalities were noted in the brain or spinal cords from the 0.10 and 0.30 mg/kg per day dosed animals.

In conclusion, the no-effect level for this study was 0.075 mg/kg bw per day. Compound-related changes, consisting primarily of tremors, ataxia and decreased activity, occurred at 0.10 and 0.30 mg/kg bw per day. Sciatic nerve degeneration was occasionally associated with clinical signs. The no-effect dose for clinical signs of toxicity for L-660,559 was similar to that previously described for emamectin benzoate. Administration of 4″*epi*-(*N*-formyl-*N*-methyl)-amino-4″-deoxy-avermectin B1 (L-660,599) was associated with sciatic nerve degeneration not observed after the administration of emamectin benzoate to CF-1 mice at doses up to 0.9 mg/kg per day, although sciatic nerve effects were seen in rat and dog (Gerson, 1993d).

## 3. Microbiological aspects

No new data were submitted on this topic.

## 4. Observations in humans

No new data were submitted on this topic.

# Comments

## Toxicological data on metabolites and/or degradates

A number of photodegradation metabolites of emamectin benzoate had been tested for acute toxicity in CD-1 mice to determine an oral median lethal dose ($LD_{50}$) in a microbial mutagenesis assay, and in a 15-day dietary study in CF-1 mice or a neurotoxicity study in dogs. The CF-1 mice used in the neurotoxicity studies lack the expression of MDR1a P-glycoprotein and are therefore not an appropriate model for human risk assessment with avermectins.

***Table 2. Summary of toxicological characterization of photodegradation products/metabolites of emamectin benzoate***

| **Compound, code and structure** | **Major rat metabolite** (> 10% AD) | **Genotoxicity assessment** (data, QSAR, RA) | **General toxicity** | **Toxicological reference values** |
|---|---|---|---|---|
| **Emamectin benzoate**<br>(MK 244;<br>L-656,748-038W) | Parent | Data<br>Not genotoxic | Full data set | ADI:<br>0–0.000 5 mg/kg bw<br>ARfD:<br>0.02 mg/kg bw |
| **L-657,831**<br>(4″*epi*-(*N*-formyl)-amino-4″-deoxy-avermectin B;<br>FAB1a) | No | Ames: negative (Sina, 1993a) | $LD_{50}$: 66 mg/kg bw (Lankas, 1995a) | Parent ADI and ARfD |
| **L-653,649**<br>(4″*epi*-amino avermectin;<br>AB1a) | No | Ames: negative (Sina, 1995) | $LD_{50}$: 100 mg/kg bw<br>15-day neurotox study comparative (including emamectin) study (dog) (Lankas & Bagdon, 1995)<br>LOAEL: 1.5 mg/kg bw per day, (only one dose tested);<br>Displayed effects were similar to emamectin (Lankas, 1992b) | Parent ADI and ARfD |
| **L-695,638**<br>(8,9-Z-4″*epi*-methylamino-avermectin B1a benzoate salt;<br>8,9-Z MAB1a;<br>NOA438376) | No | Ames: negative (Sina, 1993b) | $LD_{50}$: > 200 mg/kg bw (Lankas, 1995b) | Parent ADI and ARfD |
| **L-660,599**<br>4″*epi*-(*N*-formyl-*N*-methyl)-amino-4″-deoxy-avermectin B1;<br>MFB1a) | No | Ames: negative (Sina, 1993b) | $LD_{50}$: 31 mg/kg bw (Lankas, 1995c) | Parent ADI and ARfD |

QSAR: Quantitative structure–activity relationship; AD: Administered dose; RA: Read-across;
ADI: Acceptable daily intake; ARfD: Acute reference dose; $LD_{50}$: Median lethal dose

The Meeting concluded that for the photodegradation metabolites L-657,831, L-695,638, L-660,599 and L-653,649, the ADI and the ARfD of the parent compound should be used as the relevant values. In the case of the ADI, this is lower than the Cramer class III threshold of 1.5 µg/kg bw per day. Currently it is unknown whether the photodegradation metabolites occur as residues in commodities.

## Toxicological evaluation (addendum)

In 2011 the Meeting established an ADI of 0–0.000 5 mg/kg bw for emamectin benzoate based on the one-year and two-year rat studies, and an overall NOAEL for 14-week and 53-week toxicity studies in dogs, applying a safety factor of 100 and an additional safety factor of five based on steep dose–response curves and irreversible histopathological effects in neural tissue at the lowest-observed-adverse-effect level (LOAEL). The current Meeting concluded that the parent ADI applies also to metabolites L-657,831, L-695,638, L-660,599 and L-653,649, should they be found in food commodities.

In 2014 the Meeting established an ARfD of 0.02 mg/kg bw, on the basis of the five-week and 14-week dog studies, and applying a safety factor of 100. The current Meeting concluded that the parent ARfD applies also to metabolites L-657,831, L-695,638, L-660,599 and L-653,649, should they be found in food commodities.

*The acceptable daily intake (ADI) for emamectin, established by JMPR in 2011*

0–0.000 5 mg/kg bw*

*The acute reference dose (ARfD) for emamectin, established by the JMPR in 2014*

0.02 mg/kg bw*

** Applies to emamectin benzoate, and if necessary will apply to metabolites L-657,831, L-695,638, L-660,599 and L-653,649*

## Summary

| | Value | Study | Safety factor |
|---|---|---|---|
| ADI | 0–0.000 5 mg/kg bw [a] | One-year and two-year rat studies and 14- and 53-week toxicity studies in dogs | 500[b] |
| ARfD | 0.02 mg/kg bw [a] | Five-week and 14-week studies in dogs | 100 |

[a] Applies to emamectin benzoate and will apply to L-657,831, L-695,638, L-660,599 and L-653,649, if necessary

[b] An additional safety factor of five was applied to the standard safety factor of 100 based on the steep dose–response curves and irreversible histopathological effects in neural tissue at the lowest-observed-adverse-effect level (LOAEL)

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# Florylpicoxamid

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## Explanation

Florylpicoxamid is the International Organization for Standardization (ISO)-approved common name for (*2S*)-1,1-*bis*(4-fluorophenyl)propan-2-yl-*N*-{[3-(acetyloxy)-4-methoxypyridin-2-yl]carbonyl}-*L*-alaninate, with the Chemical Abstracts Service number 1961312-55-9. Florylpicoxamid is a picolinamid fungicide the mode of action (MOA) of which is a quinone inside inhibitor (respiration inhibitor at the cytochrome bc1 complex).

Florylpicoxamid has not previously been evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed by the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR).

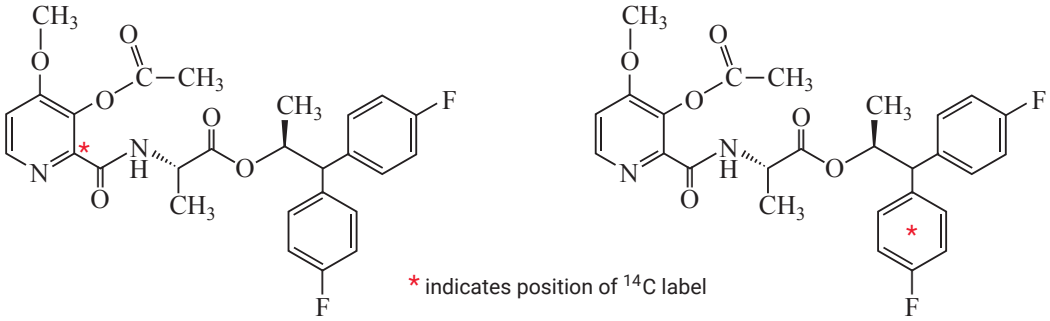
All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with relevant national or international test guidelines, unless otherwise specified. No additional information from a literature search was identified that complemented the toxicological information submitted for the current assessment.

## Evaluation for acceptable daily intake

### 1. Biochemical aspects

Five studies were conducted to investigate absorption, distribution, metabolism and excretion (ADME) of florylpicoxamid, administered by oral gavage to F344 rats, Crl: CD1 mice, New Zealand rabbits and beagle dogs. The ADME of florylpicoxamid were investigated using florylpicoxamid uniformly labelled in the phenyl ring, or labelled at the pyridine-2 position, as shown in Fig. 1. The test item in the main ADME studies was a mixture of $^{14}$C-labelled and unlabelled florylpicoxamid. Study designs for the ADME and kinetics investigations are summarized in Table 1.

***Figure 1. Structure of florylpicoxamid including position of radiolabels***




[*Pyridinyl-2-*$^{14}$C]florylpicoxamid

[*Phenyl-UL-*$^{14}$C]florylpicoxamid

***Table 1. Dosing groups for ADME experiments with $^{14}$C-labelled florylpicoxamid***

| Test group | Dose<br>[Route]<br>Number of M, F | Test compounds and collected samples |
|---|---|---|
| **Probe studies (Himmelstein, 2020)** | | |
| Rat / F344/DuCrl | 100 mg/kg bw<br>[OG, single dose]<br>1M,2F | [*Pyridinyl-2-*$^{14}$C]florylpicoxamid;<br>Excreta, blood (plasma and RBCs), expired air, volatiles and time course blood (plasma and RBCs) collected |
| Rat / F344/DuCrl | 100 mg/kg bw<br>[OG, single dose]<br>1M,1F | [*Pyridinyl-2-*$^{14}$C]florylpicoxamid;<br>Blood (plasma and RBCs) at $C_{max}$ collected |

| Test group | Dose<br>[Route]<br>Number of M, F | Test compounds and collected samples |
|---|---|---|
| Rat / F344/DuCrl | 100 mg/kg bw<br>[OG, single dose]<br>1M,2F | [*Pyridinyl-2-*$^{14}$C]X12485649<br>(Lot no. DE3-145187-97; radiochemical purity 98.7%)<br>Excreta, blood (plasma and RBCs), expired air, volatiles and time course from blood (plasma and RBCs) collected |
| Rat / F344/DuCrl | 100 mg/kg bw<br>[OG, single dose]<br>1M,1F | [*Pyridinyl-2-*$^{14}$C]X12485649<br>(Lot no. DE3-145187-97; radiochemical purity 98.7%)<br>blood (plasma and RBCs) collected at $T_{max}$ |
| Rat / F344/DuCrl | 100 mg/kg bw<br>[OG, single dose]<br>1M,1F | [*Phenyl-UL-*$^{14}$C]florylpicoxamid[a]<br>Excreta, blood (plasma and RBCs), expired air, volatiles and time course from blood (plasma and RBCs) collected |
| Rat / F344/DuCrl | 100 mg/kg bw<br>[OG, single dose]<br>1M,1F | [*Phenyl-UL-*$^{14}$C] florylpicoxamid[a]<br>Blood (plasma and RBCs) collected at $C_{max}$ |
| Mouse / Crl:CD1 (ICR) | 100 mg/kg bw<br>[OG, single dose]<br>2M,2F | [*Pyridinyl-2-*$^{14}$C] florylpicoxamid[b]<br>Excreta, blood (plasma and RBCs), expired air, volatiles collected |
| Mouse / Crl:CD1 (ICR) | 100 mg/kg bw<br>[OG, single dose]<br>2M,2F | [*Pyridinyl-2-*$^{14}$C]florylpicoxamid[b]<br>Time course from blood (plasma and RBCs) collected |
| Mouse / Crl:CD1 (ICR) | 100 mg/kg bw<br>[OG, single dose]<br>2M,2F | [*Pyridinyl-2-*$^{14}$C]florylpicoxamid[b]<br>Blood (plasma and RBCs) collected at $C_{max}$ |
| Probe study Rabbit / NZW | 100 mg/kg bw<br>[OG, single dose]<br>1F | [*Pyridinyl-2-*$^{14}$C]florylpicoxamid[b]<br>Excreta, blood (plasma and RBCs), expired air, volatiles and time course blood (plasma and RBCs) collected |
| Probe study Rabbit / NZW | 33 mg/kg bw<br>[OG, single dose]<br>1F | [*Pyridinyl-2-*$^{14}$C]florylpicoxamid[b]<br>Excreta, blood (plasma and RBCs), expired air, volatiles and time course from blood (plasma and RBCs) collected |
| **Pharmacokinetics and metabolism studies (Himmelstein, 2019a)** | | |
| Rat / Crl:CD(SD) | 25 mg/kg bw<br>[OG, single dose]<br>4M,4F | [*Pyridinyl-2-*$^{14}$C]florylpicoxamid[c]<br>Tissues, excreta, exhaled breath, cage rinse, final cage wash and time course from blood (plasma and RBCs) collected |
| Rat / Crl:CD(SD) | 250 mg/kg bw<br>[OG, single dose]<br>4M,4F | [*Pyridinyl-2-*$^{14}$C]florylpicoxamid[c]<br>Tissues, excreta, exhaled breath, cage rinse, final cage wash and time course from blood (plasma and RBCs) collected |
| Rat / Crl:CD(SD) | 25 mg/kg bw<br>[OG, repeat dose]<br>4M,4F | Unlabelled florylpicoxamid (14 doses) and single dose of [*Pyridinyl-2-*$^{14}$C]florylpicoxamid[c]<br>Tissues, excreta, exhaled breath, cage rinse, and final cage wash collected |
| Rat / Crl:CD(SD) | 25 mg/kg bw<br>[OG, single dose]<br>4M,4F | [*Phenyl-UL-*$^{14}$C]florylpicoxamid[d]<br>Tissues, excreta, exhaled breath, cage rinse, final cage wash and time course from blood (plasma and RBCs) collected |

| Test group | Dose<br>[Route]<br>Number of M, F | Test compounds and collected samples |
|---|---|---|
| **Biliary elimination/tissue distribution studies (Himmelstein, 2019b)** | | |
| ***Biliary elimination experiments*** | | |
| Rat / Crl:CD(SD)<br>cannulated bile duct | 25 mg/kg bw<br>[OG, single dose]<br>4M,4F | Single dose of [*Pyridinyl-2*-$^{14}$C]florylpicoxamid[e]<br>Bile, excreta, blood, cage wash collected |
| Rat / Crl:CD(SD)<br>cannulated bile duct | 250 mg/kg bw<br>[OG, single dose]<br>4M,4F | Single dose of [*Pyridinyl-2*-$^{14}$C]florylpicoxamid[e]<br>Bile, excreta, blood, cage wash collected |
| Rat / Crl:CD(SD)<br>cannulated bile duct | 25 mg/kg bw<br>[OG, single dose]<br>4M,4F | [*Bis-phenyl-UL*-$^{14}$C]florylpicoxamid[d]<br>Bile, excreta, blood, cage wash collected |
| ***Tissue distribution experiment*** | | |
| Rat / Crl:CD(SD) | 25 mg/kg bw<br>[OG, single dose]<br>4M,4F | [*Pyridinyl-2*-$^{14}$C]florylpicoxamid[e]<br>Tissues collected at $C_{max}$, and 24 hours post dose |
| Rat / Crl:CD(SD) | 250 mg/kg bw<br>[OG, single dose]<br>4M,4F | Single dose of [*Pyridinyl-2*-$^{14}$C]florylpicoxamid[e]<br>Tissues collected at $C_{max}$, and 24 hours post dose |
| Rat / Crl:CD(SD) | 25 mg/kg bw<br>[OG, single dose]<br>4M,4F | [*bis-Phenyl-UL*-$^{14}$C]florylpicoxamid[d]<br>Tissues collected at $C_{max}$, and 24 hours post dose |
| **In vitro metabolism in hepatocytes (Chan, 2020a)** | | |
| Human, rat (SD), mouse (CD-1), dog (beagle) and rabbit (NZW) hepatocytes | 16 µMol/L<br>[single exposure] | [*Pyridinyl-2*-$^{14}$C]florylpicoxamid[e] or<br>[*bis-Phenyl-UL*-$^{14}$C] florylpicoxamid[d] |
| **In vitro comparative metabolism using liver microsomes (Zhang, McClymont & Erskine, 2018)** | | |
| Human, rat (F344), mouse (CD-1), dog (beagle) and rabbit (NZW) liver microsomes | 2.5 µMol/L<br>[single exposure] | [*Pyridinyl-2*-$^{14}$C]florylpicoxamid[a] |

OG: Oral gavage; F: female; M: male; RBCs: red blood cells; UL: uniformly labelled; NZW: New Zealand White ; $T_{max}$: Time at which maximum concentration is attained; bw: Body weight

[a] Lot no. DE3-161026-11, radiochemical purity 97.4%;
[b] Lot no. DE3-150845-63, radiochemical purity 98.5%;
[c] Lot no. XZ7-169400-29A, radiochemical purity 99.0%;
[d] Lot no. DE3-168624-78, radiochemical purity 99.3%;
[e] Lot no. XZ7-170335-38B, radiochemical purity 99.1%

## 1.1 Absorption, distribution and excretion

### (a) Oral route

In a probe study of absorption, metabolism, and excretion of florylpicoxamid, a single oral dose of [*pyridinyl-2*-$^{14}$C]florylpicoxamid was administered by oral gavage to groups of male and female F344/DuCrl rats at 100 mg/kg body weight (bw), male and female Crl: CD1 (ICR) mice at 100 mg/kg bw and female New Zealand White rabbits at 33 or 100 mg/kg bw. In addition the absorption, metabolism, and excretion of metabolite C-X12485649 was also investigated following a single oral gavage dose of [*pyridinyl-2*-$^{14}$C] X12485649 or [*phenyl-UL*-$^{14}$C]X12485649 at 100 mg/kg bw to groups of male and female F344/DuCrl rats only. Following administration, samples were collected for a whole blood, RBC or plasma time course, as well as excretion samples (in urine and faeces in all species; volatiles and carbon dioxide in rats) for 168 hours (seven days post dosing) in rats, mice and rabbits. Tentative metabolite identification was conducted in representative blood samples (collected at $T_{max}$), as well as in representative urine samples.

The summary of kinetic parameters and excretory data on rats, mice and rabbits administered [*pyridinyl-2-*$^{14}$C]florylpicoxamid, [*phenyl-UL-*$^{14}$C]florylpicoxamid and [*pyridinyl-2-*$^{14}$C]X1485649 is shown in Table 2. Absorption was rapid. The absorption half-lives ranged from 0.1 to 5.6 hours for all test groups across species, sample types, and labelled test substances. For [*pyridinyl-2-*$^{14}$C]florylpicoxamid, the plasma maximum concentration ($C_{max}$) occurred three and two hours after administration in male and female rats, respectively, and 0.5 hours after administration in female rabbits. In mice, the blood $C_{max}$ occurred at 0.5 hours and 6 hours in male and female mice respectively. Alpha and beta plasma elimination half-lives were 4–9 hours and 39–44 hours in rats, and 9–12 hours and 41–47 hours in rabbits, respectively. The corresponding half-lives measured in the blood of mice were 3–9 hours and 21–34 hours, respectively. For pyridinyl-labelled metabolite X12485649, the alpha and beta elimination half-lives in rat plasma were 5–7 hours and 35–39 hours, and thus bioequivalent to those for the comparable pyridinyl-labelled florylpicoxamid. For phenyl-labelled florylpicoxamid the alpha and beta phase plasma half-lives in male rats were three hours and 37 hours, whilst for females they were three hours and 33 hours, respectively. Uptake and elimination from RBCs was slightly slower across all treatment groups.

The orally absorbed [*pyridinyl-2-*$^{14}$C]florylpicoxamid was rapidly excreted in urine of all species. Total urinary elimination over 168 hours accounted for 18%–33%, 19%–22%, and 75%–77% of the dose in rats, mice and rabbits, respectively. Urinary elimination was substantially complete by 48 hours in rats (89%–99%), 12 hours in mice (85%–87%), and 48 hours in rabbits (94%). Total urinary elimination of radioactivity derived from [*pyridinyl-2-*$^{14}$C]X1485649 was similar to the results for [*pyridinyl-2-*$^{14}$C]florylpicoxamid, with 21% and 40% of the dose in the male and female rats respectively, recovered by 168 hours after dosing, and the majority of this occurring within the first 24 hours (90%–93%). Total urinary elimination of radioactivity derived from [*phenyl-UL-*$^{14}$C]florylpicoxamid over 168 hours accounted for 5% and 25% of the dose in the male and female rats respectively, the majority of which occurred within the first 48 hours (78%–90%). Faecal elimination of [*pyridinyl-2-*$^{14}$C]florylpicoxamid accounted for 61%–82%, 69%–73% and 17% of the administered radioactive dose in rats, mice and rabbits, respectively. Volatiles (captured by charcoal trap) and carbon dioxide elimination were very minor routes of excretion, accounting for 0.2% of the administered radioactive dose in rats. [*pyridinyl-2-*$^{14}$C]X1485649 accounted for 54%–76% of the administered radioactive dose. Radioactivity from [*phenyl-UL-*$^{14}$C]florylpicoxamid over 168 hours accounted for 91% and 68% of the dose in male and female rats, respectively. Overall, the material balance for all groups was greater than 92% of the administered dose (AD), excluding tissues.

***Table 2. Summary of kinetic parameters and excretory data of florylpicoxamid in rats, mice and rabbits and its metabolite X12485649 in rats***

| Type of radiolabel/parameters of kinetics, absorption, excretion | Mouse | | Rat | | Rabbit | Rabbit[a] |
|---|---|---|---|---|---|---|
| | M | F | M | F | F | F |
| **[*Pyridinyl-2-*$^{14}$C] florylpicoxamid** | | | | | | |
| Plasma $C_{max}$ (µg equiv./g tissue) | NE | NE | 8.3 | 7.0 | 7.1 | 7.0 |
| Plasma $T_{max}$ (hours) | NE | NE | 3.0 | 2.0 | 0.5 | 0.5 |
| Plasma absorption $t_{1/2}$ (hours) | NE | NE | 1.1 | 0.3 | 0.4 | 0.3 |
| Plasma elimination $t_{1/2\alpha}$ (hours) | NE | NE | 4.3 | 8.7 | 11.6 | 8.8 |
| Plasma elimination $t_{1/2\beta}$ (hours) | NE | NE | 39.0 | 43.9 | 40.5 | 47.3 |
| Plasma $AUC_{0-168}$ (µg hour/g)[b] | NE | NE | 110.9 | 110.2 | 107.8 | 48.2 |
| RBC $C_{max}$ (µg equiv./g tissue) | NE | NE | 10.2 | 1.4 | 0.5 | 0.5 |
| RBC $T_{max}$ (hours) | NE | NE | 12.0 | 1.0 | 0.5 | 0.5 |
| RBC absorption $t_{1/2}$ (hours) | NE | NE | 4.8 | 0.5 | 0.5 | 0.1 |
| RBC elimination $t_{1/2}$ (hours) | NE | NE | NE | NE | NE | 10.2 |
| RBC elimination $t_{1/2\alpha}$ (hours) | NE | NE | 5.9 | 1.4 | 2.2 | NE |
| RBC elimination $t_{1/2\beta}$ (hours) | NE | NE | 74.8 | 61.1 | 59.6 | NE |
| RBC $AUC_{0-168}$ (µg hour/g)[c] | NE | NE | 235.8 | 39.4 | 13.9 | 2.1 |

| Type of radiolabel/parameters of kinetics, absorption, excretion | Mouse | | Rat | | Rabbit | Rabbit[a] |
|---|---|---|---|---|---|---|
| | M | F | M | F | F | F |
| Blood $C_{max}$ (µg equiv./g tissue) | 6.4 | 5.2 | NE | NE | 4.6 | 3.8 |
| Blood $T_{max}$ (hours) | 0.5 | 6.0 | NE | NE | 0.5 | 0.5 |
| Blood Absorption $t_{1/2}$ (hours) | 0.1 | 0.1 | NE | NE | 0.2 | 0.2 |
| Blood elimination $t_{1/2\alpha}$ (hours) | 3.2 | 9.4 | NE | NE | 4.4 | 1.9 |
| Blood elimination $t_{1/2\beta}$ (hours) | 21.5 | 33.9 | NE | NE | 29.5 | 26.2 |
| Blood $AUC_{0-168}$ (µg hour/g)[d] | 38.5 | 80.6 | NE | NE | 65.6 | 31.1 |
| Total urinary excretion 0–168 hours (% administered dose) | 19.1 | 21.3 | 18.2 | 33.5 | 75.2 | 76.8 |
| Total faecal excretion 0–168 hours (% administered dose) | 72.7 | 69.1 | 82.4 | 60.5 | 17.2 | 17.5 |
| Total volatile/$CO_2$ excretion 0–168 hours (% administered dose) | NE | NE | 0.2 | 0.2 | NE | NE |
| **[*Phenyl-UL*-$^{14}$C]florylpicoxamid** | | | | | | |
| Plasma $C_{max}$ (µg equiv./g tissue) | NE | NE | 9.3 | 6.6 | NE | NE |
| Plasma $T_{max}$ (hours) | NE | NE | 6.0 | 6.0 | NE | NE |
| Plasma absorption $t_{1/2}$ (hours) | NE | NE | 1.6 | 1.8 | NE | NE |
| Plasma elimination $t_{1/2\alpha}$ (hours) | NE | NE | 2.8 | 2.8 | NE | NE |
| Plasma elimination $t_{1/2\beta}$ (hours) | NE | NE | 37.1 | 33.0 | NE | NE |
| Plasma $AUC_{0-168}$ (µg hour $g^{-1}$)[e] | NE | NE | 134.1 | 96.0 | NE | NE |
| RBC $C_{max}$ (µg equiv./g tissue) | NE | NE | 7.1 | 1.9 | NE | NE |
| RBC $T_{max}$ (hours) | NE | NE | 12.0 | 6.0 | NE | NE |
| RBC absorption $t_{1/2}$ (hours) | NE | NE | 3.4 | 3.0 | NE | NE |
| RBC elimination $t_{1/2\alpha}$ (hours) | NE | NE | 6.5 | 8.2 | NE | NE |
| RBC elimination $t_{1/2\beta}$ (hours) | NE | NE | 57.2 | 47.7 | NE | NE |
| RBC $AUC_{0-168}$ (µg hour/g)[f] | NE | NE | 182.9 | 42.5 | NE | NE |
| **[*Pyridinyl-2*-$^{14}$C] X1485649** | | | | | | |
| Plasma $C_{max}$ (µg equiv./g tissue) | NE | NE | 10.5 | 8.7 | NE | NE |
| Plasma $T_{max}$ (hours) | NE | NE | 3.0 | 1.0 | NE | NE |
| Plasma absorption $t_{1/2}$ (hours) | NE | NE | 0.4 | 0.5 | NE | NE |
| Plasma elimination $t_{1/2\alpha}$ (hours) | NE | NE | 4.6 | 6.5 | NE | NE |
| Plasma elimination $t_{1/2\beta}$ (hours) | NE | NE | 34.5 | 39.2 | NE | NE |
| Plasma $AUC_{0-168}$ (µg hour $g^{-1}$)[e] | NE | NE | 122.9 | 103.8 | NE | NE |
| RBC $C_{max}$ (µg equiv./g tissue) | NE | NE | 6.0 | 1.3 | NE | NE |
| RBC $T_{max}$ (hours) | NE | NE | 12.0 | 1.0 | NE | NE |
| RBC absorption $t_{1/2}$ (hours) | NE | NE | 5.6 | 2.8 | NE | NE |
| RBC elimination $t_{1/2\alpha}$ (hours) | NE | NE | 7.9 | 3.8 | NE | NE |
| RBC elimination $t_{1/2\beta}$ (hours) | NE | NE | 70.8 | 129.7 | NE | NE |
| RBC $AUC_{0-168}$ (µg hour/g)[f] | NE | NE | 184.7 | 31.6 | NE | NE |

NE: Not examined; $C_{max}$: Maximum concentration achieved; $T_{max}$: Time to $C_{max}$; Source: Himmelstein, 2020

RBC: Red blood cell; AUC: Area under the concentration–time curve; $t_{1/2}$: Half-life

[a] Dosed with 33 mg/kg; [b] Plasma time course - quantifiable to: 168 h (rat), 168 h (100 mg/kg rabbit), 120 h (33 mg/kg rabbit);

[c] RBC time course - quantifiable to: 168 h (rat), 120 h (100 mg/kg rabbit), 12 h (33 mg/kg rabbit)

[d] Blood time course - quantifiable to: 96 h (mouse), 168 h (100 mg/kg rabbit), 120 h (33 mg/kg rabbit)

[e] Plasma time course - quantifiable to: 168 h (rat); [f] RBC time course - quantifiable to: 168 h (rat)

A total of five radiochemical peaks were detected in the urine samples across the profiles of rats and mice administered [*pyridinyl-2*-$^{14}$C]florylpicoxamid, with individual urine samples containing 3–5 of the total five observed radiochemical peaks. A total of four radiochemical peaks were detected in the rats dosed with [*pyridinyl-2*-$^{14}$C] X12485649. Urine samples from the rabbit with a single oral dose of [*pyridinyl-2*-$^{14}$C]florylpicoxamid at 100 mg/kg bw contained one of the five observed radiochemical peaks, while urine from the rabbit dosed orally at 33 mg/kg bw contained two peaks. Parent florylpicoxamid was not detected above the limit of detection (LOD) by radiochemical profiling in any of the three species' urine samples. The most abundant urinary metabolite in all species (13.1%–40.0%, 15.3%–17.3%, and 70.7%–76.8% of dose rats, mice and rabbits respectively) was positively identified as X12485473 following administration of [*pyridinyl-2*-$^{14}$C]florylpicoxamid or [*pyridinyl-2*-$^{14}$C] X12485649. The sum of the remaining metabolites in urine accounted for less than 5.2%, 4.7% and 4.4% of the dose in rats, mice and rabbits respectively. Label from [*phenyl-UL*-$^{14}$C]florylpicoxamid was found to be extensively metabolized to 11 metabolites. In urine samples, ten of these metabolites were observed in males and four in females samples. None of them represented more than 10% of the AD, except in one female sampling which contained a metabolite tentatively identified as a glucuronide of X12385631. Metabolite X12485649 was observed at 0.142% of AD in male rat urine.

Four radioactive peaks were detected in the $C_{max}$ blood extract profiles for rats administered [*pyridinyl-2*-$^{14}$C]florylpicoxamid or [*pyridinyl-2*-$^{14}$C] X12485649. Comparison of the two rat profiles revealed equivalent retention times for the four radioactive peaks at approximately 4.0, 43.0, 44.2 and 47.4 minutes. Comparison of the mouse and rat profiles demonstrated two radioactive peaks with identical retention times of 4.0 and 47.4 minutes. The rabbit profile showed two radioactive peaks with retention times of approximately 4.0 and 15.0 minutes, the latter of which was positively identified as X12485473. Metabolite X12485473 was subsequently quantified in all species by targeted analysis. The radioactive peak in rat blood with a retention time of ca 43.0 minutes corresponded to a tentatively identified metabolite with a molecular weight (MW) of 486. This MW was consistent with the structure of X12485649 (possibly isomer A) bearing an additional hydroxylation on the pyridine ring. The rat blood peak at 44.2 minutes corresponded to two co-eluting metabolites with MWs of 468 and 486 (possibly isomer B). The component with a MW of 468 was tentatively identified as a structure corresponding to X12485649 modified by a double bond resulting from hydroxylation and dehydration. The two possible isomers with a MW of 486 were consistent with a hydroxylated derivative of X12485649. The radioactive peak from $C_{max}$ blood samples from the rat and mouse at approximately 47.4 minutes was positively identified as X12485649, which was subsequently quantified by targeted analysis in the blood of rats and mice administered [*pyridinyl-2*-$^{14}$C]florylpicoxamid or [*pyridinyl-2*-$^{14}$C] X12485649. Florylpicoxamid was not detected above the LOD by radiochemical profiling. With the [*phenyl-UL*-$^{14}$C]florylpicoxamid, the metabolic profiles for the $C_{max}$ blood extracts provided three $^{14}$C peaks for males and four $^{14}$C peaks in the case of samples from females. Florylpicoxamid, X12485649 and X12584261 were identified as resulting from [*phenyl-UL*-$^{14}$C]florylpicoxamid.

In summary, florylpicoxamid was absorbed, highly metabolized and quickly eliminated. The overall, terminal (β) phase elimination half-lives in plasma and whole blood ranged from 22 hours to 47 hours across species, sexes and radiolabels. The pharmacokinetic parameters for [*pyridinyl-2*-$^{14}$C]florylpicoxamid and [*pyridinyl-2*-$^{14}$C] X12485649 in the rat were bio-equivalent. Recovery in urine after single oral gavage administration accounted for 18%–33% and 5%–25% in rats, for the pyridinyl- and phenyl-labelled test items respectively. Corresponding recoveries from urine were 19%–21% in mice (pyridinyl label), and 75%–77% in rabbits (pyridinyl label). In addition, dosing with [*pyridinyl-2*-$^{14}$C] X12485649 resulted in recovery of 21%–40% of the dose in rat urine, bio-equivalent to that for [*pyridinyl-2*-$^{14}$C]florylpicoxamid. Metabolite X12485649 was quantified in the blood of rats and mice and the urine of rats, and X12485473 concentrations were quantified in the blood of all species. Parent florylpicoxamid while observed in blood with phenyl labelling was not detected in urine with either phenyl or pyridinyl labelling. The overall metabolism was consistent with hydrolysis, hydroxylation, and glucuronide conjugation reactions. (Himmelstein, 2020)

Based on this study, absorption rates of florylpicoxamid in mice, rats and rabbits were considered to be approximately 21%, 34% and 77%, respectively. The absorption rate of X12485649 was approximately 40% in rats, which was similar to that of the parent.

The reasons for higher absorption rate in rabbits were not clarified. As the concentration curves

in rabbits for plasma and whole blood were flat up to 24 hours after dosing compared with those in rats or mice, one possibility suggested was that caecotrophy, a physiological strategy specialized in rabbits, allows nutrients to be gained via fermentation. This was speculated by Tulliez, Durand & Bories, (1982) and Rees Davies & Rees Davies, (2003), however, no information regarding this speculation was obtained.

The ADME of florylpicoxamid was studied in male and female rats. Pharmacokinetics for rats treated with a single dose of [*pyridinyl-2*-$^{14}$C]florylpicoxamid or [*phenyl-UL*-$^{14}$C]florylpicoxamid is summarized in Table 3. Absorption in plasma was rapid ($T_{max}$ 0.5–1.4 hours) in the low-dose groups. At the high dose, $T_{max}$ was higher than for the low-dose groups. Red blood cell pharmacokinetics were similar to those of plasma, with the exception of a higher overall β-phase half-life. Values of $C_{max}$ and area under the concentration–time curve (AUC) indicated saturation of absorption. The overall pharmacokinetics were similar for the pyridinyl and phenyl radiolabels. (Himmelstein, 2019a).

***Table 3. Plasma and blood pharmacokinetic parameters for rats given a single dose of $^{14}$C-labelled florylpicoxamid***

| Parameter | [*Pyridinyl-2*-$^{14}$C] florylpicoxamid | | | | [*Phenyl-UL*-$^{14}$C] florylpicoxamid | |
|---|---|---|---|---|---|---|
| | 25 mg/kg bw | | 250 mg/kg bw | | 25 mg/kg bw | |
| | Males | Females | Males | Females | Males | Females |
| **Plasma** | | | | | | |
| $T_{max}$ (hours) | 1.125 | 0.500 | 4.500 | 0.750 | 0.875 | 1.375 |
| $C_{max}$ (µg/g) | 4.350 | 3.885 | 9.357 | 10.097 | 3.556 | 2.940 |
| Absorption $t_{1/2}$ (hours) | 0.213 | 0.292 | 0.462 | 0.208 | 0.190 | 0.217 |
| Elimination $t_{1/2\alpha}$ (hours) | 2.591 | 2.249 | 9.402 | 6.873 | 2.400 | 2.962 |
| Elimination $t_{1/2\beta}$ (hours) | 31.824 | 30.647 | 35.286 | 22.015 | 36.166 | 30.530 |
| $AUC_{0-t}$ (µg·hour/g) | 32.212 | 16.703 | 147.944 | 98.075 | 35.483 | 22.252 |
| **Red blood cells** | | | | | | |
| $T_{max}$ (hours) | 0.625 | 0.500 | 6.500 | 3.500 | 1.625 | 1.250 |
| $C_{max}$ (µg/g) | 0.702 | 0.538 | 2.469 | 2.234 | 0.747 | 0.456 |
| Absorption $t_{1/2}$ (hours) | 0.164 | 0.157 | 1.738 | 1.315 | 0.286 | 0.238 |
| Elimination $t_{1/2\alpha}$ (hours) | 4.032 | 1.301 | 6.533 | 4.184 | 10.365 | 5.941 |
| Elimination $t_{1/2\beta}$ (hours) | 166.361 | 13.564 | 132.838 | 25.753 | 87.404 | 63.744 |
| $AUC_{0-t}$ (µg·hour/g) | 13.823 | 10.558 | 76.386 | 32.206 | 20.898 | 7.505 |

$C_{max}$: Maximum concentration achieved; $T_{max}$: Time to $C_{max}$; $t_{1/2}$: Half-life Source: Himmelstein, 2019a

AUC: Area under the concentration–time curve

The Meeting noted that the toxicokinetic parameters of florylpicoxamid in SD rats were qualitatively and quantitatively similar to those in F344 rats (Himmelstein, 2020). The data on tissue levels at around the $T_{max}$ were obtained from results for the SD strain only.

All mean tissue concentrations were equal to 1.21 µg equiv./g or greater at 168 hours after dose administration. Tissue plasma ratios indicated a very low potential for accumulation. The highest tissue plasma ratios were generally observed in tissues such as the gastrointestinal (GI) tract and liver. At the high dose, tissue/organ concentrations were higher than at the low dose, and tissue concentrations did not scale proportionately to the administered dose. However, there were no major differences in the pattern of tissue distribution between doses. Female rats tended to have lower tissue $^{14}$C residue levels retained at 168 hours than male rats (see Table 4) (Himmelstein, 2019a).

***Table 4. Mean tissue concentration of radioactivity 168 hours after oral administration of $^{14}$C-labelled florylpicoxamid (µg equiv./g)***

| | Dose [*Pyridinyl-2-*$^{14}$C] florylpicoxamid | | | | | | Dose [*Phenyl-UL-*$^{14}$C] florylpicoxamid | |
|---|---|---|---|---|---|---|---|---|
| | 25 mg/kg bw | | 25 mg/kg bw[a] | | 250 mg/kg bw | | 25 mg/kg bw | |
| **Tissue** | **Males** | **Females** | **Males** | **Females** | **Males** | **Females** | **Males** | **Females** |
| Plasma | 0.009188 | NE | 0.0108 | NE | 0.0605 | NE | 0.0200 | 0.00545 |
| RBCs | 0.0460 | NA | 0.0548 | NA | 0.197 | NA | 0.0339 | 0.0120 |
| Blood | 0.0244 | 0.00757 | 0.0256 | NA | 0.115 | NA | 0.0292 | 0.0106 |
| Adrenals | 0.0167 | NA | 0.0179 | NA | 0.101 | NA | 0.0472 | 0.0247 |
| Bone | NE | NE | NE | NE | NE | NE | NE | NE |
| Bone marrow | NE | NE | NE | NE | NE | NE | 0.0177 | NE |
| Brain | NE | NE | 0.00827 | NE | NE | NE | 0.0109 | NE |
| Lymph nodes | 0.00927 | NE | 0.0109 | NE | 0.0470 | NE | 0.0498 | 0.0303 |
| Uterus | NA | 0.00156 | NA | 0.00247 | NA | 0.0113 | NA | 0.00488 |
| Carcass | 0.00910 | NE | 0.0578 | NE | NE | NE | 0.0218 | 0.0140 |
| Fat | 0.0165 | NE | 0.0118 | 0.00810 | 0.0949 | 0.0499 | 0.201 | 0.177 |
| GI tract | 0.0708 | 0.00625 | 0.0214 | 0.00960 | 0.282 | 0.363 | 1.21 | 0.131 |
| Heart | 0.0100 | 0.00190 | 0.0112 | 0.00235 | 0.0477 | 0.0141 | 0.0155 | 0.00583 |
| Kidney | 0.0690 | 0.0162 | 0.0638 | NE | 0.296 | 0.105 | 0.0693 | 0.0143 |
| Liver | 0.223 | 0.0170 | 0.236 | 0.0193 | 0.782 | 0.140 | 0.235 | 0.0337 |
| Lungs | 0.0108 | 0.00238 | 0.0131 | 0.00322 | 0.0484 | 0.0208 | 0.0282 | 0.0119 |
| Muscle | 0.00821 | NE | 0.00705 | NE | NE | NE | NE | NE |
| Ovaries | NA | NA | NA | 0.00580 | NA | NA | NA | 0.0174 |
| Pancreas | 0.00827 | 0.00138 | 0.00934 | 0.00218 | 0.0391 | 0.0139 | 0.0405 | 0.0175 |
| Pituitary | NE | NE | NE | NE | NE | NE | NE | NE |
| Skin | 0.0121 | NE | 0.0223 | 0.00868 | 0.0555 | 0.0895 | 0.0425 | 0.0209 |
| Spleen | 0.00961 | 0.00128 | 0.0161 | 0.00325 | 0.0368 | 0.0115 | 0.0121 | 0.00400 |
| Testes | 0.00758 | NA | 0.00762 | NA | 0.131 | NA | 0.0107 | NA |
| Thymus | 0.00593 | NE | 0.00652 | 0.00195 | 0.0285 | 0.0103 | 0.0151 | 0.00499 |
| Thyroid | NE | NE | 0.0212 | NE | NE | NE | 0.0249 | NE |
| Bladder | 0.00940 | 0.0146 | 0.0128 | 0.00534 | 0.0606 | NE | 0.0257 | 0.0199 |

NA: Not Applicable; NE: Not examined; GI: Gastrointestinal; Source: Himmelstein, 2019a

[a] Fourteen daily doses of unlabelled florylpicoxamid followed by a single oral dose of [*pyridinyl-2-*$^{14}$C] florylpicoxamid

In an excretion assay performed with 25 and 250 mg/kg bw of [*pyridinyl-2-*$^{14}$C]florylpicoxamid and [*Phenyl-UL-*$^{14}$C] florylpicoxamid for excretion/mass balance the overall mean material balance across all dose groups ranged from 85.3% to 96.3% of AD. The majority of the administered radioactivity was recovered in the faeces. By 168 hours after dosing, across the low-, high-, and repeat-dose groups (with pyridinyl label), the mean percentages of AD recovered in faeces ranged from 73.3% to 77.6% for males and from 52.2% to 58.8% for females. The mean recoveries for rats receiving phenyl ring-labelled florylpicoxamid were 85.2% and 80.1% for male and female rats respectively. Recovery in urine ranged from 9.80% to 19.2% for males and from 17.5% to 40.4% for females given the pyridinyl-labelled florylpicoxamid; comparable values for the phenyl-labelled compound were 7.14% and 12.4% in male and female rats respectively. In exhaled breath (carbon dioxide traps) the mean recoveries for low doses (25 mg/kg bw) of the pyridine labelled compound were 0.185% and 0.0318% in male and female rats respectively. (Himmelstein, 2019a)

Biliary elimination and tissue distribution excretion assays were conducted in rats. In the

biliary elimination experiment where bile duct-cannulated rats were treated with 25 or 250 mg/kg bw of [*pyridinyl-2*-$^{14}$C]florylpicoxamid or [*phenyl-UL*-$^{14}$C] florylpicoxamid, the mean values obtained ranged from 79.3% to 93.1% of AD recovered across sexes, doses and radiolabels. The majority of the recovered dose was associated with the faeces. At 48 hours after oral administration of pyridinyl- or phenyl-labelled florylpicoxamid at doses of 25 or 250 mg/kg bw, 1.46%–9.47% of AD was found from radioactivity in the urine, 4.35%–21.3% of AD in bile, and 56.0%–76.0% of AD in the faeces. Maximum absorption under conditions of the study occurred at the low dose and this was similar across sexes and radiolabels; mean values ranged from 19.3% to 23.8% of AD. Saturation of absorption occurred with a two-fold decline in absorption at the high dose. Tissue distribution was investigated following a single administration ($T_{max}$ and 24 hours) of radiolabelled florylpicoxamid at 25 or 250 mg/kg bw. The highest mean tissue concentrations, with the exception of the GI tract tissue, were 41.0 and 22.4 µg equiv./g of bladder tissue in male and female rats respectively. These values were observed at $T_{max}$ after high-dose administration of [*pyridinyl-2*-$^{14}$C]florylpicoxamid. Other tissues from male rats given the high dose that exhibited concentrations in the range of approximately 10–40 µg equiv./g tissue were adrenals, bladder, kidney, liver and pituitary. Although a similar pattern was observed in female rats at the high dose, the concentrations were generally lower than those observed in males. After low doses of either radiolabelled florylpicoxamid, maximum tissue concentrations ranged from approximately 0.1 to 10 µg equiv./g (Himmelstein, 2019b).

**(b) Dermal route**

The dermal penetration and absorption of [*pyridinyl-2*-$^{14}$C]florylpicoxamid (radiochemical purity 99.1%, Lot XZ7-170335-38B) formulated as GF-3840 was measured in vitro through human skin mounted in a flow-through diffusion cell. The results showed absorption of the 100 g/L concentrate (given as "EC"; probably "emulsifiable concentrate") to be 0.23%, while absorption from the spray dilutions of this concentrate was 7%. These data indicate a low level of concern regarding dermal absorption. (Whitfield, 2019)

## 1.2 Biotransformation

**(a) In vitro studies**

Two in vitro studies were conducted to compare species differences in the metabolism of florylpicoxamid.

The in vitro comparative metabolism of radiolabelled florylpicoxamid was investigated in human, rat, mouse, dog and rabbit hepatocytes. Florylpicoxamid was extensively metabolized during hepatocyte incubation by hepatocytes of all five test species. The amount of florylpicoxamid remaining at the 120-minute incubation time point across both labels in humans (3.88%–12.5%) was similar to the other species (2.39%–12.2%). Across time points and labels, five major metabolites (X12485473, X12641325, X12485631, X12584261 and X12485649) were quantified in the human hepatocyte samples at above 5% of the applied radioactive dose. All five of these metabolites were also quantified in the rat, mouse and rabbit experiments. Of the five major human metabolites, three (X12485473, X12584261 and X12485649) were quantified in the dog while two were absent (X12641325 and X12485631) by $^{14}$C detection. A full scan liquid chromatography–mass spectrometry (LC/MS) signal indicating X12641325 was seen in the dog experiment, consistent with rapid biotransformation of X12485631 to the *O*-glucuronide conjugate X12641325. Overall, no unique human metabolites were identified in the hepatocyte samples when compared to the rat, mouse, dog and rabbit hepatocyte incubations (Chan, 2020a). In this study, chiral analysis was conducted to investigate possible interconversion of stereoisomeric ratios of florylpicoxamid and its stereoisomers (X12519017, X12518891 and X12519015), and between the deacetylated florylpicoxamid metabolite X12485649 and its stereoisomers (X12504939, X12506173 and X12506171) in diluted dose stock solution, dosed incubation buffer, and in rat, rabbit, dog, mouse and human hepatocytes incubated with $^{14}$C-labelled florylpicoxamid. Dosed incubation buffer, diluted dose stock samples and hepatocytes from five species (human, rat, mouse, dog and rabbit) were found to contain only 100.00% florylpicoxamid or X12485649. The results from this study indicated that no interconversion of stereoisomers was occurring with hepatocytes from any of the species tested (Chan, 2020a).

In vitro metabolism of $^{14}$C-labelled florylpicoxamid was investigated using liver microsomes from mice, rats, rabbits, dogs and human donors. Eleven radiochemical peaks were detected in the incubations containing $^{14}$C-labelled florylpicoxamid, cofactors and microsomes from all species. Most metabolites formed in human liver microsomes, including all metabolites that represented more than 5% of the applied dose in the incubations, were also formed in rat, mouse and rabbit liver microsomes. Fewer metabolites were formed in dog liver microsomal incubations. The five major peaks found in incubations (microsomes with florylpicoxamid and cofactors) representing more than 5% of the applied dose, were identified as:

- X12485473 formed by deacetylation and esterase hydrolysis (accounting for an average of 3.34%–65.4% of the applied dose),
- a metabolite formed by deacetylation, two CYP hydroxylations, and one dehydration (accounting for an average of 10.8%–23.9% of the applied dose),
- a metabolite formed by deacetylation and one hydroxylation on the pyridine ring (accounting for an average of 2.19%–7.19% of the applied dose),
- a metabolite formed by deacetylation and aliphatic CYP hydroxylation followed by dehydration (accounting for an average of 14.4–64.4% of the applied dose), and
- metabolite X12485649, formed by deacetylation (accounting for an average of 2.15%–6.01% of the applied dose).

Metabolites of X12485473 and X12485649 are consistent with metabolic fate of florylpicoxamid observed in previous in vivo metabolism studies in rats.

Overall, florylpicoxamid was highly metabolized in liver microsomes from mouse, rat, rabbit, dog and from human donors. No unique metabolites were formed by human microsomal incubations that were not also seen with mouse, rat, rabbit and dog liver microsomes (Zhang, McClymont & Erskine, 2018).

**(b) In vivo studies**

In the pharmacokinetics and metabolism study, the metabolite profile of $^{14}$C-labelled florylpicoxamid was qualitatively similar across sexes and doses, and bridge cleavage metabolites were observed resulting in different metabolites from the two radiolabels. The metabolite profiles of excreta from rats dosed with florylpicoxamid $^{14}$C-labelled in the pyridinyl and phenyl moieties are shown in Table 5. Florylpicoxamid and the 11 identified metabolites in faeces accounted for 42.9%–67.6% of the dose, whilst urine accounted for 4.0%–38.9% of the dose, this across both radiolabels and all dose groups. For urine, two cleaved metabolites (X12485473, and to a lesser extent X12641325) were present at equal or greater than the 10% of absorbed dose, which was considered to be ca 34% (Himmelstein, 2020). Most of the significant metabolites present at equal or greater than 10% of the absorbed dose at low dose, were observed in faeces, namely: X12485473, X12493055, X12632407, X12485631 and X12485649.

In this study chiral analysis was conducted on selected faecal samples for known enantiomers of parent florylpicoxamid and its proximal deacetylated metabolite X12485649. The chiral analysis of florylpicoxamid and X12485649 in faeces indicated no shift in stereoisomers (Table 6; Himmelstein, 2019a).

***Table 5. Metabolite profile in urine and faeces of rats dosed with $^{14}$C-labelled florylpicoxamid (results in bold indicate ≥10% absorbed dose at low dose in urine)***

| | Percentage of administered dose | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | [*Pyridinyl-2-*$^{14}$C] florylpicoxamid | | | | | | [*Phenyl-UL-*$^{14}$C] florylpicoxamid | |
| | 25 mg/kg bw | | 25 mg/kg bw[a] | | 250 mg/kg bw | | 25 mg/kg bw | |
| | Males | Females | Males | Females | Males | Females | Males | Females |
| **Urine** | | | | | | | | |
| Florylpicoxamid | no | no | no | no | < 0.1 | < 0.1 | no | no |
| X696476 | 1.9 | 0.9 | 2.1 | 0.9 | 0.9 | 0.6 | NA | NA |
| X12485473 | **7.6** | **22.1** | **10.1** | **38.0** | 5.5 | 15.5 | NA | NA |
| X12714411 | NA | NA | NA | NA | NA | NA | 1.1 | 0.4 |
| X12641325 | NA | NA | NA | NA | NA | NA | 2.4 | **8.4** |
| X12493055 | NA | NA | NA | NA | NA | NA | 0.1 | 0.1 |
| X12632407 | no | no | no | no | no | no | 0.2 | 0.4 |
| X12641685 | no | no | no | no | < 0.1 | < 0.1 | < 0.1 | no |
| X12485631 | NA | NA | NA | NA | NA | NA | 0.1 | 0.3 |
| X12584261 | no | no | no | no | no | no | no | no |
| X12629973 | no | no | no | no | no | no | no | < 0.1 |
| X12485649 | no | no | no | < 0.1 | < 0.1 | 0.1 | no | < 0.1 |
| **Total identified** | 9.5 | 22.9 | 12.2 | 38.9 | 6.5 | 16.3 | 4.0 | 9.6 |
| Unknown 1 | 1.6 | 0.2 | 2.5 | 0.1 | 0.9 | < 0.1 | NA | NA |
| Unknown 2 | 1.8 | 0.4 | 2.3 | 0.2 | 0.9 | 0.2 | NA | NA |
| Unknown 3 | 0.6 | 0.2 | 0.5 | no | 0.3 | 0.1 | NA | NA |
| **Total** | 14.6 | 24.0 | 18.8 | 39.4 | 9.5 | 17.3 | 5.1 | 10.8 |
| **Faeces** | | | | | | | | |
| Florylpicoxamid | 1.3 | 0.8 | 1.0 | no | 31.2 | 19.8 | 0.9 | 1.3 |
| X696476 | 2.3 | 2.5 | 1.7 | 3.7 | 3.9 | 0.7 | NA | NA |
| X12485473 | 14.7 | 18.0 | 20.5 | 24.3 | 2.3 | 12.6 | NA | NA |
| X12714411 | NA | NA | NA | NA | NA | NA | 0.6 | 0.4 |
| X12641325 | NA | NA | NA | NA | NA | NA | 0.1 | 0.1 |
| X12493055 | NA | NA | NA | NA | NA | NA | 5.0 | 1.4 |
| X12632407 | 8.3 | 1.8 | 5.7 | 0.3 | 3.2 | 0.2 | 6.0 | 1.3 |
| X12641685 | 2.2 | < 0.1 | 2.5 | 0.8 | 0.6 | no | 1.5 | 0.5 |
| X12485631 | NA | NA | NA | NA | NA | NA | 21.0 | 43.5 |
| X12584261 | 1.9 | 0.9 | 1.6 | 0.5 | 0.7 | 0.5 | no | no |
| X12629973 | 2.9 | 1.7 | 2.1 | 1.3 | 0.8 | 0.6 | 2.6 | 2.0 |
| X12485649 | 15.7 | 19.7 | 16.3 | 12.0 | 24.9 | 20.1 | 21.9 | 15.9 |
| **Total identified** | 49.4 | 45.6 | 51.5 | 42.9 | 67.6 | 54.5 | 59.6 | 66.5 |
| Unknown 1 | 0.7 | no | 0.6 | < 0.1 | 0.2 | no | NA | NA |
| Unknown 2 | 0.4 | no | no | no | no | no | NA | NA |
| Unknown 3 | no | no | no | no | no | no | NA | NA |
| **Total** | 71.7 | 55.2 | 72.6 | 51.7 | 75.7 | 58.1 | 77.6 | 74.2 |

no: Not observed; NA: Not applicable to the radiolabelled test substance Source: Himmelstein, 2019a

[a] Fourteen daily doses of unlabelled florylpicoxamid followed by a single oral dose of [*pyridinyl-2-*$^{14}$C]florylpicoxamid;

*Table 6. Stereoisomers of $^{14}$C-labelled florylpicoxamid and $^{14}$C-labelled X12485649 in faeces*

| | Stereoisomers of florylpicoxamid (percentage of total MS area response) | |
|---|---|---|
| | [*pyridinyl-2-*$^{14}$C] florylpicoxamid | |
| | 250 mg/kg bw | |
| | Males | Females |
| Florylpicoxamid | 97.72 | 97.81 |
| X12519017 | 2.27 | 2.16 |
| X12518891 | 0.01 | 0.03 |
| X12519015 | ND | ND |

| | Stereoisomers of X12485649 (percentage of total MS area response) | | | | |
|---|---|---|---|---|---|
| | [*pyridinyl-2-*$^{14}$C] florylpicoxamid | | | [*phenyl-UL-*$^{14}$C]florylpicoxamid | |
| | 25 mg/kg bw | 25 mg/kg bw [a] | | 25 mg/kg bw | |
| | Males | Males | Females | Males | Females |
| X12485649 | 99.34 | 99.33 | 99.24 | 98.87 | 98.53 |
| X12506173 | 0.01 | ND | ND | 0.17 | 0.19 |
| X12504939 | 0.66 | 0.67 | 0.76 | 0.96 | 1.23 |
| X12506171 | ND | ND | ND | ND | ND |

ND: Not detected; MS: Mass spectroscopy/spectrometry Source: Himmelstein, 2019a

[a] Fourteen daily doses of unlabelled florylpicoxamid followed by a single oral dose of [*phenyl-UL-*$^{14}$C]florylpicoxamid

In the study of biliary elimination and tissue distribution in rats, metabolism was investigated. Metabolite profiles in bile (with or without glucuronidase) and plasma are summarized in Table 7. A total of 12 components were identified and quantified in rat bile without glucuronidase incubation. Florylpicoxamid was not detected. The two major metabolites identified at 10% or more of the absorbed dose in bile (without glucuronidase incubation) were [M+H]-647 and X12641325 (Table 7). In these calculations the absorption rate was considered to be ca 34% (Himmelstein, 2020). A total of eight components were identified and quantified in rat bile after glucuronidase incubation. The three metabolites identified at 10% or more of the absorbed dose in bile with glucuronidase incubation were X12485473, X12485631 and X12485649. A total of 12 components were identified and quantified in rat plasma. Florylpicoxamid was not detected. Two metabolites were found to be present individually at greater than 1 µg equiv./g in plasma; these were X12485473 and [M+H]-473 (Table 8).

Chiral analysis was conducted to investigate possible interconversion of florylpicoxamid and its stereoisomers (X12519017, X12518891 and X12519015) and the florylpicoxamid deacetylated metabolite X12485649 and its stereoisomers (X12504939, X12506173 and X12506171) in dose preparations, and pooled samples of bile, plasma, liver and fat of rats administered $^{14}$C-labelled florylpicoxamid. Results were compared with those previously obtained from faeces samples. In summary, florylpicoxamid isomer ratios observed in faeces, liver and fat were mostly retained as florylpicoxamid. Overall, the chiral analyses showed detectable percentage ratios of X12504939 at 0.76%–8.36%, 5.75%–30.07%, 1.06%–21.91% and 1.01%–5.28% in bile, plasma, liver, and fat samples respectively. These percentages were increased above ratios for X12504939 found in the dose preparations (0.39%–0.74%) or faeces (0.66%–1.23%). The apparent enrichment of this minor isomer was most likely due to stereoselective metabolism of X12485649 to downstream metabolites, which had occurred more rapidly than the corresponding metabolism for X12504939.

*Table 7. Metabolite profile in the bile of rats dosed with $^{14}$C-labelled florylpicoxamid (bold indicates that 10% or more of the absorbed dose was found in bile)*

| | Percentage of administered dose | | | | | |
|---|---|---|---|---|---|---|
| | [*pyridinyl-2-*$^{14}$C]Florylpicoxamid | | | | [*bis-phenyl-UL-*$^{14}$C] Florylpicoxamid | |
| | 25 mg/kg bw | | 250 mg/kg bw | | 25 mg/kg bw | |
| | Males | Females | Males | Females | Males | Females |
| **Without glucuronidase** | | | | | | |
| **Metabolite** | | | | | | |
| X12485473 | 1.73 | 1.73 | 0.416 | 0.558 | NA | NA |
| [M+H]-663-a | 0.412 | 0.264 | 0.136 | 0.0931 | 0.419 | 0.288 |
| [M+H]-439 | NA | NA | NA | NA | 0.559 | 0.272 |
| [M+H]-663-b | 0.464 | 0.422 | 0.130 | 0.155 | 0.333 | 0.383 |
| [M+H]-647 | **5.46** | **5.60** | 1.84 | 2.43 | **4.22** | **4.57** |
| [M+H]-633-a | 0.260 | 0.436 | 0.191 | 0.205 | 0.276 | 0.247 |
| X12641325 | NA | NA | NA | NA | **9.26** | **10.7** |
| [M+H]-649 | 1.33 | 0.568 | 0.653 | 0.147 | 0.365 | 0.720 |
| [M+H]-679 | 0.327 | ND | 0.0545 | ND | 0.389 | ND |
| [M+H]-663-b | 1.64 | 1.62 | 0.675 | 0.678 | 1.52 | 1.11 |
| [M+H]-663-c | 0.199 | 0.228 | ND | ND | 0.316 | 0.151 |
| X12485649 | 0.205 | 0.110 | 0.0905 | 0.0658 | 0.0974 | 0.167 |
| **With glucuronidase** | | | | | | |
| **Metabolite** | | | | | | |
| X12485473 | **4.33** | **4.46** | 0.624 | 1.70 | NA | NA |
| [M+H]-647 | 0.708 | 1.11 | ND | ND | ND | 0.614 |
| X12632407 | 0.361 | ND | 0.153 | ND | 0.628 | ND |
| X12493055 | NA | NA | NA | NA | 1.11 | 0.459 |
| [M+H]-487-a | 1.11 | 0.695 | 0.215 | ND | 1.32 | 0.683 |
| X12485631 | NA | NA | NA | NA | **9.90** | **12.4** |
| X12584261 | 0.354 | ND | 0.264 | ND | 0.313 | ND |
| X12485649 | **5.33** | **4.83** | 2.93 | 2.63 | **4.17** | **4.47** |

ND: Not detected/identified; NA: Not applicable to the radiolabelled test substance; Source: Himmelstein, 2019b

*Table 8. Metabolite concentrations in plasma of rats dosed with $^{14}$C-labelled florylpicoxamid*

| | Metabolite concentration (µg equiv./g) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | [*pyridinyl-2*-$^{14}$C]Florylpicoxamid | | | | [*bis-phenyl-UL*-$^{14}$C]Florylpicoxamid | | | |
| | 25 mg/kg bw | | 250 mg/kg bw | | 25 mg/kg bw | | 25 mg/kg bw | |
| | Males[a] | Females[b] | Males[c] | Females[d] | Males[a] | Females[a] | Males[e] | Females[e] |
| **Metabolite** | | | | | | | | |
| X12485473 | 0.253 | 0.653 | 0.685 | 1.52 | NA | NA | NA | NA |
| [M+H]-336 | NA | NA | NA | NA | ND | ND | 0.0171 | ND |
| [M+H]-439 | NA | NA | NA | NA | ND | ND | 0.0426 | ND |
| X12641325 | NA | NA | NA | NA | 0.198 | 0.118 | 0.136 | 0.0955 |
| [M+H]-649 | 0.0771 | ND | ND | ND | NA | NA | NA | NA |
| [M+H]-473 | 0.218 | ND | 1.47 | ND | 0.245 | ND | ND | ND |
| X12493055 | NA | NA | NA | NA | ND | ND | 0.0401 | 0.0195 |
| [M+H]-487-a | ND | 0.0523 | 0.411 | ND | ND | ND | 0.0162 | ND |
| [M+H]-503-a,b | 0.349 | ND | ND | ND | 0.190 | 0.141 | ND | ND |
| X12485631 | NA | NA | NA | NA | 0.109 | 0.237 | 0.0333 | 0.102 |
| X12584261 | 0.214 | 0.0417 | 0.599 | ND | 0.154 | 0.0286 | 0.0153 | ND |
| X12485649 | 0.116 | 0.0895 | ND | 0.561 | 0.0646 | 0.0931 | ND | ND |

ND: Not detected/identified; NA: Not applicable to the radiolabelled test substance; Source: Himmelstein, 2019b

[a] Sample was collected one hour post dose;

[b] Sample was collected 0.5 hour post dose;

[c] Sample was collected 4.5 hour post dose;

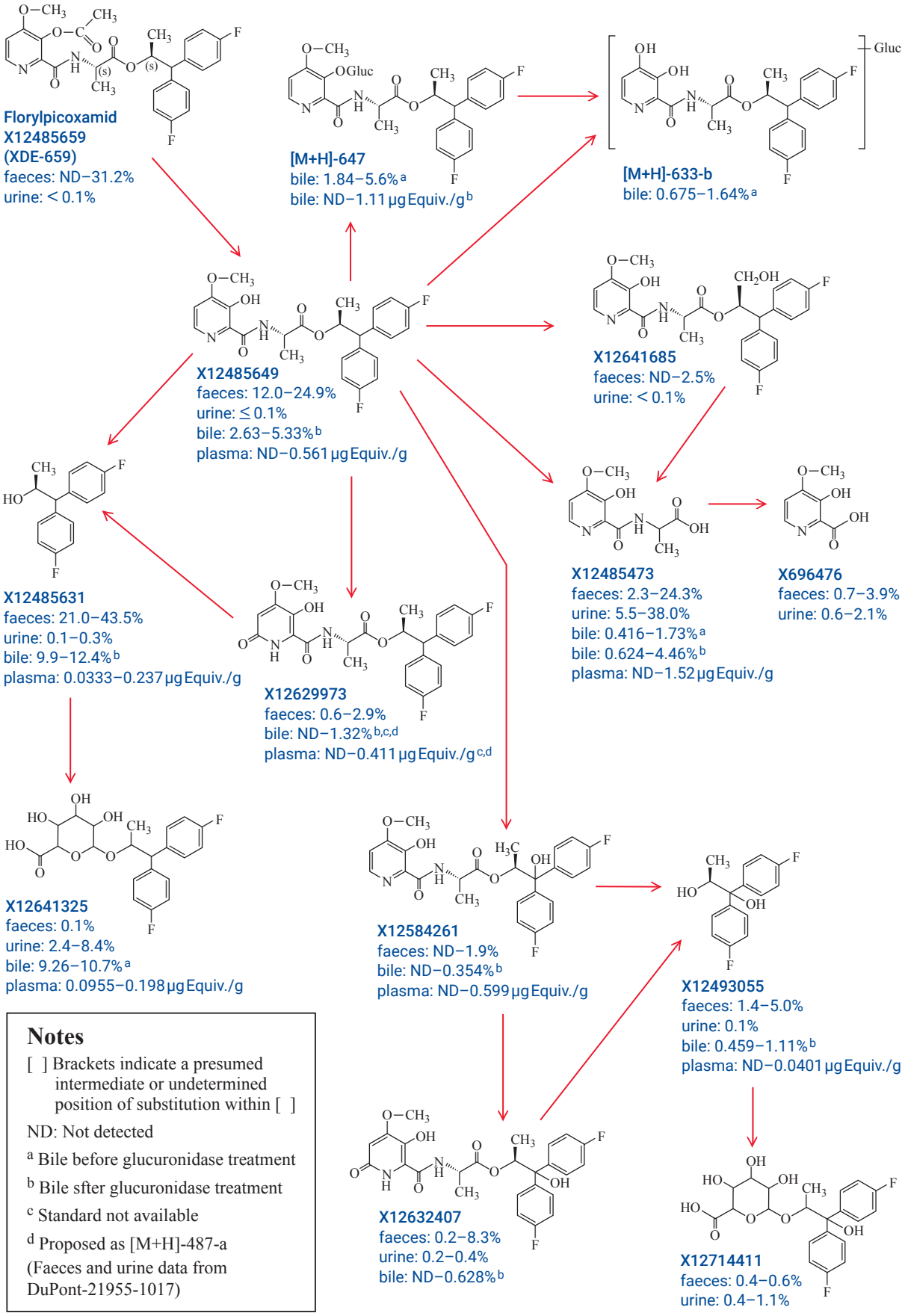
[d] Sample was collected 0.75 hour post dose;

[e] Sample was collected 24 hours post dose

In conclusion, the key initial metabolic step in the metabolism of florylpicoxamid involved deacetylation to form X12485649. Subsequent reactions involved aromatic oxidation, aliphatic and aromatic hydroxylations, ester bridge formation or amide cleavages, *O*-demethylation, and phase II glucuronide conjugation. Stereoselective metabolism of X12485649 was evident based on increased ratios of X12504939 in bile, plasma, liver and fat relative to faeces and dose preparations. Metabolites identified in bile and plasma were generally consistent with those previously identified in faeces and urine, and confirm the role of first-pass hepatic metabolism contributing to biliary elimination and systemic uptake as key processes in the extensive metabolism and elimination of florylpicoxamid. (Himmelstein, 2019b)

The proposed metabolic pathway for florylpicoxamid in rats is shown in Fig. 9.

*Figure 2. Proposed metabolic pathway for florylpicoxamid in rats*




**Notes**

[ ] Brackets indicate a presumed intermediate or undetermined position of substitution within [ ]

ND: Not detected

[a] Bile before glucuronidase treatment

[b] Bile sfter glucuronidase treatment

[c] Standard not available

[d] Proposed as [M+H]-487-a

(Faeces and urine data from DuPont-21955-1017)

Redrawn from Himmelstein, 2019b

## 1.3 Effects on enzymes and other biochemical parameters

Information on toxicokinetics from each feeding study is summarized below and in Table 9. More detailed information is provided in Annex 2.

### (a) Toxicokinetic analysis of florylpicoxamid and metabolites in feeding studies on 90-day toxicity, reproductive and development toxicity in mice, rats and rabbits

In addition to the required regulatory ADME studies on florylpicoxamid using radiolabelled test materials, toxicokinetic (TK) analyses were integrated into the toxicology testing program for florylpicoxamid. This integrated approach allowed the generation of critical TK data without using additional animals. It also allowed a comprehensive assessment of systemic toxicity and overall interpretation of the studies. Blood and urine samples were analyzed in all general toxicity studies from all the species tested. Blood samples were taken and analyzed from rat and rabbit fetuses and rat neonates in reproductive and developmental toxicity studies. Milk samples from the rat reproductive toxicity studies were also analyzed. Toxicokinetic samples from the general toxicity studies (blood, urine) were analyzed for florylpicoxamid, as well as metabolites X12485649, X12485473, X12584261 and X12530093. Toxicokinetic samples from the developmental and reproductive toxicity studies (blood and milk) were analyzed for florylpicoxamid, as well as metabolites X12485649, X12485473, X12584261, X12641325 and X12530093. Toxicokinetic data were used to estimate systemic exposure (diurnal area-under-the-curve, $AUC_{24}$) and to enable TK comparisons across doses, sexes, duration of exposure, species and life stages at steady-state exposure levels. Studies that investigated TK, the study parameters for each study and their authors are all shown in Table 9.

***Table 9. Details of toxicity studies involving toxicokinetic analysis***

| Study | Species Life stage | Dose (ppm) | Sample type | Time points | Toxicokinetic parameter | Analytes |
|---|---|---|---|---|---|---|
| Development probe study (Passage, 2020) | Rats Adult, fetus | 0, 3000 | Blood | GD 20: 07:00, 09:00, 13:00 | AUC, AUC/TMI | A |
| Development toxicity study (Passage, 2018a) | Rats Adult, pup | 0, 300, 1000 or 4000 | Blood | GD 21: terminal | Blood concentration | A |
| Palatability probe study (Passage, 2018b) | Rabbits Adult | 0, 350, 750 or 1250 | Blood | GD 27: 10:00, 13:00, 15:00 | AUC, AUC/TMI | A |
| Developmental toxicity study (Passage, 2018c) | Rabbits Adult, fetus | 0, 125, 250 or 750 | Blood | GD 27: 10:00, 13:00, 15:00 | AUC, AUC/TMI | A |
| Reproductive/ Developmental toxicity study (Passage, 2018d) | Rats Adult, pup | 0, 300, 1000 or 3000 | Blood | Male/female: TD 14: 09:00, 12:00, 14:00 | AUC, AUC/TMI | A |
| | | | Milk | LD 4 | Milk : Blood concentration ratio | A |
| 90-day toxicity study (Mukerji, 2019) | Rats Adult | 0, 300, 1000 or 3000 | Blood | Male/Female: TD 90: 09:00, 12:00, 14:00 | AUC, AUC/TMI, $t_{1/2}$ | B |
| | | | Urine | TD 85 for 24 hours | Mole % of dietary intake from total excretion in urine | B |

| Study | Species Life stage | Dose (ppm) | Sample type | Time points | Toxicokinetic parameter | Analytes |
|---|---|---|---|---|---|---|
| 90-day toxicity study (Mukerji, 2018) | Mice Adult | 0, 150, 500 or 1500 | Blood | TD 90: 08:00, 11:00, 13:00 | AUC, AUC/TMI, $t_{1/2}$ | B |
| | | | Urine | Spot urine: TD 91 (male) and TD 92 (female) | Urine concentration | B |
| Reproductive toxicity study (Passage, 2019) | Rats Adult, pup | 0, 300, 1000, 3000 or 4000 | Blood | TD 70: 07:00. 09:00, 13:00 | AUC, AUC/TMI | A |
| | | | Milk | LD 4 | Milk : Blood concentration ratio | A |

GD: Gestation day; TD: Treatment day; LD: Lactation day; Source: Chan, 2019

AUC: Area under the concentration–time curve; TMI: Test material intake;

Analyte A, florylpicoxamid, X12485649, X12485473, X12584261, X12641325 and X12530093;

Analyte B, florylpicoxamid, X12485649, X12485473, X12584261 and X12530093.

***Developmental toxicity probe study in female Crl: CD(SD) rats***

This TK evaluation of florylpicoxamid associated with continuous daily dietary feeding indicated rapid absorption, hydrolysis of the parent florylpicoxamid to X12485649, and further metabolism to X12485473, X12584261 and X12641325. Quantitation of X12485473 and X12641325 in blood confirmed these were prominent metabolites in rats and fetuses exposed to florylpicoxamid, followed by X12485649 and X12584261. The analysis of rat blood and fetus blood at terminal sacrifice indicated minimal maternal transfer and systemic exposure of fetuses to X12485649, X12485473 and X12641325.

***Dietary developmental toxicity study in Crl:CD(SD) rats***

The quantitation of X12485473 and X12641325 in blood confirmed that these are prominent metabolites in rats and pups exposed to florylpicoxamid, followed in importance by X12485649 and X12584261. The results suggested maternal transfer of X12485649, X12485473, X12584261 and X12641325 to the pups through blood.

***Palatability probe study in NZW rabbit***

This TK evaluation of florylpicoxamid associated with continuous daily dietary feeding in New Zealand White rabbits indicated rapid absorption, hydrolysis of parent florylpicoxamid to X12485649 and further metabolism to X12485473, X12584261 and X12641325. Quantitation of X12485473 and X12641325 in blood confirmed these analytes are prominent metabolites in rabbits exposed to florylpicoxamid. Formation of these metabolites generally departed from dose-proportionality with increasing dietary concentration due to saturation of formation, as measured by dose-normalized $AUC_{24}$ values.

***Dietary developmental toxicity study in NZW rabbit***

The TK evaluation of florylpicoxamid associated with continuous daily dietary feeding, indicated rapid absorption, hydrolysis of parent florylpicoxamid to X12485649 and further metabolism to X12485473, X12584261 and X12641325. Quantitation of X12485473 and X12641325 in blood confirmed these analytes were prominent in rabbits exposed to florylpicoxamid. Formation of these metabolites departed from dose-proportionality with increasing dietary concentration due to saturation of formation, as measured by dose-normalized $AUC_{24}$ values. Blood analysis at terminal sacrifice indicated no apparent direct transfer of florylpicoxamid or metabolites (X12485649, X12584261 and X12530093) from mother to fetus via the blood. Systemic exposure to X12485473 and X12641325 was between 2- and 6-fold higher in adult rabbits than in fetuses.

***Dietary reproduction/developmental toxicity screening test in Crl: CD(SD) rats***

This toxicokinetic evaluation of florylpicoxamid associated with continuous daily dietary feeding indicates rapid absorption, hydrolysis of florylpicoxamid to X12485649 and further metabolism to X12485473, X12584261, and X12641325. Formation of these metabolites generally departed from dose-proportionality with increasing dietary concentration due to saturation of elimination and/or formation as measured by dose-normalized $AUC_{24}$ values during the premating phase. Post-natal analysis of dam blood and milk (LD4) and pup blood (PND4) indicated minimal lactational transfer and systemic exposure of pups to X12485473 and X12641325 at 1 to 2 orders of magnitude lower than observed in the dams.

***A 90-day dietary toxicity study with a 28-day recovery period in F344/DuCrl rats with an assessment of immunotoxicity, neurotoxicity, in vivo genetic toxicity and toxicokinetics***

The TK evaluation of florylpicoxamid associated with continuous daily dietary feeding in rats indicated a balance between absorption and metabolic clearance of the parent, and clearance of the resulting metabolites. Florylpicoxamid appears to undergo absorption and metabolism to X12485649, X12584261 and X12485473 based on measurements taken in blood and excreted in urine. Formation of these metabolites generally departed from dose-proportionality with increasing dietary concentration due to saturation of elimination and/or of formation as measured by dose-normalized $AUC_{24}$ values. Quantitation of X12485473 in blood and urine confirmed that it is a prominent metabolite in rats exposed to florylpicoxamid.

***A 90-day dietary toxicity study in Crl: CD1 (ICR) mice***

The TK evaluation of florylpicoxamid associated with continuous daily dietary feeding in mice indicated rapid absorption, hydrolysis of parent florylpicoxamid to X12485649 and further metabolism to X12485473, X12584261 and X12641325. Formation of these metabolites generally departed from dose-proportionality with increasing dietary concentration due to saturation of elimination and/or of formation, as measured by dose-normalized $AUC_{24}$ values. Quantitation of X12485473 in blood and urine confirmed X12485473 as a prominent metabolite in mice exposed to florylpicoxamid.

***Two-generation dietary reproduction toxicity study in Crl: CD (SD) rats***

This TK evaluation of florylpicoxamid in P1 and P2 rats associated with continuous daily dietary feeding indicated rapid absorption, hydrolysis of parent florylpicoxamid to X12485649 and further metabolism to X12485473, X12584261 and X12641325. Formation of these metabolites generally departed from dose-proportionality with increasing dietary concentration due to saturation of elimination and/or of formation as measured by dose-normalized $AUC_{24}$ values during the premating phase. Postnatal analysis of dam blood and milk (LD 4) and pup blood (PND 4) indicated some lactational transfer and systemic exposure of pups to X12485473 and X12641325 at one-tenth to one-hundredth of the level observed in the dams.

Overall, the blood TK data obtained across various rat and mouse toxicology studies supported nearly complete metabolism of florylpicoxamid to X12485649, followed by ester bridge cleavage to give X12485473 and X12641325. Low levels of X12584261 were also found in blood samples. Little to no parent florylpicoxamid, X12485649, X12484261 or X12530093 were found in urine samples, with X12485473 as the predominant urinary TK biomarker in rats and mice. Across the reproductive and development toxicity studies, in utero exposure to florylpicoxamid metabolites in fetuses was generally lower than exposure in dams, based on the major TK blood biomarkers X12485473 and X12641325. Lactational exposure of neonatal rats to florylpicoxamid and/or metabolites was found to consist primarily of X12485649 and X12485473, with lower levels of X12584261 and X12641325 present in milk, and little to no parent florylpicoxamid or X12530093 in this sample matrix. The resulting systemic exposure to PND 4 pups was lower than corresponding dam blood levels for both the major blood TK biomarkers of X12485473 and X12641325 and the minor TK markers, florylpicoxamid, X12485649 and X12584251.

In summary, the toxicokinetic evaluation of florylpicoxamid associated with continuous daily dietary feeding indicated rapid absorption, hydrolysis of parent florylpicoxamid to X12485649 and

further metabolism to X12485473, X12641325 and X12584261. Quantitation of X12485473 and X12641325 in blood, urine and/or milk confirmed these are major metabolites in animals exposed to florylpicoxamid (Chan, 2019).

**(b) Toxicokinetic analysis in the long-term studies**

Numerical data in mice (Albretsen, 2019a) and rats (Albretsen, 2019b) are described at Annex 2.

***Mouse***

The TK evaluation of florylpicoxamid associated with continuous daily dietary feeding in mice indicated rapid absorption, hydrolysis of parent florylpicoxamid to X12485649 and further metabolism to X12485473, X12584261 and X12641325. Of these, X12641325 and X12485473 are cleaved metabolites representing the *bis*-phenyl and pyridine rings of florylpicoxamid. Quantitation of X12485473 and X12641325 in blood and urine confirmed these analytes as prominent metabolites in mice exposed to florylpicoxamid (Chan, 2020b).

***Rat***

The TK evaluation of florylpicoxamid associated with continuous daily dietary feeding in mice and rats indicated rapid absorption, hydrolysis of parent florylpicoxamid to X12485649 and further metabolism to X12485473, X12584261 and X12641325. Florylpicoxamid and certain metabolites (X12485649 or X12584261) were not quantifiable, or were below the limit of quantitation (LOQ) in nearly all blood samples across diet concentration groups in both males and females. However, florylpicoxamid and three metabolites (X12485649, X12641325 and X12485473) were quantifiable in urine. These, combined with the very small fraction of florylpicoxamid detected, accounted for up to 15.7% (in males) and 22.6% (in females) of the daily doses ingested, that is 300, 1000, or 3000 ppm for males, 300, 1000 or 4000 ppm for females. Florylpicoxamid in urine is likely to have occurred by direct low-level transfer into urine on diet particles, because it undergoes complete deacetylation when absorbed. Metabolites X12641325 and X12485473 are cleaved metabolites representing the *bis*-phenyl and pyridine rings of florylpicoxamid. They are both known significant urinary metabolites observed following oral gavage. (Chan, 2020a)

# 2. Toxicological studies

## 2.1 Acute toxicity

Acute toxicities of florylpicoxamid were summarized in Table 10.

***Table 10. Summary of acute toxicity studies on florylpicoxamid***

| Route; Method | Species; strain (sex) | End-point | Purity; Batch/lot number | Reference |
|---|---|---|---|---|
| Oral; Gavage | Rat; Wistar (F) | $LD_{50}$ > 2000 mg/kg bw | 95.1%; ENBK-147245-067 | Verma, 2015 |
| Dermal; Topical, 24 hour semi-occlusive exposure | Rat; Wistar (M/F) | $LD_{50}$ > 2000 mg/kg bw | 97.1%; YN4-156527-095 (TSN311927) | Patel, 2016a |
| Inhalation; Nose only[a], four hours | Rat; Wistar (M/F) | $LC_{50}$ > 5.48 mg/L air | 97.1%; YN4-156527-095 (TSN311927) | Patel, 2016b |
| Dermal; Topical, four hours, semi-occlusive | Rabbit; NZW (M) | Not irritant to skin of rabbits | 97.1%; YN4-156527-095 (TSN311927) | Patel, 2016c |

| Route; Method | Species; strain (sex) | End-point | Purity; Batch/lot number | Reference |
|---|---|---|---|---|
| Eye; Instillation – washing after 24 hour post-application | Rabbit; NZW (M) | Slight irritating effect on rabbit eyes. Complete recovery after 24 hours | 97.1%; YN4-156527-095 (TSN311927) | Patel, 2016d |
| Dermal; Topical – local lymph node assay, LLNA | Mouse; CBA/J (F) | Not a dermal sensitizer | 97.1%; YN4-156527-095 (TSN311927) | Patel, 2016e |

M: Males; F: Females; NZW: New Zealand White; $LD_{50}$: Median lethal dose; $LC_{50}$: Median lethal concentration

[a] The time-weighted average exposure concentration of florylpicoxamid in air for rats was 5.48 mg/L The mass median aerodynamic diameter (MMAD) of florylpicoxamid aerosols was 3.59 µM with an average geometric standard deviation (GSD) of 1.60

### (a) Lethal doses

#### *Oral*

In an acute oral toxicity study, no clinical signs and no mortality were observed in rats treated with 2000 mg/kg bw. Normal body weight gain was observed in all the rats. External and visceral examination of terminally sacrificed rats did not reveal any lesion of pathological significance. The acute oral median lethal dose ($LD_{50}$) of florylpicoxamid in Wistar rats was greater than 2000 mg/kg bw (Verma, 2015).

#### *Dermal*

In an acute dermal toxicity study, there were no effects on clinical signs of toxicity, body weights or gross pathology. In the absence of any pathological lesion in terminally euthanized rats, it is concluded that the test item did not produce any treatment-related effects at the dose level used in this study. The acute dermal $LD_{50}$ of florylpicoxamid in Wistar male and female rats was greater than 2000 mg/kg bw (Patel, 2016a).

#### *Inhalation*

In an acute inhalation toxicity study no mortality occurred following treatment with florylpicoxamid at 5.48 mg/L air. No treatment-related clinical sings were observed. A slight decrease in body weight occurred following dosing on days 1 and 3 in all animals treated at 5.48 mg/L. Recovery of body weight effects occurred by day 7. No abnormalities were noted at gross pathology. The acute inhalation $LC_{50}$ of florylpicoxamid in rats was greater than 5.48 mg/L air (Patel, 2016b).

### (b) Dermal irritation

In a primary (acute) dermal irritation study, three young adult male New Zealand White rabbits were dermally exposed for four hours to 500 mg of florylpicoxamid moistened with 0.5 mL of distilled water to approximately, over an area of 6 $cm^2$ area of intact dorsal skin. Animals then were observed for 72 hours. Irritation was scored by the method of Draize.

None of the rabbits showed clinical signs indicating systemic toxicity. Very slight erythema (score 1) was observed in all rabbits at one hour after patch removal. Recovery was completed in all rabbits by 24 hours after patch removal. The mean dermal irritation scores at 24, 48 and 72 hours after patch removal were: mean erythema scores, 0.00, 0.00, 0.00; mean oedema scores, 0.00, 0.00, 0.00. No signs of toxicity that would indicate systemic toxicity were observed and all animals gained body weight throughout the study. Florylpicoxamid was considered non-irritant (Patel, 2016c).

### (c) Ocular irritation

In a primary (acute) eye irritation study, florylpicoxamid was instilled into the conjunctival sac of three young adult male NZW rabbits, the rabbits receiving 44.6, 46.8 and 45.3 mg, respectively. Eyes were washed 24 hours after instillation. Animals then were observed for 72 hours. Irritation was scored by the method of Draize.

Conjunctival redness, characterized as some blood vessels being hyperaemic (injected) (score 1) was evident in all rabbits one hour after treatment; recovery occurred by 24 hours in all rabbits. Examination with fluorescein dye and a cobalt blue filter after florylpicoxamid application revealed no disruption of the corneal epithelium layer in any rabbits at 24 hours. The three animals' average eye irritation scores (mean of scores observed at 24, 48 and 72 hours post florylpicoxamid application) were all 0.00 for corneal opacity, iris inflammation, conjunctival redness and conjunctival chemosis. Recovery was completed in all rabbits by 24 hours after instillation, however, due to conjunctival redness at one hour after application, florylpicoxamid was considered to cause slight irritation in rabbit eyes (Patel, 2016d).

**(d) Dermal sensitization**

In a dermal sensitization study using florylpicoxamid in an acetone : olive oil mixture (4:1 v/v) as a vehicle, groups of five female CBA/J mice were tested using the local lymph node assay, as described in OECD TG 429. The positive control was α-hexylcinnamaldehyde 25% (v/v) in the same vehicle.

There were no indications of clinical signs, body weight effects or local irritation in animals treated with 5% or 25% (w/v) of florylpicoxamid. A local reaction consisting of very slight erythema (score 1) was observed from days 2 to 4 with florylpicoxamid at 75%. Stimulation indices for the groups treated with florylpicoxamid at 5%, 25% and 75% were 1.21, 1.56 and 2.14, respectively. Florylpicoxamid was not identified as a dermal sensitizer (Patel, 2016e).

## 2.2 Short-term studies of toxicity

**(a) Oral administration**

***Mouse***

A 14-day dietary range-finding study was conducted in female Crl:CD1 (ICR) mice and female F344/DuCrl rats (the rat study is described below) to evaluate the potential palatability and toxicity of florylpicoxamid (purity 95.1%; Lot no. ENBK-147245-067 [TSN309924]).

Three female mice per group were administered florylpicoxamid in the diet at concentrations of 0 or 2500 ppm for 14 days, and other groups 500 or 1250 ppm for seven days. Dietary concentrations of 0. 500, 1250 and 2500 ppm corresponded to 0, 96.6, 245 and 491 mg/kg bw per day, respectively. The 2500 ppm group underwent body weight loss from day 1 to day 10. The 500 and 1250 ppm groups showed decreased body weight and body weight gain compared to controls at the beginning of treatment. Body weights in all groups recovered over the treatment period, with the 500 and 1250 ppm groups showing earlier improvement in body weight gain than the 2500 ppm group. At study termination, body weights in all treatment groups were similar to those of controls, however body weight gains remained lower than for controls. In all treatment groups, and most prominently in the 2500 ppm group, the mice scratched their food, likely indicating the test material was unpalatable. Measured food consumption values were similar to controls in all dose groups. Additionally, treatment-related clinical observations consisted of decreased faeces in mice given 2500 ppm for the majority of the study. There were no treatment-related gross lesions at necropsy. The 2500 ppm dose group displayed absolute and relative liver weight increases of 24.9% and 22.0%, respectively compared to controls. All mice within the 2500 ppm dose group had very slight centrilobular hepatocellular hypertrophy with altered tinctorial properties which was associated with treatment. Histopathology was not performed on the 500 and 1250 ppm groups.

Based upon the results of this study unpalatability and body weight loss were observed at 2500 ppm. (Anderson, 2020).

A thirteen-week dietary toxicity study was conducted. Ten male and ten female Crl CD1 (ICR) mice per group were given florylpicoxamid (purity 96.3%; Lot no. EXP-16-BH2635, TSN312284) in the diet at concentrations of 0, 150, 500 or 1500 ppm (equal to 0, 19.1, 59.7 and 192 mg/kg per day for males, 0, 21.9, 68.3 and 201 mg/kg per day for females) for at least 90 days. Additional sets of animals (ten per sex), given either 0 or 1500 ppm for 90 days, were maintained on control food for an additional 28 days to assess the reversibility of any treatment-related effects. Parameters evaluated were daily cage-side observations, weekly detailed clinical observations, ophthalmic examinations, body weights,

food consumption, haematology, clinical chemistry, selected organ weights. Gross and histopathological examinations were conducted and samples of blood and urine collected for toxicokinetic analyses.

No treatment-related effects at any dose were apparent as clinical signs, nor from ophthalmic observations, haematology or clinical chemistry parameters. In females at 1500 ppm, body weights were slightly lowered (by less than 7% at most measurement time points) and with no statistical significance in the latter half of the study. Females in this dose showed a slight decrease in body weight gain (by approximately 10% compared to controls) with no statistical significance throughout the study. Food consumptions at this dose was also slightly lowered (by ca 10% at most points measured) with statistical significance compared to controls. These decreases were very slight but considered to be adverse because the effects were observed in rats and dogs. There were no treatment-related changes in body weight, body weight gain or food consumption in males at 1500 ppm. Treatment-related increases in relative liver weight were observed in males at 1500 ppm and females at 500 ppm and above. The increases correlated with histopathological observations of very slight to slight hepatocellular hypertrophy. Based on no changes in hepatotoxicity parameters, the liver hypertrophy was considered adaptive. For animals in the recovery groups, no treatment-related effects were seen in clinical signs, body weight, food consumption, ophthalmic observations, organ weights, gross examinations, or histopathological examinations of the liver. this indicated that all treatment-related effects were fully reversed within four weeks of cessation of treatment.

The main changes observed in this study are summarized in Table 11.

***Table 11. Summary of findings in the 13-week dietary toxicity study in mice***

| | **Dietary dose (ppm)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Males** | | | | **Females** | | | |
| | **0** | **150** | **500** | **1500** | **0** | **150** | **500** | **1500** |
| ***Body weight (g); mean ± standard deviation*** | | | | | | | | |
| *N* | 20 | 10 | 10 | 20 | 20 | 10 | 10 | 20 |
| Day 1 | 31.2 ± 1.9 | 31.7 ± 2.1 | 30.9 ± 1.9 | 31.3 ± 2.1 | 24.9 ± 1.2 | 24.8 ± 1.3 | 24.7 ± 1.2 | 24.9 ± 1.7 |
| Day 4 | 32.1 ± 2.0 | 32.2 ± 2.0 | 31.5 ± 2.1 | 31.7 ± 2.2 | 25.8 ± 1.3 | 25.2 ± 1.0 | 25.6 ± 1.3 | 25.4 ± 2.0 |
| Day 29 | 36.0 ± 2.3 | 36.2 ± 1.8 | 35.4 ± 2.7 | 35.4 ± 2.4 | 29.4 ± 2.5 | 29.4 ± 1.4 | 29.1 ± 1.6 | 29.3 ± 2.5 |
| Day 57 | 39.0 ± 2.5 | 39.7 ± 2.9 | 38.1 ± 3.2 | 38.1 ± 2.8 | 32.3 ± 3.8 | 31.5 ± 1.7 | 32.0 ± 2.9 | 31.2 ± 3.0 |
| Day 92/93[a] | 40.7 ± 2.5 | 41.1 ± 3.3 | 39.8 ± 3.0 | 40.0 ± 2.8 | 36.2 ± 6.1 | 33.2 ± 1.8 | 33.6 ± 3.2 | 34.1 ± 2.6 |
| ***Body weight gain (g); mean ± standard deviation*** | | | | | | | | |
| *N* | 20 | 10 | 10 | 20 | 20 | 10 | 10 | 20 |
| Day 1–4 | 0.9 ± 0.3 | 0.54 ± 0.4 | 0.6 ± 0.7 | 0.5 ± 0.5 | 0.9 ± 0.7 | 0.4 ± 0.6 | 1.0 ± 0.7 | 0.6 ± 0.7 |
| Day 1–29 | 3.8 ± 1.0 | 3.7 ± 1.6 | 3.6 ± 1.2 | 3.4 ± 1.0 | 4.5 ± 1.6 | 4.6 ± 1.0 | 4.5 ± 1.3 | 4.4 ± 1.4 |
| Day 1–57 | 7.8 ± 1.5 | 8.0 ± 2.6 | 7.2 ± 2.1 | 6.9 ± 2.2 | 7.4 ± 2.8 | 6.8 ± 1.1 | 7.4 ± 2.5 | 6.3 ± 1.6 |
| Day 1–92/93[a] | 9.5 ± 2.1 | 9.4 ± 2.9 | 8.9 ± 1.8 | 8.8 ± 2.3 | 10.8 ± 5.0 | 8.5 ± 1.7 | 8.9 ± 2.8 | 8.3 ± 1.8 |
| ***Food consumption (g/mouse per day); mean ± standard deviation*** | | | | | | | | |
| *N* | 20 | 10 | 10 | 20 | 20 | 10 | 10 | 20 |
| Day 1–4 | 4.6 ± 0.3 | 4.7 ± 0.2 | 4.2* ± 0.2 | 4.6 ± 0.3 | 4.2 ± 0.2 | 4.1 ± 0.5 | 4.2 ± 0.3 | 3.9* ± 0.3 |
| Day 25–26 | 4.7 ± 0.4 | 4.9 ± 0.4 | 4.4* ± 4.7 | 4.7 ± 0.3 | 4.6 ± 0.4 | 4.6 ± 0.5 | 4.3 ± 0.3 | 4.2* ± 0.4 |
| Day 53–57 | 4.6 ± 0.5 | 4.9 ± 0.4 | 4.4 ± 0.3 | 4.7 ± 0.5 | 4.4 ± 0.4 | 4.3 ± 0.5 | 4.4 ± 0.5 | 4.1 ± 0.3 |
| Day 88–92/92[a] | 4.4 ± 0.6 | 4.4 ± 0.5 | 4.1 ± 0.3 | 4.6 ± 0.5 | 4.1 ± 0.5 | 3.8 ± 0.3 | 3.5 ± 0.1 | 3.6 ± 0.3 |

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | 0 | 150 | 500 | 1500 | 0 | 150 | 500 | 1500 |
| ***Relative organ weight*** | | | | | | | | |
| *N* | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Liver (g/100 bw) | 6.649 ± 0.349 | 4.877 ± 0.787 | 4.712 ± 0.240 | 5.050 ± 0.492 | 4.188 ± 0.314 | 4.345 ± 0.286 | 4.592* ± 0.374 | 4.672* ± 0.413 |
| ***Histopathology – hepatocellular hypertrophy increased eosinophilia, centrilobular/midzonal*** | | | | | | | | |
| *N* | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Total | 2 | 3 | 2 | 10 | 2 | 2 | 7 | 10 |
| Very slight | 2 | 3 | 2 | 7 | 2 | 2 | 7 | 10 |
| Slight | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 |

*N*: Number of mice examined; bw: Body weight; Source: Mukerji, 2018

[a] day 92 for males and day 93 for females;

* Different from control mean with statistical significance at $p < 0.0.5$

In conclusion, the no-observed-adverse-effect level (NOAEL) in 13-week toxicity study in mice was 500 ppm (equal to 68.3 mg/kg bw per day) based on a slight decreases in body weights, body weight gains and food consumption in females at 1500 ppm (equal to 201 mg/kg bw per day). The NOAEL in males was 1500 ppm (equal to 192 mg/kg bw per day), the highest dose tested.

The study author had proposed the NOAEL was 1500 ppm (equal to 192 mg/kg bw per day), the highest dose tested in the report (Mukerji, 2018).

***Rat***

A 14-day dietary range-finding study was conducted in female Crl:CD1 (ICR) mice (see above) and female F344/DuCrl rats. The methods of this study have already been described in the mouse study above. The results observed in this study for rats were as follows.

Three female F344/DuCrl rats per group were administered florylpicoxamid in the diet at concentrations of 0 or 5000 ppm for 14 days and other groups 1000 and 2500 ppm for seven days. Corresponding time-weighted, average doses for 0, 1000, 2500 and 5000 ppm were equal to 0, 77.9, 186 and 374 mg/kg bw per day, respectively. Compared to controls, all groups displayed decreased body weights, body weight gains and food consumption at the beginning of treatment, probably due to decreased palatability of the test diet. Body weights and body weight gains in all groups, along with food consumption, improved over the treatment period. The 1000 and 2500 ppm groups showed earlier improvements in body weight gain than the 5000 ppm group, however the majority of the body weight gains and food consumption values in all groups remained lower than controls throughout treatment period. Treatment-related clinical observations consisted of sporadic occurrences of decreased faeces throughout the study in the animals at 5000 ppm. No treatment-related gross lesions were seen at necropsy. At 5000 ppm, absolute and relative liver weight was slightly increased compared to controls, by 8.9% and 12.9% respectively. All rats at 5000 ppm displayed very slight centrilobular to midzonal hepatocellular hypertrophy with altered tinctorial properties. Histopathology was not performed at on the 1000 and 2500 ppm group rats (Anderson, 2020).

Two 28-day dietary toxicity studies were conducted using different strain of rat.

A 28-day dietary toxicity study was conducted using F344/DuCrl rats. Five male and five female rats were given florylpicoxamid (purity 92.5%; Lot no. YJ0-156745-090 [TSN310553]) in the diet for 29 days at concentrations of 0, 300, 1000 or 3000 ppm (equal to 0, 24.9, 84.0 and 230 mg/kg bw per day for males, 0, 26.1, 85.2 and 243 mg/kg per day for females). Parameters recorded were clinical observations, ophthalmic examinations, body weight, food consumption, haematology, prothrombin time (PT), urinalysis, clinical chemistry, selected organ weights, and gross and histopathological examinations. Additionally, toxicokinetic assessments were performed on urine and blood samples.

There were no treatment-related effects from ophthalmic and detailed clinical observations, in PT times, or urinalysis parameters. Body weights of males showed a slight decrease (by ca 5%; no statistical significance) at 3000 ppm from week 1 after the start of treatment to termination. The difference in body weight gain of males compared with controls was not statistically significant, but was lowered during treatment by 34% and 14% in the periods days 1–4 and days 23–29, respectively. This slight, but delayed, occurrence of decreased body weight gain in males was considered to be adverse rather than due to low palatability. In females, body weight and body weight gain were comparable to those at 3000 ppm except for a decrease in body weight gain on day 1, when it was lower by 26% than in the control group. This transient decrease was not considered adverse as it was quickly recovered. Food consumptions in both sexes was decreased by ca 10% throughout the study. The decreased food consumption in both cases was considered to be due to the toxicity of florylpicoxamid rather than low palatability since the effect was observed throughout the study, not transiently. A similar effect to this, at a similar dose was also observed in a long-term rat study.

Males given 3000 ppm florylpicoxamid had lower RBC counts (by 10%), haematocrit (by 11.75%), and haemoglobin concentration (by 13.5%) compared to the controls; all statistically significant. Reticulocyte counts were slightly higher (17.6%) than in controls, although this was not statistically significant. These changes were considered to be treatment-related adverse effect due to the consistency of similar effects found in males at the same dose level in the 90-day study (Mukerji, 2019). There were no changes in clinical chemistry parameters related to treatment.

Males and females at 3000 ppm had treatment-related, higher relative liver weights (males by 9.9%, females by 9.4%; both statistically significant) and higher relative kidney weights (males by 10%, females by 7.9%) when compared to controls. The higher liver weights correlated with the microscopic observation of very slight centrilobular/midzonal hypertrophy; their incidences were 0/5, 0/5, 0/5, and 5/5 in both sexes at 0, 300, 1000 and 3000 ppm respectively. The observed liver hypertrophy without hepatotoxic changes was considered adaptive. The slight increases in the kidney weight were not considered to be toxicological significant because there were no corresponding histopathological alterations, nor signs from clinical chemistry and urinalysis.

Under the conditions of this study, the NOAEL in 28-day toxicity study in F344/DuCrl rats was 1000 ppm (equal to 84.0 mg/kg bw per day) based on decreases in food consumption and body weight gain and decreases in red blood cell mass in males at 3000 ppm (equal to 230 mg/kg bw per day) (Murphy et al., 2018).

A separate 28-day dietary toxicity study was conducted using CD1:Crf rats. Florylpicoxamid (purity 96.3%; Lot no. EXP-16-BH2635) was administered for 28 consecutive days to five males and five females CD [Crl:CD®SD] (Sprague Dawley) rats per group in the diet at concentrations of 0, 1000, 3000, 4000 or 5000 ppm (equal to 0, 69.9, 206, 278 and 345 mg/kg bw per day for males, 0, 75.8, 247, 320 and 355 mg/kg bw per day for females). This study also served as a bridging study since previous subchronic toxicity studies with the test material had been performed in Fischer 344 rats (Mukerji, 2019) and SD rats had been planned for use in the future chronic study. Assessment of toxicity was based on mortality, clinical observations, body weight, food and compound consumption and clinical and anatomical pathology.

All animals survived until scheduled necropsy. No dose-related clinical signs were observed. At 5000 ppm body weights were slightly decreased (by ca 6% and 7% in males and females respectively) during the treatment period. At 4000 ppm and below, dose-related changes in body weight were not observed. Food consumption was reduced at 3000 ppm above in males, and 4000 ppm and above in females during the first week, by 8%, 9% and 18% at 3000, 4000 and 5000 ppm respectively in males, and by 6% or 21% at 4000 and 5000 ppm respectively in females, respectively. The reduction in food consumption in females at 5000 ppm was considered to be adverse due to the effect continuing throughout the study, although degree of reduction varied. Transient decreases in food consumption in males at 3000 ppm above, and in females at 4000 ppm were not considered to be adverse due to their inconsistency. Minimal to mild decreases (by 10%) in erythrocytes, haemoglobin (Hb), and haematocrit (Ht), collectively red cell mass, were observed in males at 5000 ppm. Due to the consistency of these changes in haematological parameters with those observed in the 28-day toxicity study in male F344 rats at 3000 ppm, the changes in haematological parameters, although slight, were considered to be due to the toxicity of florylpicoxamid. Dose-related increases in liver weight in males at 3000 ppm and

above, along with minimum to mild hepatocellular hypertrophy were observed in males at 3000 ppm and above; their incidences were 0/5, 0/5, 4/5, 5 and 5/5 at 0, 1000, 3000, 4000 and 5000 ppm respectively. The grades of hepatocellular hypertrophy were seen to increase with dose. An incidence of minimal hypertrophy of the thyroid follicular cells was increased in males in a dose-related manner at 4000 and 5000 ppm; incidence was 0/5, 0/5, 0/5, 3/5 and 4/5 at 0, 1000, 3000, 4000 and 5000 ppm, respectively. Morphologically, a minor reduction in colloid in some of the follicle lumens was associated with the follicular hypertrophy. The liver hypertrophy with minimum histopathological change, but without increased thyroid effect was considered to be a marginal adverse effect. Similar thyroidal effects were not found in the same strain of rats at 3000 ppm, even when treatment duration was longer, such as in the long-term study. Treatment-related effects were not observed in the liver or thyroid of females.

In conclusion, the NOAEL for the 28-day dietary toxicity study in Sprague Dawley rats was 3000 ppm (equal to 206 mg/kg bw per day) based on marginal effects on liver and thyroid in males at 4000 ppm (equal to 278 mg/kg bw per day) (Heward, 2018).

Doses and toxicity observed in F344 rats and Sprague Dawley rats during 28-day dietary studies are compared in Table 12. Sex differences of treatment-related effects are also compared between the two studies.

***Table 12. Comparison of treatment and effects observed in F344 and Sprague Dawley rats in 28-day dietary toxicity studies***

| | Males | | Females | |
|---|---|---|---|---|
| **Effects observed** | **F344** | **SD** | **F344** | **SD** |
| ***Lower body weight*** | | | | |
| NOAEL/LOAEL (ppm) | 1000/3000 | 4000/5000 | NE | 4000/5000 |
| Difference from control values | Continuous decrease by 5% | Continuous decrease by 6% | | Continuous decrease by 7% |
| ***Food consumption*** | | | | |
| NOAEL/LOAEL (ppm) | 1000/3000 | NE | 1000/3000 | 4000/5000 |
| Difference from control values | Continuous decrease by 10% | | Continuous decrease by 10% | Continuous decrease by 21% to 4% |
| ***Haematological effects*** | | | | |
| NOAEL/LOAEL (ppm) | 1000/3000 | 4000/5000 | NE | NE |
| Difference from control values | Decrease by 10% | Decrease by 10% | | |
| ***Liver hypertrophy*** | | | | |
| NOAEL/LOAEL (ppm) | 1000/3000 | 1000/3000 | 1000/3000 | NE |
| Difference from control values | Increased up 10% | Increased by 14%, 26% and 26% at 3000, 4000 and 5000 ppm, respectively | Increased by 10% | |
| Grade of hepatocellular hypertrophy | Very slight | Minimal to mild | Very slight | |
| Adaptive or adverse? | Adaptive due to no indication of related change | Adaptive at 3000 ppm, Adverse at ~4000 ppm due to thyroid change also | Adaptive due to no indication of related change | |
| ***Thyroidal hypertrophy*** | | | | |
| NOAEL/LOAEL (ppm) | NE | 3000/4000 | NE | NE |
| Intensity of change | | Minimal | | |

NE: No effect observed; Source: Murphy et al., 2018; Heward, 2018

NOAEL: No-observed-adverse-effect level; LOAEL: Lowest-observed-adverse-effect level;

The comparison of treatment-related effects indicated that both rat strains showed qualitatively similar effects, except for thyroid follicular cell hypertrophy. The most plausible MOA of the thyroid effect was considered to be as a secondary effect of liver hypertrophy. In both strains. male rats were more sensitive to induction of the effects than females. In conclusion, the treatment-related effects observed in both strains were considered to be similar.

A 13-week dietary toxicity study was conducted in rats. Ten male and ten female F344/DuCrl rats per group were given florylpicoxamid (purity 96.8%; Lot no. YN4-156527-094, TSN311503) in the diet for 90 days at concentrations of 0, 300, 1000 or 3000 ppm (equal to 0, 18.2, 59.4, or 177 mg/kg bw per day for males, 0, 18.8, 63.0 and 185 mg/kg bw per day for females) to evaluate general toxicity, neurotoxicity, immunotoxicity, genotoxicity, toxicokinetics, and RNA-Seq-based transcriptome profiles. Additional groups of 10 male and 10 female rats were first given 0 or 3000 ppm for the 90-day treatment period, then switched to control feed for another 28 days to evaluate recovery from any potential treatment-related effects. Parameters evaluated as end-points were daily cage-side observations, weekly detailed clinical observations, ophthalmological examinations, functional observational battery (FOB; cage-side, hand-held and open-field observations, rectal temperature, fore- and hind-limb grip performance, landing foot splay and motor activity), body weight, body weight gain, food consumption, haematology, PT, urinalysis, clinical chemistry, selected organ weights, and gross and histopathological examinations which included a specifically detailed review of the nervous system. The study also included toxicokinetic analysis of blood and urine, RNA-Seq-based transcriptome profiles of liver tissue, micronucleus frequency in peripheral blood (genotoxicity), and an assessment of the sheep red blood cell (sRBC) antibody response (immunotoxicity). The last two activities included two groups of animals treated with cyclophosphamide (CPA) to serve as positive controls for these end-points.

There were no treatment-related alterations in cage-side or clinical observations, weekly detailed clinical observations, ophthalmic examinations or the FOB. At 3000 ppm males showed treatment-related decreases in body weight, body weight gain and food consumption compared to controls throughout treatment period. At the end of treatment, body weights, body weight gains and food consumption were reduced compared to controls by 8.1%, 13.3%, and 9.8% respectively, in males at 3000 ppm. No changes in these parameters were seen in females of any treated group, nor in males at 150 or 500 ppm.

As regards haematology, males at 3000 ppm showed a statistically significant decrease in mean RBC count, Hb concentration, and Ht. These decrements were slight but considered to be adverse because of their consistency with-the findings in other rat studies. There were no treatment-related alterations in PT. In males at 3000 ppm lower alanine transaminase (ALT), alkaline phosphatase (ALP), aspartate transaminase (AST) and triglyceride values were not considered toxicologically significant due to the reductions being opposite to the direction that would indicate adversity in these parameters. Increased incidence of bilirubin in the urine of males at 3000 ppm (0/10 in controls, 5/10 at 3000 ppm) was not considered adverse because no other changes related renal toxicity were seen at this dose.

From immunotoxicity examinations, data derived from anti-sRBC IgM in enzyme-linked immunosorbent assays (ELISA) did not reveal statistically significant differences between the three dose levels of florylpicoxamid and the vehicle control. The positive control CP produced a statistically significant and greater than 99% reduction in the anti-sRBC IgM response.

Examination of micronucleus frequency in peripheral blood, to investigate genotoxicity, revealed no treatment-related or statistically identified differences in micronucleus frequencies in the peripheral blood of any of the dose groups when compared to their respective controls. A significant increase in the frequency of micronucleated cells was observed in the CP positive control group. Therefore, under the conditions of this study, florylpicoxamid was considered to be negative in the rat peripheral blood micronucleus test.

Compared with controls, absolute and relative liver weights were increased (by 11.2% and 17.5%, respectively; statistically significant) in males at 3000 ppm. The higher liver weights corresponded to increases in the incidence of very slight hypertrophy of centrilobular/midzonal hepatocytes with increased cytoplasmic eosinophilia. In females, relative liver weight was increased by 8.4% at 1000 ppm, and absolute and relative liver weights increased by 9.0% and 9.5%, respectively, at 3000 ppm. Liver hypertrophy, in males at 3000 ppm and in females at 1000 ppm and above, was adaptive due to no other changes that indicated hepatotoxicity being observed at these doses. Absolute and relative kidney

weights were increased at 3000 ppm compared to controls in males by 9.1% and 15.5% respectively, and in females by 6.7% and 7.4% respectively. The increases were not considered adverse because no related changes were observed that indicated renal toxicity.

A summary of general toxicity in this 13-week rat study is shown in Table 13.

***Table 13. The summary of general toxicities in 13-week dietary toxicity study in rats***

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | **0** | **300** | **1000** | **3000** | **0** | **300** | **1000** | **3000** |
| ***Body weight (g); mean ± standard deviation*** | | | | | | | | |
| *N* | 20 | 10 | 10 | 20 | 20 | 10 | 10 | 20 |
| Day 1 | 124.2 ± 10.4 | 123.1 ± 10.1 | 125.4 ± 10.3 | 123.8 ± 11.2 | 103.1 ± 6.3 | 103.5 ± 6.7 | 102.3 ± 4.8 | 102.5 ± 5.4 |
| Day 4 | 138.6 ± 2.2 | 137.0 ± 11.1 | 139.9 ± 11.1 | 135.3 ± 11.7 | 111.0 ± 6.6 | 111.2 ± 6.9 | 109.6 ± 5.2 | 108.6 ± 5.4 |
| Day 29 | 225.7 ± 15.2 | 218.2 ± 10.4 | 223.1 ± 14.1 | 211.3 ± 13.2 | 143.3 ± 6.4 | 144.1 ± 8.4 | 139.5 ± 4.9 | 138.7 ± 7.8 |
| Day 57 | 272.2 ± 19.7 | 262.1 ± 13.9 | 265.2 ± 16.4 | 252.2 ± 17.7 | 156.1 ± 6.9 | 159.5 ± 10.6 | 152.9 ± 4.4 | 153.7 ± 9.3 |
| Day 91 | 309.1 ± 23.5 | 296.6 ± 16.4 | 301.5 ± 22.6 | 284.1 ± 20.6 | 169.3 ± 8.0 | 172.7 ± 10.7 | 165.9 ± 5.0 | 168.2 ± 11.3 |
| ***Body weight gain (g); mean ± standard deviation*** | | | | | | | | |
| *N* | 20 | 10 | 10 | 20 | 20 | 10 | 10 | 20 |
| Days 1–4 | 14.34 ± 2.2 | 13.94 ± 1.9 | 14.54 ± 1.9 | 11.6 ± 1.8 | 7.9 ± 1.7 | 7.7 ± 1.1 | 7.2 ± 1.6 | 6.2 ± 1.6 |
| Days 1–29 | 101.5 ± 11.3 | 95.1 ± 6.1 | 97.7 ± 6.5 | 87.5 ± 11.5 | 40.2 ± 4.9 | 40.6 ± 4.9 | 37.2 ± 4.4 | 36.3 ± 5.3 |
| Days 1–57 | 148.0 ± 15.5 | 139.0 ± 9.6 | 139.8 ± 8.6 | 128.5 ± 18.3 | 52.9 ± 4.9 | 56.0 ± 7.0 | 50.6 ± 5.0 | 51.2 ± 7.3 |
| Days 1–91 | 184.9 ± 19.3 | 173.5 ± 11.4 | 176.1 ± 15.6 | 160.4 ± 22.0 | 66.2 ± 6.5 | 69.2 ± 8.7 | 63.6 ± 4.9 | 65.8 ± 9.1 |
| ***Food consumption (g/rat per day); mean ± standard deviation*** | | | | | | | | |
| *N* | 20 | 10 | 10 | 20 | 20 | 10 | 10 | 20 |
| Days 1–4 | 12.1 ± 0.7 | 12.0 ± 0.7 | 12.0 ± 0.5 | 9.6* ± 0.5 | 9.3 ± 0.2 | 9.2 ± 0.4 | 8.9 ± 0.1 | 7.3 ± 0.5 |
| Days 29–36 | 14.2 ± 0.7 | 14.0 ± 0.4 | 13.7 ± 0.3 | 13.3* ± 0.6 | 9.3 ± 0.4 | 9.1 ± 0.6 | 9.3 ± 0.2 | 8.9 ± 0.3 |
| Days 57–64 | 14.6 ± 0.8 | 13.9 ± 0.8 | 14.1 ± 0.7 | 13.4* ± 0.7 | 9.2 ± 0.3 | 9.3 ± 0.4 | 9.0 ± 0.1 | 8.9* ± 0.3 |
| Days 85–91 | 14.2 ± 0.8 | 13.5* ± 0.4 | 13.5* ± 0.7 | 12.9 ± 0.7* | 8.4 ± 0.7 | 8.1 ± 0.3 | 8.1 ± 0.4 | 8.3 ± 0.4 |

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | 0 | 300 | 1000 | 3000 | 0 | 300 | 1000 | 3000 |
| ***Haematology*** | | | | | | | | |
| *N* | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| RBC ($10^6$/µL) | 9.53 ± 0.27 | 9.66 ± 0.17 | 9.50 ± 0.21 | 8.93 ± 0.30 | 8.70 ± 0.22 | 8.67 ± 0.26 | 8.68 ± 0.27 | 8.65 ± 0.19 |
| Hb (g/dL) | 15.3 ± 0.4 | 15.5 ± 0.2 | 15.2 ± 0.3 | 14.3 ± 0.4 | 15.1 ± 0.3 | 15.1 ± 0.4 | 15.0 ± 0.4 | 14.8 ± 0.4 |
| Ht (L/L) | 47.5 ± 1.1 | 48.0 ± 0.8 | 47.1 ± 0.9 | 44.1 ± 1.3 | 45.7 ± 0.9 | 45.6 ± 1.2 | 45.3 ± 1.2 | 44.9 ± 1.0 |
| MCV (fL) | 49.9 ± 0.6 | 49.7 ± 0.4 | 49.6 ± 49.4 | 49.4 ± 0.4 | 52.6 ± 0.3 | 52.6 ± 0.5 | 52.2 ± 0.4 | 51.9 ± 0.2 |
| Reticulocytes ($10^9$/L) | 195.5 ± 22.4 | 190.8 ± 17.6 | 211.1 ± 195.4 | 195.4 ± 31.0 | 209.4 ± 39.2 | 194.8 ± 37.8 | 211.9 ± 36.3 | 224.3 ± 46.2 |
| ***Relative organ weight*** | | | | | | | | |
| *N* | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Liver (g/100) | 2.575 ± 0.262 | 2.578 ± 0.200 | 2.672 ± 0.213 | 3.026 ± 0.217 | 2.556 ± 0.094 | 2.644 ± 0.142 | 2.770 ± 0.155 | 2.800 ± 0.109 |
| ***Histopathology – hepatocellular hypertrophy increased eosinophilia, centrilobular/midzonal*** | | | | | | | | |
| *N* | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Total | 3 | 2 | 3 | 8 | 0 | 0 | 0 | 0 |
| Very slight | 2 | 2 | 3 | 1 | 0 | 0 | 0 | 0 |
| Slight | 1 | 0 | 0 | 7 | 0 | 0 | 0 | 0 |

*N*: Number of rats examined; RBC: Erythrocyte count; Hb: Haemoglobin concentration; Source, Mukerji, 2019
Ht: Haematocrit; MCV: Mean corpuscular volume;

* Different from control mean with statistical significance at $p < 0.0.5$

Resulting from the neurotoxicity examination, there were no treatment-related histopathological changes in the central or peripheral nervous system nor any significant changes observed in the FOB. Florylpicoxamid showed no potential for neurotoxicity.

The potential of recovery from treatment-related effects from a 90-day exposure to 3000 ppm was evaluated in males and females which had received control food for 28 days following the last exposure to florylpicoxamid. Body weight and body weight gain in males at 3000 ppm was still decreased compared to controls (by 6.1% and 11.5% respectively) at the end of the recovery period. All other treatment-related effects, including haematology, clinical chemistry, organ weight and histopathological changes, had fully recovered at the end of 28 days.

The liver whole transcriptome responses in male and female rats treated with florylpicoxamid were qualitatively similar. At 3000 ppm the number of differentially expressed genes in male and female liver was 736 and 311, respectively. Of the four nuclear receptor activation biomarker genes (Cyp1a1, Cyp2b1, Cyp3a1 and Cyp4a1), Cyp2b1 showed the highest induction and was increased 20.5-fold and 79.2-fold at 3000 ppm in male and female liver, respectively. A treatment-related increase in Cyp1a1 expression (2.9-fold) was observed in males, but not females, at 3000 ppm. A treatment-related increase in Cyp3a1expression was observed in both males (2.1-fold) and females (2.5-fold) at 3000 ppm. Florylpicoxamid induced metabolic enzymes in the liver of both sexes via CAR/PXR activation.

Table 14 shows a summary of nuclear receptor-related Cyp gene expression in the rat liver.

*Table 14. The summary of nuclear receptor related Cyp gene expression in rat liver (values shown are group mean fold change relative to the control group mean = 1)*

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| Gene expression | 0 | 300 | 1000 | 3000 | 0 | 300 | 1000 | 3000 |
| Cyp1a1 | 1a | 1.2 | 1.5 | **2.9** | 1 | 0.8 | 1.4 | 1.8 |
| Cyp2b1 | 1 | **2.1** | **3.6** | **20.5** | 1 | **2.5** | **7.1** | **79.2** |
| Cyp3a23/3a1 | 1 | 1.2 | 1.3 | **2.1** | 1 | 1.2 | 1.2 | **2.5** |
| Cyp4a1 | 1 | 1 | 1 | 0.9 | 1 | 1 | 1 | 0.9 |

Values in bold were considered to be treatment-related. Source: Mukerji, 2019

In conclusion, the NOAEL for general toxicity in male rats was 1000 ppm (equal to 59.4 mg/kg bw per day) based on decreased body weight and body weight gain, and lowered red blood cell parameters at 3000 ppm (equal to177 mg/kg bw per day). The NOAELs for both neurotoxicity and immunotoxicity were 177 mg/kg bw per day, the highest dose tested. Florylpicoxamid was considered to be negative in the rat peripheral blood micronucleus test (Mukerji, 2019).

### *Dog*

A preliminary dose range-finding study was conducted to investigate the toxicity of florylpicoxamid by oral gavage in male and female beagle dog. This study was composed of two experiments (Phases A and B). The same female dogs from Phase A were used in Phase B after a four-day washing period. Male dogs were used in Phase B only. Florylpicoxamid (purity 96.3%, Lot no. EXP-16-BH2635) was administered by oral capsule for three consecutive days at 100, 300, 500 or 750 mg/kg bw per day to two female dogs per dose level in the Phase A experiment. In Phase B two male and two female dogs were given florylpicoxamid for 21 consecutive days at doses of 250 or 500 mg/kg bw per day, but the 500 mg/kg bw per day dose was reduced to 400 mg/kg bw par day on day 8. On day 8 food availability was increased to 12 hours per day, and animals were given some canned food (approximately one-quarter can) on days 8–12 to stimulate the appetite. Animals were observed for morbidity, mortality and injury twice daily. Observations for clinical signs were conducted daily. Body weight and food consumption were measured daily. Haematology and clinical chemistry parameters were measured on all Phase B animals on day 8 and prior to scheduled terminal necropsy. Urine analysis was performed on all Phase B animals on day 21. Gross pathological and histopathological examinations were carried out on all Phase B animals.

There were no effects on mortality. Faecal changes, such as soft, watery, and/or mucoid stools were continuously observed in females at all dose levels in Phase A and in both sexes in Phase B. In Phase B, sporadic occurrence of vomitus/emesis and a continuous occurrence of salivation were noted in either or both sexes at 250 and 500/400 mg/kg bw per day. These clinical signs were considered to be treatment-related and the possibility of florylpicoxamid eliciting a local effect such as vomitus/emesis or salivation was not excluded due to its slight irritating effects on rabbits' eyes (Patel, 2016d).

As in Phase B, body weights in both sexes at 500 mg/kg bw per day decreased by ca 10% during the first weeks the dose was decreased to 400 mg/kg bw per day. At 250 mg/kg bw per day, at the end of the 21 days of dosing, body weight remained the same or increased when compared to day −1 values, while at 500/400 mg/kg bw per day body weights remained 0.8% to 8.8% lower than on day −1 until the end of the dosing period.

Food consumption in Phase A was generally lower during dosing than for the same animals during predose or washout periods. In Phase B at 250 mg/kg bw per day, food consumption was generally slightly lower in both sexes during the first week of dosing compared to pretest values. After the prolonging food availability period, food consumption values increased. During the first week of dosing food consumption values in dogs given 500 mg/kg bw per day were generally much lower than pretest values. By day 12, food consumption values in this group were similar to the pretest values.

A relationship between the dose-related decreases in body weight and food consumption in Phase

B was considered. The decreases in food consumption started just after the first treatment by gavage, and the decreases lessened from eight days or 12 days onwards, after prolonging the food time food was available. The changes in body weights corresponded to changes in food consumption, indicating that the changes in the body weight (until the extended food availability took effect) were related to lower palatability. However the possibility of a treatment-related adverse effects on body weight was not excluded due to continuously lower body weights being recorded at 250 and 500/400 mg/kg bw per day after that time. Changes in body weight and food consumptions on an individual basis are shown in Table 15.

***Table 15. Individual data on feed intakes and body weights in dogs (Phase B experiment)***

| | **Dietary dose (mg/kg bw)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Males** | | | | **Females** | | | |
| **Dose** | **250** | | **500/400[a]** | | **250** | | **500/400[a]** | |
| **Animal code** | **403** | **404** | **407** | **408** | **401** | **402** | **405** | **406** |
| ***Food intake (g)*** | | | | | | | | |
| Day −3 to −2 | 308 | 334 | 275 | 294 | 180 | 205 | 180 | 205 |
| Day −2 to −1 | 167 | 357 | 317 | 256 | 231 | 199 | 231 | 199 |
| Day −1 to 1 | 219 | 369 | 334 | 274 | 238 | 222 | 238 | 222 |
| Day 1 to 2 | 203 | 248 | 203 | 137 | 259 | 218 | 259 | 218 |
| Day 2 to 3 | 174 | 224 | 175 | 197 | 145 | 112 | 145 | 112 |
| Day 3 to 4 | 175 | 260 | 146 | 87 | 115 | 165 | 115 | 165 |
| Day 4 to 5 | 121 | 184 | 145 | 125 | 113 | 95 | 113 | 95 |
| Day 5 to 6 | 152 | 186 | 88 | 77 | 155 | 132 | 155 | 132 |
| Day 6 to 7 | 150 | 154 | 132 | 75 | 153 | 105 | 153 | 105 |
| Day 7 to 8 | 122 | 199 | 127 | 80 | 171 | 132 | 171 | 132 |
| Day 8 to 9 | 430 | 463 | 226 | 96 | 246 | 167 | 246 | 167 |
| Day 9 to 10 | 396 | 466 | 291 | 153 | 272 | 162 | 272 | 162 |
| Day 10 to 11 | 392 | 402 | 228 | 225 | 220 | 219 | 220 | 219 |
| Day 11 to 12 | 353 | 339 | 271 | 181 | 306 | 197 | 306 | 197 |
| Day 12 to 13 | 395 | 442 | 248 | 364 | 328 | 179 | 328 | 179 |
| Day 13 to 14 | 222 | 359 | 259 | 254 | 302 | 262 | 302 | 262 |
| Day 14 to 15 | 339 | 362 | 375 | 426 | 285 | 238 | 285 | 238 |
| Day 15 to 16 | 315 | 427 | 309 | 333 | 276 | 283 | 276 | 283 |
| Day 16 to 17 | 431 | 274 | 323 | 413 | 280 | 318 | 280 | 318 |
| Day 17 to 18 | 241 | 520 | 300 | 236 | 301 | 243 | 301 | 243 |
| Day 18 to 19 | 188 | 258 | 275 | 315 | 260 | 235 | 260 | 235 |
| Day 19 to 20 | 403 | 388 | 251 | 272 | 248 | 199 | 248 | 199 |
| Day 20 to 21 | 331 | 264 | 363 | 327 | 242 | 234 | 242 | 234 |
| ***Body weight (kg)*** | | | | | | | | |
| Day −3 | 10.90 | 9.35 | 9.20 | 9.05 | 6.75 | 6.75 | 6.90 | 5.95 |
| Day −2 | 11.20 | 9.70 | 9.45 | 9.30 | 7.05 | 7.05 | 7.20 | 6.15 |
| Day −1 | 11.15 | 9.45 | 9.10 | 8.95 | 6.90 | 6.85 | 6.95 | 6.00 |
| Day 1 | 10.95 | 9.40 | 9.00 | 9.05 | 7.25 | 7.15 | 7.15 | 6.00 |
| Day 2 | 10.60 | 9.20 | 8.80 | 8.80 | 6.85 | 6.85 | 6.90 | 5.90 |
| Day 3 | 10.75 | 9.30 | 8.45 | 8.45 | 6.60 | 6.65 | 6.75 | 5.75 |
| Day 4 | 10.75 | 9.30 | 8.50 | 8.45 | 6.60 | 6.70 | 6.75 | 5.65 |
| Day 5 | 10.75 | 9.10 | 8.40 | 8.25 | 6.70 | 6.65 | 6.65 | 5.65 |

| | Dietary dose (mg/kg bw) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| Dose | 250 | | 500/400[a] | | 250 | | 500/400[a] | |
| Animal code | 403 | 404 | 407 | 408 | 401 | 402 | 405 | 406 |
| Day 6 | 10.70 | 9.25 | 8.20 | 8.30 | 6.60 | 6.55 | 6.55 | 5.70 |
| Day 7 | 10.60 | 9.25 | 8.30 | 8.15 | 6.80 | 6.55 | 6.50 | 5.60 |
| Day 8 | 10.40 | 8.90 | 7.95 | 8.05 | 6.50 | 6.50 | 6.35 | 5.45 |
| Day 9 | 10.60 | 9.15 | 8.05 | 8.10 | 6.70 | 6.50 | 6.50 | 5.50 |
| Day 10 | 10.85 | 9.25 | 8.15 | 8.15 | 6.70 | 6.55 | 6.65 | 5.50 |
| Day 11 | 10.80 | 9.55 | 8.35 | 8.15 | 6.70 | 6.75 | 6.55 | 5.65 |
| Day 12 | 10.70 | 9.35 | 8.05 | 8.10 | 6.75 | 6.60 | 6.20 | 5.45 |
| Day 13 | 10.80 | 9.25 | 8.05 | 8.25 | 6.80 | 6.60 | 6.25 | 5.85 |
| Day 14 | 10.85 | 9.35 | 8.05 | 8.05 | 6.65 | 6.60 | 6.60 | 5.75 |
| Day 15 | 11.00 | 9.55 | 8.25 | 8.50 | 6.90 | 6.80 | 6.60 | 5.80 |
| Day 16 | 10.90 | 9.50 | 8.10 | 8.40 | 6.55 | 6.95 | 6.40 | 5.80 |
| Day 17 | 10.95 | 9.40 | 8.30 | 8.65 | 6.85 | 6.85 | 6.65 | 6.00 |
| Day 18 | 11.05 | 9.85 | 8.25 | 8.60 | 7.05 | 6.85 | 6.65 | 6.00 |
| Day 19 | 11.25 | 9.65 | 8.30 | 8.80 | 7.10 | 7.25 | 6.70 | 6.00 |
| Day 20 | 11.25 | 9.65 | 8.20 | 8.70 | 7.10 | 6.90 | 6.65 | 5.95 |
| Day 21 | 11.20 | 9.60 | 8.30 | 8.85 | 7.10 | 6.85 | 6.65 | 5.95 |

Source: Heward, 2017

[a] The dose of 500 mg/kg bw per day was lowered to 400 mg/kg bw per day on day 8;
On day 8, food availability was increased to 12 hours per day and animals were given some canned food (approximately one-quarter can) until day 12 to stimulate the appetite

No effects were observed on ophthalmology, haematology, clinical chemistry or urine analysis parameters, nor from gross and histopathological examinations.

In conclusion, the treatment with florylpicoxamid increased occurrences of soft/watery faeces, vomitus/emesis and salivation at 250 and 500/400 mg/kg bw per day, suggesting effects on the GI tract , but without corresponding histopathological changes. Local effects or low-palatability of this compound might related to these clinical signs. Treatment also resulted in decreased body weight and food consumption in both sexes at 250 and 500/400 mg/kg bw per day. A dose level of 500 mg/kg bw per day was considered to be excessive for dogs. (Heward, 2017).

In a 90-day toxicity study, groups of four beagle dogs of each sex were administered florylpicoxamid (purity 98.1%; Lot no. EXP-17-BN7923 [TSN313275]) in capsules at doses of 0, 60, 150 or 400 mg/kg bw per day. All dogs were given a basal diet of Lab Diet® (Certified Canine Diet #5007, pMI Nutrition International, Inc.) ad libitum for a period of 12 hours ± 60 minutes per day, except during fasting periods or select husbandry functions. On dosing days, feed was offered 30 to 60 minutes prior to dosing. On occasions, animals were offered supplemental feed as part of standard veterinary care. Animals were observed for morbidity, mortality and injury twice daily and for clinical signs weekly. Body weight and food consumption were measured weekly. Ophthalmoscopic examinations were performed in the pretest period and prior to scheduled necropsy. Samples were taken for haematology, clinical chemistry and urinalysis parameters to be were measured pretest and at weeks 7 and 13. Organ weight measurement, gross pathological and histopathological examinations were performed on all groups

The major treatment-related effects are summarized in Table 16.

There were no effects on mortality. Dose-related increases in the occurrence of soft faeces were observed in both sexes at all doses. The occurrence of watery faeces increased in males at 150 and 400 mg/kg bw per day and in females at 400 mg/kg bw per day. The soft and/or watery faeces were observed throughout the study, did not worsen as the treatment period progressed. No other clinical

observations related to florylpicoxamid were observed. The increased occurrence of soft or watery faeces was considered to be a treatment-related effect on the GI tract, and the possibility of mild local effect was not excluded as its cause.

Body weights were continuously lower than for controls in both sexes at 400 mg/kg bw per day (within 10% in males, or approximately 10% in females) and in males at 150 mg/kg bw per day (within 5%) Body weights in these groups were more markedly lower during the first two weeks. Body weight gains in these groups appeared to be suppressed in line with the changes in body weight. Body weights and body weight gains in males at 60 mg/kg bw per day and in females at 60 and 150 mg/kg bw per day were comparable to control values.

Food consumption was decreased in males and females at 400 mg/kg bw per day. Decreases were observed in the first seven weeks, especially the first two weeks. Reductions in consumption had recovered by the latter half of the treatment. Food consumption in males at 150 mg/kg bw per day was reduced in the first two weeks but quickly recovered to be comparable to control values. Food consumption values in females at 60 and 150 mg/kg bw per day were similar, and both lower than controls after seven weeks. Food consumption in the three female animals comprising the control group were high and similar to values for control males during the latter period of the study.

No effect was observed on haematology, clinical chemistry or urinalysis except a slight decrease (by ca 10%) in albumin and an increase in ALP in both sexes at 400 mg/kg bw per day. The decrease in albumin was slight but this change may be related to the increased occurrence of soft or watery faeces and the lower body weight seen in this group. The increase in ALP was considered to be treatment-related but not adverse because no other parameters indicating hepatotoxicity were observed. The increase in liver weight without changes in other hepatotoxic parameters was considered to be adaptive. There were no treatment-related gross pathological or histopathological findings, including findings in the GI tract.

The changes in clinical signs, body weight, body weight gain and food consumption were interpreted as follows.

- Integrating the results obtained, the increased occurrence of soft and watery faeces in males and females at 400 mg/kg bw per day was considered to be adverse due to several related changes suggesting effects on the GI tract, such as the lowered body weights and the decreases in serum albumin observed in this group. The effect on the GI tract was considered to be mild because of no corresponding morphological change or serum electrolytes. The occurrence of soft/watery faeces did not worsen with increased treatment duration.
- Continuous decreases in body weight, body weight gain and food consumption in both sexes at 400 mg/kg bw per day were considered adverse due to their consistency with the preliminary study (Heward, 2017) although the possibility was not excluded that the decreases at the beginning of the study were due to low palatability.
- As there were too few animals in these dog studies for useful statistical analysis, individual data from males at 150 mg/kg bw per day were checked. One male dog (#3002) clearly showed much lower body weight (ca 1.9 kg decrease), decreased body weight gain (ca 1kg decrease) and decreased food consumption (by ca 20% to 50%). The occurrence of soft or watery faeces was not, however, worse in #3002. The changes of these parameters in other three dogs at the same dose were comparable to changes seen in the controls at termination, but their body weights were slightly but continuously lower than control values in the first half of the study. The difference in food consumption from controls was not clear in these three dogs. Integrated, the changes in body weight and body weight gain in males at 150 mg/kg bw were considered adverse. The possibility that lower food consumption in one dog at 150 mg/kg bw per day was adverse could not be excluded.

*Table 16. Major toxicities observed in the 90-day toxicity study in dogs*

| | Dose (mg/kg bw per day) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Male | | | | Females | | | |
| | **0** | **60** | **150** | **400** | **0** | **60** | **150** | **400** |
| | | | | **Clinical signs** | | | | |
| ***Faeces, soft*** | | | | | | | | |
| *N* of times | 8 | 72 | 227 | 274 | 11 | 73 | 63 | 128 |
| *N* of dogs | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| ***Faeces, watery*** | | | | | | | | |
| *N* of times | 2 | 2 | 79 | 83 | 6 | 2 | 0 | 45 |
| *N* of dogs | 2 | 2 | 4 | 4 | 4 | 2 | – | 4 |
| ***Mean body weight (kg); mean ± standard deviation*** | | | | | | | | |
| −1 week | 7.588 ± 0.7157# | 7.91 ± 0.8826 | 7.65 ± 0.6494 | 7.73 ± 1.0331 | 6.588 ± 0.7052 | 6.325 ± 0.5694 | 6.600 ± 0.7561 | 6.675 ± 0.4873 |
| 1 week | 7.90 ± 0.8226 | 8.08 ± 0.8420 | 7.500 ± 1.0008 | 7.513 ± 1.2154 | 6.713 ± 0.6395 | 6.463 ± 0.5297 | 6.575 ± 0.8912 | 6.400 ± 0.5244 |
| 4 weeks | 8.550 ± 1.0528 | 8.600 ± 1.2104 | 7.938 ± 1.0419 | 7.663 ± 0.9936 | 7.125 ± 0.6551 | 6.713 ± 0.6369 | 6.938 ± 0.9801 | 6.288 ± 0.8320 |
| 9 weeks | 9.138 ± 1.4109 | 9.625 ± 1.4027 | 8.900 ± 0.9687 | 8.388 ± 0.8740 | 7.788 ± 0.7261 | 7.300 ± 0.9028 | 7.488 ± 1.1521 | 7.238 ± 0.7487 |
| 13 weeks | 9.625 ± 1.4517 | 10.125 ± 1.5135 | 9.100 ± 0.7937 | 8.600 ± 0.9083 | 8.263 ± 0.7016 | 7.700 ± 1.0685 | 7.650 ± 1.1569 | 7.738 ± 0.5498 |
| ***Mean body weight gain (kg); mean ± standard deviation*** | | | | | | | | |
| Weeks −1 to 1 | 0.313 ± 0.1315 | 0.175 ± 0.1190 | −0.150 ± 0.4546 | -0.225 ± 0.2533a | 0.125 ± 0.1190 | 0.138 ± 0.1315 | −0.025 ± 0.1500 | −0.275 ± 0.1041b |
| Weeks 1–2 | 0.100 ± 0.3082 | 0.088 ± 0.0854 | 0.025 ± 0.1190 | -0.150 ± 0.2160 | 0.125 ± 0.1323 | 0.163 ± 0.0750 | 0.175 ± 0.1500 | 0.200 ± 0.0913b |
| Weeks 3–4 | 0.175 ± 0.1658 | 0.075 ± 0.2327 | 0.138 ± 0.2626 | 0.038 ± 0.2720 | 0.088 ± 0.2287 | −0.013 ± 0.1652 | −0.038 ± 0.0854 | −0.013 ± 0.1797 |
| Weeks 12 to 13 | 0.113 ± 0.1750 | 0.075 ± 0.1190 | 0.013 ± 0.2287 | 0.000 ± 0.1080 | 0.300 ± 0.1472 | −0.038 ± 0.2496 | 0.075 ± 0.0500 | 0.000 ± 0.1581 |
| ***Mean food consumption (g/animal per day); mean ± standard deviation*** | | | | | | | | |
| Week −1 | 233.7 ± 24.53 | 261.6 ± 33.65 | 204.8 ± 67.75 | 177.4 ± 53.54 | 201.5 ± 13.35 | 181.0 ± 18.04 | 163.0 ± 32.73 | 130.8 ± 20.16** |
| Weeks 1–2 | 258.6 ± 31.65 | 266.1 ± 28.10 | 186.9 ± 80.32 | 164.9 ± 79.63 | 225.6 ± 20.67 | 220.6 ± 44.76 | 197.8 ± 46.11 | 129.9 ± 19.58** |
| Weeks 3–4 | 243.5 ± 4.31 | 275.5 ± 32.51 | 247.6 ± 50.61n | 192.6 ± 68.96 | 252.8 ± 31.52 | 207.3 ± 40.23 | 191.5 ± 34.25 | 153.5 ± 52.86* |
| Weeks 8–9 | 270.2 ± 46.38 | 296.2 ± 28.74 | 269.0 ± 52.72 | 217.0 ± 41.59 | 276.3 ± 27.63 | 206.5 ± 16.01* | 200.0 ± 37.59** | 201.0 ± 33.34** |
| Weeks 12 to 13 | 249.1 ± 6.67n | 317.9 ± 38.39n | 302.7 ± 35.30n | 248.5 ± 21.81n | 305.9 ± 40.00 | 228.7 ± 15.91* | 220.5 ± 38.11** | 236.8 ± 26.35* |
| | | | | **Blood biochemistry** | | | | |
| ***Alkaline phosphatase (U/L); mean ± standard deviation*** | | | | | | | | |
| Day 48 | 106.3 ± 16.11 | 143.3 ± 52.25 | 112.3 ± 37.09 | 154.3 ± 40.40 | 87.5 ± 14.71 | 87.8 ± 11.62 | 99.5 ± 17.14 | 175.8 ± 63.57** |
| Day 90 | 77.8 ± 8.96 | 111.8 ± 44.33 | 91.5 ± 31.71 | 137.8 ± 42.44 | 70.0 ± 14.79 | 71.0 ± 7.48 | 86.8 ± 35.51 | 145.8 ± 48.84* |

| | Dose (mg/kg bw per day) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Male | | | | Females | | | |
| | 0 | 60 | 150 | 400 | 0 | 60 | 150 | 400 |
| ***Albumin (g/dL)*** | | | | | | | | |
| Day 48 | 3.08 ± 0.096 | 3.03 ± 0.050 | 2.75 ± 0.300 | 2.63 ± 0.050* | 3.10 ± 0.115 | 3.05 ± 0.191 | 2.98 ± 0.150 | 2.60 ± 0.200 |
| Day 90 | 3.20 ± 0.000 | 3.08 ± 0.126 | 2.88 ± 0.263* | 2.75 ± 0.129** | 3.10 ± 0.115 | 3.10 ± 0.141 | 3.00 ± 0.216 | 3.00 ± 0.082 |
| **Organ weights** | | | | | | | | |
| Liver/body weight (percentage); mean ± standard deviation | | | | | | | | |
| | 2.69 ± 0.25 | 2.88 ± 0.28 | 2.93 ± 0.37 | 3.26 ± 0.39 | 2.89 ± 0.25 | 2.94 ± 0.17 | 2.92 ± 0.19 | 3.19 ± 0.30 |

N: Number; n: Inappropriate for statistical analysis; Source: Heward, 2019

Statistically different from control values with significance: * $p < 0.05$, ** $p < 0.01$

In conclusion, the NOAEL for the 90-day oral capsule study in dogs was 60 mg/kg bw per day based on the lower body weights, decreased body weight gains and reduced food consumption in male dogs at 150 mg/kg bw per day (Heward, 2017).

**(b) Dermal application**

To evaluate the toxicity of florylpicoxamid (purity 98.1%; Lot no. EXP-17-BN7923 [TSN313275]) when administered dermally to male and female rats, four groups of young adult male and female Crl: CD(SD) rats (10/sex per group) were administered the test substance dermally for approximately six hours per day, for 29 days, at doses of 0, 100, 300 or 1000 mg/kg bw per day.

No deaths occurred and no clinical or ophthalmological observations were attributed to exposure to the test substance. There were no adverse or test substance-related effects on body weight or food consumption, clinical pathology parameters, organ weights, gross pathology or microscopic pathology.

Under the conditions of this study, the NOAEL for toxicity by dermal application in rats was 1000 mg/kg per day, the highest dose tested (Anderson, 2019).

**(c) Exposure by inhalation**

There were no available data on exposure by inhalation.

## 2.3 Long-term studies of toxicity and carcinogenicity

***Mouse***

To assess the oncogenic potential of florylpicoxamid, 50 male and 50 female Crl:CD1 (ICR) mice per group were treated with florylpicoxamid (purity 98.1% : Lot no. EXP-17-BN7923) at dietary concentrations of 0, 150, 500 or 1500 ppm for 18 months, except females at 1500 ppm where remaining mice were euthanized at week 46 due to high mortality in this group. The actual intakes (except 1500 ppm in females) were equal to 0, 16.4, 55.3 and 172 mg/kg bw per day for males, 0, 20.4 and 72.2 mg/kg bw per day for females. The test item intake at 45 weeks in females in the 1500 ppm group was 230 mg/kg bw per day. Parameters and end-points evaluated in this study were: mortality, clinical observations, body weight, food consumption, food efficiency, compound consumption, neurobehavioural examinations, clinical pathology (haematology), gross necropsy findings, organ weights, and histopathological examinations. Florylpicoxamid and its metabolites (X12485649, X12584261, X12485473 and X12641325) were quantified in mouse blood and urine to evaluate systemic exposure and urinary excretion. Blood and urine samples were collected as part of the 18-month dietary oncogenicity study in which mice consumed diets containing 0, 150, 500 or 1500 ppm of florylpicoxamid. The kinetics of florylpicoxamid and its metabolites were investigated (Chan, 2020b).

All groups except females at 1500 ppm were necropsied as scheduled during week 79. Amongst females at 1500 ppm survival was lower than the control females and less than 50% at week 45; the numbers of surviving female mice at 0, 150, 500 and 1500 ppm were 50, 48, 48 and 23 at week 45. The study report explains that since female mice did not tolerate 1500 ppm, suffering excessive mortality, surviving females at 1500 ppm were euthanized at week 46. As a result no further information, including pathology, was obtained for this group beyond week 46.

No test article-related early deaths or differences in survival rate were observed amongst male groups, or in female groups receiving 150 or 500 ppm when compared to control groups. Survivals were 44/50, 43/50, 44/50 and 47/50 for males, 38/50, 36/50 and 36/50 for females at week 77 for groups receiving 0, 150, 500 or 1500 ppm respectively (1500 ppm for males alone). Specific clinical signs associated with the treatment were not observed in any treated group. Thin appearance (1/50, 5/50, 0/50, 9/50 at 0. 150, 500 and 1500 ppm, respectively) was considered treatment-related in females at 1500 ppm and was associated with the moribund condition in some of the animals that died or were euthanized at week 46. No treatment-related neurobehavioural findings were observed in any treated group of either sex, including females at 1500 ppm (these last up to week 46).

At 1500 ppm, body weights were slightly lower (by 10% or less, but consistently) than those of controls between week 33 and termination in males, and between weeks 12 and 46 in females. Throughout the study body weights were comparable between groups receiving 0, 150 and 500 ppm in males, and between these groups of females. There were no consistent differences in food consumption, in either sex throughout the study for any of the treated groups.

No treatment-related changes in haematology values were observed in either sex. Amongst organ weights recorded it was observed that liver weight relative to body weight was statistically increased in males at 1500 ppm when compared to controls. The increase was considered to be adaptive because no parameter that would indicate hepatotoxicity was observed in this group. No other treatment-related changes in organ weights were observed in either sex. Pathological examinations revealed no dose-dependent, increased incidences of either neoplastic or non-neoplastic change in males at 150, 500 and 1500 ppm, nor in females at 150 or 500 ppm. Significant results for this study are summarized in Table 17.

***Table 17. Major changes observed in the 18-month oncogenicity and toxicity study in mice***

| | | **Dietary concentration (ppm)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **Males** | | | | **Females** | | | |
| | | **0** | **150** | **500** | **1500** | **0** | **150** | **500** | **1500** |
| **Week/s** | | ***Body weight (g)*** | | | | | | | |
| 45 | Mean | 46.1 | 44.6* | 45.9 | 43.7** | 38.9 | 36.3 | 38.4 | 36.0 |
| | SD | 3.90 | 5.16 | 4.56 | 3.77 | 4.87 | 3.80 | 5.26 | 3.24 |
| | *N* | 48 | 47 | 50 | 49 | 50 | 48 | 48 | 23 |
| 53 | Mean | 46.1 | 44.7* | 46.0 | 43.8** | 39.3 | 38.9 | 39.0 | T |
| | SD | 4.78 | 5.45 | 4.56 | 3.79 | 4.62 | 3.96 | 5.33 | T |
| | *N* | 46 | 47 | 50 | 48 | 49 | 46 | 45 | T |
| 77 | Mean | 47.2 | 44.7* | 46.5 | 43.9** | 39.8 | 4.12 | 39.3 | T |
| | SD | 4.64 | 5.73 | 4.92 | 4.18 | 5.37 | 4.95 | 4.96 | T |
| | *N* | 44 | 43 | 44 | 47 | 38 | 36 | 36 | T |
| | | ***Food consumption (g/mouse)*** | | | | | | | |
| 25–26 | Mean | 5.0 | 4.7 | 5.2 | 4.7 | 4.9 | 5.3 | 5.1 | 4.5* |
| | SD | 0.76 | 1.11 | 0.69 | 0.70 | 1.06 | 0.82 | 0.83 | 0.82 |
| 44–45 | Mean | 4.5 | 4.3 | 4.5 | 4.4 | 4.6 | 4.5 | 4.5 | 4.6 |
| | SD | 0.58 | 0.65 | 0.44 | 0.41 | 0.95 | 0.63 | 0.76 | 1.14 |
| 77–78 | Mean | 5.5 | 5.1 | 4.8 | 5.0 | 5.1 | 5.1 | 5.1 | T |
| | SD | 0.76 | 1.08 | 0.77 | 0.53 | 1.49 | 0.88 | 0.66 | T |

| | | Dietary concentration (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | Females | | | |
| | | 0 | 150 | 500 | 1500 | 0 | 150 | 500 | 1500 |
| | | | | | *Liver weight* | | | | |
| Absolute (g) | Mean | 2.53 | 2.37 | 2.78 | 2.65 | 2.06 | 2.21 | 2.00 | T |
| | SD | 0.80 | 0.92 | 1.12 | 0.80 | 0.38 | 0.60 | 0.36 | T |
| | *N* | 40 | 41 | 43 | 47 | 37 | 34 | 33 | T |
| Relative to body weight (%) | Mean | 5.53 | 5.42 | 5.98 | 6.08** | 5.23 | 5.42 | 5.24 | T |
| | SD | 1.58 | 2.06 | 2.10 | 1.70 | 0.69 | 1.30 | 0.69 | T |

*N*: Number of mice examined; SD: Standard deviation; T: Terminated week 46 Source: Albretsen, 2020

Statistically different from control values with significance: * $p < 0.05$, ** $p < 0.01$

The Meeting noted that information relating to carcinogenic potential, such as histopathological results, was not obtained for females at 1500 ppm, therefore carcinogenic potential in females at 1500 ppm could not be judged.

In conclusion, the NOAEL for chronic toxicity in mice was 500 ppm (equal to 55.3 mg/kg bw per day) based on lower body weight in males at 1500 ppm (equal to 172 mg/kg bw per day). The NOAEL for carcinogenicity in male mice was 1500 ppm (equal to 172 mg/kg bw per day), the highest dose test. In females, a dose of 1500 ppm caused excess mortality that prevented proper evaluation of carcinogenicity. No increased cancer incidence was found at 500 ppm in females (equal to 72.2 mg/kg bw per day) (Albretsen, 2020),

***Rat***

To investigate chronic toxicity and carcinogenicity, 80 male and 80 females CD rats per group were administered florylpicoxamid (purity 97%; Lot nos EXP-17-BN7913_A, and EXP-17-BN7913_B) in the diet for 24 months. Concentrations for males were 0, 300, 1000 or 3000 ppm (equal to 0, 11.9, 39.7 and 123 mg/kg bw per day), and for females 0, 300, 1000 or 4000 ppm (equal to 0, 14.0, 46.9 and 200 mg/kg bw per day. At 12 months, 10 male and 10 female rats per group were allocated for interim sacrifice. Parameters and end-points evaluated in this study were: mortality, clinical observations, body weight, food consumption, test material intake and food efficiency, and examinations of neurobehaviour, ophthalmology, haematology, clinical chemistry, and pathology. Since the number of surviving females at 1000 ppm had fallen to 12 by week 102, all remaining females were euthanized at week 103. All remaining males were euthanized during week 105. No significant differences in the survival rate were noted in males or females when compared to the control groups. The premature termination, by two weeks for males and one week for females, with respect to the study plan was not judged to have any influence on assessment of carcinogenicity. The kinetics of florylpicoxamid and its metabolites was investigated. Liver samples were collected from the lateral lobe of animals only at the chronic terminal necropsy (day 365).

No treatment-related changes were observed in clinical observations, palpable mass observations, neurobehaviour or ophthalmology examinations. Females at 4000 ppm showed slightly lower body weights (by ca 10%, but with statistical significance), throughout the study. The slight but continuously lower body weights at 4000 ppm were considered a treatment-related and adverse effect. Lower body weights were also observed in males at 3000 ppm, however the change in this case was not considered to be adverse because of its size (ca 5%) and because it was mostly observed during the earlier half of the treatment period. Body weight gains in 3000 ppm males were comparable to the controls. The decreases in food consumption that were observed several times in females at 4000 ppm were not considered treatment-related due to their inconsistency and their extent; within 10% of controls. There were no treatment-related effects on body weight or food consumption in females at 300 or 1000 ppm, nor in males at any dose level.

Florylpicoxamid did not elicit any effects relating to clinical pathology end-points during the chronic toxicity (3, 6, and 12 months) or carcinogenicity (18 and 24 months) phases.

Liver weights, both absolute and relative to body weight, were slightly (by 20% or less) increased at 3000 ppm in both sexes at 12 months and in males at termination. Microscopically observed incidences of hepatocellular hyperplasia were increased in males and females at 12 months, and in females at 24 months. Since no other finding indicating hepatotoxicity was observed at the highest dose, the liver hypertrophy in males and females at the highest dose was judged to be adaptive.

Histopathological examination of non-neoplastic changes revealed an incidence of minimal or mild pelvic mineralization of the kidneys was much lower in females at 4000 ppm than at lower doses in the two-year carcinogenicity group, where its incidence/rats examined was 52/70, 52/70, 53/70 and 23/70 at 0, 300, 1000 and 4000 ppm. There were no findings relating to this change at 4000 ppm. The decrease was not considered to be toxicologically significant, since a decrease was the opposite to what would be considered an adverse effect on urinary system.

The incidence of basal cell adenoma in the two-year carcinogenicity group was slightly higher in males at 3000 ppm than in other groups (0, 1, 1 and 4 rats at 0, 300, 1000 and 3000 ppm, respectively). This tumour was found at random anatomical sites: ear, dorsal lumber region, ventral thorax or muzzle each in one rat, and right-hind limb in two rats. Basal cell carcinoma, a malignant type of basal cell adenoma, was not observed in males. In females, one basal cell adenoma and one basal cell carcinoma were observed, but only in the 0 ppm group. Whereas the incidence in males at 3000 ppm was slightly above the upper range of historical controls (0/70 to 3/70), the increase was not considered to be treatment-related for the following reasons:

- The tumours' occurrences at random sites indicated that these tumours originated incidentally as gross lesions, but were not found in routine sampling of skin for histopathological examination at necropsy. Tissue components of skin, such as epidermal appendages, typically show variations dependent on the site (for example dorsal or abdominal skin). In this study, no treatment-related changes were seen in skin tissue dissected for routine examination. Since there was no site specificity in the occurrence of tumours, and a lack of any treatment-related findings in the skin with the same components as the random sites where tumours occurred, these tumours were considered incidental.
- Incidences related to basal cell tumour induction, such as hyperplasia, proliferation of basal cells, inflammatory changes or dysplasia, were not observed microscopically at any of the skin sites. This compound does not have any effect on immune function, such as is known to be induced by anticancer drugs (Greaves, 2012a).
- Although rodent basal cell tumours are traditionally considered epidermal neoplasms it appears much more likely that most of them originate from hair germ epithelium (Mecklenburg et al., 2013). In this study, other tumours originating from surface epidermis or the epidermal appendages were not increased at 3000 ppm.

The incidence of malignant astrocytoma was slightly higher in males at 3000 ppm than other groups. The brain tumours were located in the cerebrum or olfactory lobe. No other types of neuroectodermal tumour, such as oligodendroglioma, ependymoma or benign astrocytoma, were observed. The slightly higher incidence of malignant astrocytoma was not considered to be treatment-related for the following reasons:

- The incidence of preneoplastic lesions, including gliosis (Kaufmann et al., 2012), was not increased.
- Florylpicoxamid did not induce an inflammatory reaction or continuous stimulation that led to proliferative activity in the central nervous system.
- Malignant astrocytoma is known to be a frequent finding, but with a low incidence, in the central nervous system (CNS) of male SD rats. Nagatani et al., (2013) conducted a comprehensive analysis of the occurrence of spontaneous CNS tumours including astrocytoma, oligodendroglioma and granular cell tumours in male and female F344 and SD rats, based on over 1000 rats. The results indicated that the rate of incidence of malignant astrocytoma was high among rat CNS tumours. According to this reference, its incidence was consistently higher in males than females in both strains, and higher in SD male rats (mean 2.1%, range 0%–6.7%) than F344 male rats (mean 0.7%, range 0%–4.0%). The

authors also pointed out that occurrence of astrocytoma, oligodendroglioma and granular cell tumour in SD rats was sometimes more than 1%.

- It has been recommended that carcinogenicity of neuroectodermal tumours in rodents be evaluated grouped together as glioma, which includes astrocytoma, oligodendroglioma and ependymoma (Greaves, 2012b). There was no occurrence of other types of glioma at 3000 ppm, suggesting no increase in gliomas as such was caused by the treatment.

Major findings in the long-term study in rats are summarized below in Table 18.

***Table 18. The summary of finding in a long-term study in rats treated with florylpicoxamid***

| | | Dietary concentration (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | Females | | | |
| | | **0** | **300** | **1000** | **3000** | **0** | **300** | **1000** | **4000** |
| Rats at start of study | | 70 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| **Number of survivals:** | | | | | | | | | |
| Week 89 | | 34 | 36 | 39 | 32 | 34 | 29 | 13 | 16 |
| Week 101 | | 22 | 22 | 20 | 19 | 26 | 15 | 13 | 16 |
| **Body weight (g)** | | | | | | | | | |
| Week 1 | Mean | 322.4 | 316.5 | 318.1 | 313.0** | 217.5 | 214.5 | 214.1 | 210.7 |
| | SD[b] | 16.31 | 17.21 | 16.83 | 15.63 | 15.22 | 18.66 | 15.24 | 15.72 |
| Week 13 | Mean | 594.7 | 585.7 | 573.4** | 679.4** | 314.2 | 315.6 | 365.2 | 298.6** |
| | SD | 46.43 | 46.89 | 43.29 | 56.11 | 28.77 | 34.21 | 26.81 | 25.33 |
| Week 29 | Mean | 725.4 | 713.3 | 703.5 | 679.4 | 360.8 | 360.6 | 352.3 | 336.4** |
| | SD | 62.39 | 64.70 | 61.39 | 56.11** | 42.15 | 47.23 | 41.53 | 34.15 |
| Week 53 | Mean | 839.9 | 821.4 | 841.5 | 786.9** | 427.4 | 425.6 | 420.9 | 387.6** |
| | SD | 73.97 | 106.62 | 83.15 | 80.67 | 6.97 | 70.03 | 64.15 | 56.04 |
| Week 77 | Mean | 901.4 | 884.7 | 886.2 | 844.6 | 499.2 | 480.6 | 469.5 | 444.2** |
| | SD | 85.32 | 129.92 | 91.98 | 101.67 | 86.46 | 82.43 | 83.91 | 83.73 |
| Week101 | Mean | 865.6 | 894.5 | 886.5 | 855.9 | 582.0 | 534.5 | 471,3** | 485.1* |
| | SD | 101.59 | 133.62 | 131.65 | 91.51 | 95.22 | 91.33 | 109.87 | 121.15 |
| **Food consumption (g)** | | | | | | | | | |
| Week 1–2 | Mean | 23.6 | 23.8 | 23.3* | 23.9 | 15.5 | 15.5 | 14.7** | 14.0** |
| | SD | 3.17 | 1.99 | 1.64 | 1.69 | 1.38 | 1.77 | 1.20 | 1.12 |
| Week 12–13 | Mean | 25.3 | 25.1 | 25.0 | 24.5 | 16.2 | 16.1 | 16.2 | 1.52* |
| | SD | 3.37 | 3.42 | 2.82 | 4.46 | 2.25 | 2.22 | 2.36 | 2.60 |
| Week 28–29 | Mean | 26.4 | 26.2 | 25.9 | 26.1 | 16.2 | 15.8 | 15.9 | 16.5 |
| | SD | 3.15 | 2.75 | 2.42 | 3.69 | 2.76 | 2.03 | 1.70 | 3.24 |
| Week 52–53 | Mean | 25.0 | 24.6 | 25.9 | 25.3 | 16.5 | 15.7 | 16.5 | 16.2 |
| | SD | 5.33 | 4.38 | 2.77 | 3.40 | 2.74 | 3.00 | 2.07 | 2.25 |
| Week 76–77 | Mean | 24.8 | 24.9 | 25.0 | 25.2 | 17.3 | 16.3 | 15.3 | 16.6 |
| | SD | 3.30 | 6.27 | 3.75 | 5.56 | 3.07 | 4.58 | 4.97 | 4.54 |
| Week 100–101 | Mean | 22.9 | 22.9 | 23.2 | 25.1 | 19.0 | 16.8 | 16.5 | 16.6 |
| | SD | 4.60 | 5.61 | 6.20 | 5.03 | 2.99 | 5.24 | 4.18 | 3.15 |

| | | Dietary concentration (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | Females | | | |
| | | 0 | 300 | 1000 | 3000 | 0 | 300 | 1000 | 4000 |
| **Liver weight, relative to body weight (%)** | | | | | | | | | |
| 12 months | N | 10 | 10 | 10 | 9 | 10 | 10 | 10 | 10 |
| | Mean | 2.50 | 2.50 | 2.69 | 2.88 | 2.74 | 2.78 | 3.02 | 3.02 |
| | SD | 0.43 | 0.20 | 0.24 | 0.33 | 0.24 | 0.31 | 0.43 | 0.29 |
| 24 months | N | 15 | 18 | 16 | 16 | 25 | 15 | 12 | 15 |
| | Mean | 2.48 | 2.57 | 2.58 | 2.92 | 2.62 | 2.59 | 3.03 | 2.71 |
| | SD | 0.40 | 0.49 | 0.45 | 0.42 | 0.92 | 0.33 | 0.75 | 0.34 |
| **Histopathology (incidence/rats examined)** | | | | | | | | | |
| ***Hepatocellular hypertrophy*** | | | | | | | | | |
| 12 months | | 1/10 | 0/10 | 1/10 | 6/10 | 1/10 | 0/10 | 1/10 | 8/10 |
| 24 months | | 2/70 | 1/70 | 1/70 | 3/70 | 0/70 | 0/70 | 0/70 | 13/70* |
| ***Skin*** | | | | | | | | | |
| Basal cell adenoma | | 0/70 | 1/70[a] | 1/70[a] | 4/70[a] | 1/70[a] | 0/70 | 0/70 | 0/70 |
| Basal cell carcinoma | | 0/70 | 0/70 | 0/70 | 0/70 | 1/70[b] | 0/70 | 0/70 | 0/70 |
| Keratoacanthoma | | 2/70 | 3/70 | 3/70 | 4/70 | 0/70 | 0/70 | 0/70 | 0/70 |
| Papilloma | | 2/70 | 3/70 | 0/70 | 0/70 | 0/70 | 0/70 | 0/70 | 0/70 |
| Hyperplasia, focal epiderma, head | | 1/70 | 0/70 | 0/70 | 0/70 | 0/70 | 0/70 | 0/70 | 0/70 |
| Hyperplasia, focal epiderma, lip | | 0/70 | 0/70 | 1/70 | 0/70 | 0/70 | 0/70 | 0/70 | 0/70 |
| Hyperplasia, focal epiderma, lumber | | 0/70 | 0/70 | 0/70 | 0/70 | 0/70 | 0/70 | 0/70 | 1/70 |
| Hyperplasia, focal epiderma, tail | | 0/70 | 0/70 | 0/70 | 0/70 | 0/70 | 1/70 | 0/70 | 0/70 |
| ***Neuroectodermal neoplasms and preneoplastic findings in CNS (brain and spinal cord)*** | | | | | | | | | |
| Brain, astrocytoma, malignant | | 1/70[c] | 1/70 (f) | 0/70 | 3/70[c,d,e] | 0/70 | 0/70 | 0/70 | 1/70[c] |
| Cervical spinal cord, astrocytoma malignant | | 0/70 | 0/70 | 0/70 | 0/70 | 1/70i | 0/70 | 0/70 | 0/70 |
| Cerebrum, gliosis focal | | 0/70 | 0/70 | 0/70 | 0/70 | 1/70 | 0/70 | 0/70 | 0/70 |
| Medulla, gliosis focal | | 0/70 | 0/70 | 0/70 | 0/70 | 0/70 | 0/70 | 0/70 | 1/70 |
| Olfactory lobe, gliosis focal | | 0/70 | 0/70 | 0/70 | 1/70 | 0/70 | 0/70 | 0/70 | 0/70 |

SD: Standard deviation; Source: Albretsen, 2019a

[a] The basal cell adenoma was not a fatal tumour;

[b] Fatal tumour;

[c] Each tumour at 0, 300 or 3000 ppm located in the olfactory lobe was not metastasized or fatal;

[d] One tumour at 3000 ppm located in the olfactory lobe was not metastasized but was considered fatal;

[e] One tumour at 3000 ppm located in the cerebrum was not metastasized or fatal;

Statistically different from control values with significance: * $p < 0.05$, ** $p < 0.01$

In conclusion, the NOAEL for chronic toxicity was 1000 ppm equal to 46.9 mg/kg bw per day based on the low body weight in females at 4000 ppm. The NOAEL for carcinogenicity was 3000 ppm equal to 123 mg/kg bw per day, the highest dose tested. Florylpicoxamid was not carcinogenic in rats. (Albretsen, 2019a)

## 2.4 Genotoxicity

Experimental data from in vitro and in vivo studies are summarized in Table 19.

***Table 19. Summary of in vitro and in vivo genotoxicity studies on florylpicoxamid***

| Test | Test system | Concentration and test substance | End-point | Reference |
|---|---|---|---|---|
| **In vitro genotoxicity tests** | | | | |
| Bacterial reverse mutation | *Salmonella typhimurium* TA1537, TA1535, TA98, TA100, TA102 | Florylpicoxamid ± S9 activation at:<br>Trial 1: 0, 156.25, 312.5, 625, 1250, 2500, 5000 µg/plate<br>Trial 2: 0, 51.2, 128, 320, 800, 2000, 5000 µg/plate | Non-mutagenic | Tendulkar, 2016a |
| Bacterial reverse mutation | *Salmonella typhimurium* TA 1537, TA 1535, TA 98, TA 100 *Escherichia coli* WP2 *uvr*A | Florylpicoxamid ± S9 activation at:<br>0, 333, 667, 1000, 3333 and 5000 µg/plate | Non-mutagenic | Myhre, 2019a |
| Mammalian chromosomal aberration | Human peripheral blood lymphocytes | Florylpicoxamid ± S9 activation at:<br>3 h 30 minutes with/without S9: 0, 7.8125, 15.625, 31.25, 62.5, 125, 250 µg/mL<br>24 h 30 minutes without S9: 0, 7.8125, 15.625, 31.25, 62.5, 125, 250 µg/mL | Non-clastogenic | Tendulkar, 2016b |
| Mammalian cell micronucleus | Human peripheral blood lymphocytes | Florylpicoxamid ± S9 activation at:<br>4 hours, with S9: 0, 2.0, 4.0, 6.0, 10, 20, 30, 45, 60 µg/mL<br>4 hours, without S9: 0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20 µg/mL<br>24 hours, without S9: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0 µg/mL | Neither clastogenic nor aneugenic | Xie, 2019a |
| Mammalian forward mutation | CHO/HPRT | Florylpicoxamid ± S9 activation at:<br>0, 62.5, 125, 250, 500, 1000, 2000 µg/mL | Non-mutagenic | Vashi, 2017 |
| Mammalian forward mutation | CHO/HPRT | Florylpicoxamid :<br>With S9 at:<br>0, 7.81, 15.6, 31.3, 62.5, 125, 250 µg/mL<br>Without S9 at:<br>0, 1.96, 3.91, 7.81, 15.6, 31.3, 62.5 µg/mL | Non-mutagenic | Aquino, 2019 |
| **In vivo genotoxicity tests – somatic cells** | | | | |
| Integrated 90-day dietary toxicity study with in vivo micronucleus test | Rat F344/DuCrl, Males and Females | Florylpicoxamid administered via the diet at 0, 300, 1000 and 3000 ppm<br>Males: 0, 18.2, 59.4, 177 mg/kg bw/day<br>Females: 0, 18.8, 63.0, 185 mg/kg bw/day | Neither clastogenic nor aneugenic | Mukerji, 2019 |

CHO: Chinese hamster ovary; HPRT: Hypoxanthine–guanine phosphoribosyl transferase;
S9: Rat liver supernatant fraction obtained by centrifuging at 9000 g

### (a) In vitro studies

Reverse mutation in *Salmonella typhimurium* (strains TA1537, TA1535, TA98, TA100 and TA102) was evaluated in a bacterial reverse mutation test. Florylpicoxamid (purity 97.1%; Lot no. YN4-156527-095, 9) was tested in two independent trials, with and without metabolic activation. Bacterial cultures were exposed to florylpicoxamid at six concentrations (three replicate plates per concentration) between 51.2 and 5000 µg/plate. From a preliminary cytotoxicity test, 5000 µg/plate was selected as the top dose as indicated in the guidelines for testing and showing moderate cytotoxicity. After 48 hours of incubation at 37 ± 1°C, any revertant colonies were scored.

Florylpicoxamid did not induce any significant increase in the number of revertants in any of the five tester strains in either trial, with or without S9 mix. Values for negative controls were all within historical control ranges for the laboratory, and positive controls showed an increase in the number of revertant colonies, thus demonstrating the efficiency of the test system. All criteria for a valid study were met as described in the protocol. From the results of this study, under the specified experimental conditions, florylpicoxamid was concluded to be non-mutagenic in the bacterial reverse mutation assay using *Salmonella typhimurium* (Tendulkar, 2016a).

Reverse mutation in *Salmonella typhimurium* (strains TA1537, TA1535, TA98 and TA100) and Escherichia coli strain WP2 uvrA was evaluated in a bacterial reverse mutation test. Florylpicoxamid (purity, 85.9%; Lot no. ENBK-172341-001 [TSN400570]) was tested in two independent trials, with and without metabolic activation. In the toxicity-mutation trial, bacterial cultures were exposed to florylpicoxamid at eight concentrations (two replicate plates per concentration) between 33.3 and 5000 µg/plate. Based on the toxicity-mutation test, 5000 µg/plate was selected for the mutagenicity test, as the recommended top dose indicated by following the guidelines. In the mutagenicity trial, bacterial cultures were exposed to florylpicoxamid at five concentrations (three replicate plates per concentration) between 333 and 5000 µg/plate. After 50–71 hours of incubation at 37 ± 2°C, revertant colonies were scored.

Florylpicoxamid did not induce any significant increase in the number of revertants in any of the five tester strains in either trial, with or without S9 mix. The values for negative controls were all within historical control ranges for the laboratory, and positive controls showed an increase in the number of revertant colonies, thus demonstrating the efficiency of the test system. All criteria for a valid study were met as described in the protocol. From the results of this study, under the specified experimental conditions, florylpicoxamid was concluded to be non-mutagenic in the bacterial reverse mutation assay using *Salmonella typhimurium* and *Escherichia coli*. (Myhre, 2019a),

In a mammalian chromosome aberration test, florylpicoxamid (purity 97.1%; Lot no. YN4-156527-095) was tested in two independent experiments, with and without metabolic activation; a 2% (v/v) mix of rat liver supernatant fraction obtained by centrifuging at 9000 g (S9). Human peripheral blood lymphocyte cultures were exposed to florylpicoxamid at six dose levels (two cultures for each dose level) between 7.81 and 250 µg/mL of test article.

Florylpicoxamid did not induce statistically significant or biologically relevant increases in the number of cells with chromosome aberrations in the absence or presence of S9 mix in either of the two independently conducted experiments. No effect on the number of polyploid cells or number of cells with endoreduplicated chromosomes was observed either in the absence or presence of S9-mix. All negative controls were within the historical limits and positive controls showed an increase in the incidence of cells with chromosome aberrations. All criteria for a valid study were met as described in the protocol. From the results of this study it was concluded that florylpicoxamid did not show potential to induce chromosomal aberrations in either the absence or presence of a metabolic activation system under the described experimental conditions, and was negative for clastogenicity (Tendulkar, 2016b).

In a mammalian cell micronucleus assay, human peripheral blood lymphocytes were exposed to florylpicoxamid (purity 85.9%; Lot no. ENBK-172341-001 [TSN400570]) at different concentrations, both in the absence and presence of an Aroclor-induced S9 activation system. The test substance was formulated in dimethyl sulfoxide (DMSO) and formed a clear solution at 500 mg/mL, the highest stock concentration tested. Based upon results of the preliminary study, the doses chosen for the definitive micronucleus assay were 2.5, 5, 7.5, 10, 12.5, 15, 17.5 and 20 µg/mL for the nonactivated four-hour group, 2.0, 4.0, 6.0, 10, 20, 30, 45 and 60 µg/mL for the S9 activated four-hour exposure group, and 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8 and 1.0 µg/mL for the nonactivated 24-hour group.

Visible precipitate was observed at doses of 45 and 60 µg/mL at the beginning of treatment in the S9-activated, four-hour exposure group. Cytotoxicity, to the extent of 350% of cytokinesis-blocked proliferation index (CBPI) relative to the vehicle control, was observed at doses of 10, 12.5, 15, 17.5 and 20 µg/mL in the nonactivated four hour exposure group, at doses of 30, 45 and 60 µg/mL in the S9-activated four hour exposure group, and at doses 0.6, 0.8 and 1.0 µg/mL in the nonactivated 24-hour exposure group. The doses selected for evaluation of micronuclei were 2.5, 5 and 10 µg/mL for the nonactivated four-hour group, 10, 20 and 30 µg/mL for the activated four hour group, and 0.2,

0.4 and 0.6 µg/mL for the nonactivated 24 hour group. No significant or dose-dependent increases in micronuclei induction were observed at any dose in any treatment group, with or without activation ($p$ was greater than 0.05 in Fisher's exact and Cochran–Armitage tests). All positive and vehicle control values were within acceptable ranges, and all criteria for a valid assay were met. From the results of this study, it is concluded that florylpicoxamid was negative in the in vitro mammalian cell micronucleus assay in human peripheral blood lymphocytes under the conditions and according to the criteria of the test protocol (Xie, 2019a).

A mammalian cell gene mutation assay [*hprt* locus], in Chinese hamster ovary (CHO) CHO-K1 cells cultured in vitro, was conducted in two independent experiments. Cultures were exposed to florylpicoxamid (purity 97,1%; Lot no. YN4-156527-095 [TSN311927]) at six concentration levels (two replicate cultures per level) between 62.5 and 2000 µg/mL of culture medium, values selected from a preliminary cytotoxicity test, both in the absence and presence of metabolic activation, a 2% (v/v) mix of S9 mix, for a period of four hours.

No significant concentration-related increase in the mutation frequency was observed at any of the treatment concentrations between 62.5 and 2000 µg/mL in either the absence and presence of metabolic activation, and the induced mutation frequency was comparable to that in the negative control group. All negative controls were within the historical limits and positive controls showed an increase in mutant frequency. During both trials neither pH nor osmolality were observed to influence metabolic activity, whether in the presence or absence of metabolic activation. All criteria for a valid study as described in the protocol were met. Based on the results from this study, it is concluded that florylpicoxamid did not have potential to induce gene mutations at the *hprt* locus of CHO-K1 cells, neither in the absence nor presence of metabolic activation under the present experimental conditions. (Vashi, 2017)

In a mammalian cell gene mutation assay (*hprt* locus) of florylpicoxamid (purity 85.9%; Lot no. ENBK-172341-001 [TSN400570]), CHO-K1-BH4 cells cultured in vitro were tested. The vehicle was DMSO. As contamination of the doses was observed during the preliminary toxicity assay, it was repeated. In this retest, carried out with metabolic activation, the concentrations tested were 3.91, 7.81, 15.6, 31.3, 62.5, 125, 250, 500, 1000 and 2000 µg/mL. The maximum concentration evaluated approximated to the limit dose for this assay. Visible test substance precipitation was observed at concentrations of 62.5 µg/mL and above with S9, µg/mL 31.3 µg/mL and above without S9 at the beginning of treatment; at the end of treatment these values were 125 µg/mL and above with S9, and 31.3 µg/mL and above without S9. Adjusted relative survival was 34.60% and 13.88% at concentrations of 125 µg/mL and 62.5 µg/mL, with and without S9 respectively. Adjusted relative survival was approximated 0% at all higher concentrations with or without S9. Based upon these results the concentrations chosen for the definitive mutagenicity assay were 7.81, 15.6, 31.3, 62.5, 125 and 250 µg/mL with S9, and 1.96, 3.91, 7.81, 15.6, 31.3 and 62.5 µg/mL without S9.

In the initial definitive mutagenicity assay, visible precipitate was observed at concentrations of 15.6 µg/mL and above at the beginning of treatment, and at the end of treatment, at 250 µg/mL and above with S9 activation, and 15.6 µg/mL and above without S9 activation. The average absolute cloning efficiency of vehicle controls was greater than 60% (without S9). Therefore, this condition was repeated with the same dose levels.

Cultures treated at all concentrations with S9 were chosen for mutant selection. No dose-dependent increases or statistical trends in mutant frequency, as compared to the concurrent vehicle controls, were observed at any concentration evaluated with S9 ( $p$ = 0.01 or greater). All values were within the historical control 95% confidence interval.

In the repeat definitive mutagenicity assay without S9, visible precipitation was observed at concentrations of 15.6 µg/mL and above at the beginning of treatment, and by the end of treatment at 31.3 µg/mL and above. The average adjusted relative survival was 13.84% at a concentration of 62.5 µg/mL without S9. Concentration values of 1.96, 3.91, 7.81, 15.6 and 31.3 µg/mL were chosen for mutant selection. No dose-dependent increases or statistical trends in mutant frequency, as compared to the concurrent vehicle controls, were observed at any concentration evaluated without S9 ( $p$ = 0.01 or greater). All values were within the historical control 95% confidence interval.

All negative controls were within the historical limits and positive controls showed an increase

in mutant frequency. During both trials neither pH nor osmolality were observed to influence metabolic activity, whether in the presence or absence of metabolic activation

All criteria for a valid study were met as described in the protocol. Based on the results from this study, it was concluded that under the present experimental conditions florylpicoxamid did not have potential to induce gene mutations at the *hprt* locus of CHO-K1-BH4 cells, either in the absence or presence of metabolic activation (Aquino, 2019).

**(b) In vivo studies**

One in vivo micronucleus assay performed in peripheral blood reticulocytes was included in a 90-day dietary toxicity study in rats. For the micronucleus part of the study five male and five female F344/DuCrl rats per group were given test diets containing florylpicoxamid (purity 96.8%; Lot no. YN4-156527-094, TSN311503) at concentrations of 0, 300, 1000 or 3000 ppm for at least 90-days. Peripheral blood was collected from five animals of each sex per dose and four blood smears were made. Wedge smears were fixed in methanol and stained with acridine orange, then anonymized and manually scored for micronuclei under the microscope. All slides were scored according to OECD GD 474 (2014).

Compared to controls, males in the 3000 ppm group showed treatment-related decreases in body weight, body weight gain and food consumption throughout treatment period. There were no treatment-related or statistically identified differences in micronucleated reticulocyte frequencies in any of the dose groups when compared to their respective controls. A significant increase in the frequency of micronucleated cells was observed in the CPA positive control group. All criteria for a valid study were met as described in the protocol. Based on the results of this study it was concluded that florylpicoxamid does not have potential to induce in vivo clastogenic or aneugenic effects (Mukerji, 2019).

## 2.5 Reproductive and developmental toxicity

**(a) Multigeneration studies**

In a screening study for reproduction toxicity, groups of ten male and ten female Crl: CD(SD) rats were administered florylpicoxamid (purity 96.3%; Batch no. EXP-16-BH2635 [TSN312284]) in the diet at concentrations of 0, 300, 1000 or 3000 ppm (equal to 0, 24.3, 79.9 and 238 mg/kg bw per day for males). Time-weighted average doses in females were calculated as 0, 23.9, 75.9 or 227 mg/kg bw per day during prebreeding, 0, 23.2, 75.1 or 222 mg/kg bw per day during the gestation, and 0, 26.4, 86.3 or 269 mg/kg bw per day during lactation. Females were exposed for two weeks prior to breeding, through breeding (two weeks), gestation (three weeks), and lactation (three weeks). Females were necropsied during the period postpartum day (PPD) 22–24. The males were exposed for two weeks prior to breeding and exposure continued through breeding (two weeks) up until necropsy on day 31 of the study. Effects on gonadal function, mating behaviour, conception, development of the conceptus, parturition and early postnatal growth, and survival were evaluated. In addition the following examinations were conducted in adults: thyroid hormone measurement, gross necropsy, and histopathology with an emphasis on organs of the reproductive system. As for examinations of the offspring, assessments were made of litter size, survival, sex, body weight, and the presence of gross external morphological alterations. Blood and milk were collected for measurement of florylpicoxamid and related metabolites. Collections included: blood in males and females near the end of the prebreeding period, adult female blood and milk on lactation day (LD) 4, and pup blood on postnatal day (PND) 4 to evaluate systemic exposure.

There were no treatment-related differences in body weight for males or females at any exposure level tested throughout the duration of the study. Similarly, there were no treatment-related differences in the body weight gain of females at any exposure level tested during the gestation or lactation periods. Males at 3000 ppm and females at 1000 and 3000 ppm had statistically significant decreases in food consumption compared to controls on test days 1–4, possibly due to a slight palatability effect. There were no treatment-related differences in the amount of food consumed by any of the treated males or females when compared to their respective controls throughout the remainder of the study.

There were no treatment-related effects at any dose level on mating, conception, fertility, time to mating, gestation length, post-implantation loss, pup survival, or the sex ratio. There were no treatment-related effects on litter size or pup body weight at any dose level tested. There were

no treatment-related differences in thyroxine (T4), triiodothyronine (T3) or thyroid-stimulating hormone (TSH) for males or females on test day 8 at any dose level tested. Males at 1000 or 3000 ppm had treatment-related higher (but not statistically significant) mean relative liver weights (by 9.7% and 8.9%, respectively) compared to controls. Females at 3000 ppm had a higher mean absolute liver weight (by 7.1%) compared to controls. The higher liver weights corresponded to increases in the incidence of very slight hypertrophy of hepatocytes in centrilobular/midzonal areas, with increased cytoplasmic eosinophilia in males at 1000 and 3000 ppm, and in females at 3000 ppm. The liver hypertrophy without hepatotoxic indicators at 1000 and 3000 ppm was considered to be adaptive.

The NOAELs for parental, reproductive and offspring toxicity were all 3000 ppm (equal to 222 mg/kg bw per day), the highest dose tested (Passage, 2018d)

A main two-generation dietary reproduction toxicity study was conducted. Groups of 25 male and 25 female rats were fed diets containing 0, 300, 1000, 3000 ppm (males only) or 4000 ppm (females only) florylpicoxamid (purity 98.1%; Lot no. EXP-17-BN7923 [TSN313275]) for approximately 10 weeks prior to breeding, and through breeding, gestation, and lactation for two generations. From the corresponding time-weighted average doses, 0, 17.5, 58.4, and 179 mg/kg bw per day for males and 0, 19.3, 65.3 and 255 mg/kg bw per day during gestation of the first generation parent (F0) were selected, based on the established guidance (WHO, 2017). In-life parameters included clinical observations, food consumption, body weights, reproductive performance, pup survival, pup body weights, anogenital distance, and puberty onset. Estrous cyclicity was checked daily for all F0 and F1 parental females for three weeks prior to mating and during cohabitation until each female was sperm- or plug-positive, or until the two-week mating period ended. On the day of scheduled necropsy, vaginal cytology was checked for all F0 and F1 parental female rats. In adults, gross pathology, histopathology, organ weights, oocyte quantitation, and sperm count, motility, and morphology were evaluated. Examinations for urinalysis, haematology and blood chemistry were conducted using weanling offspring of the F1 generation. The rats used were chosen, one of each sex per litter, the total number of rats examined being 10 of each sex per group. Among offspring, postmortem evaluations in weanlings included gross pathology, histopathology of the offspring of F0 parents only, and organ weights. Preputial separation and vaginal opening were checked for indicators of puberty onset in F1 and F2 pups. In offspring of F1 females (10 per group), vaginal cytology from vaginal opening to first estrus was checked to investigate the first ovulation.

Blood and milk were collected for toxicokinetic analysis of florylpicoxamid and related metabolites. Collections included: blood in males and females near the end of the prebreeding period, adult female blood and milk on LD 4, and pup blood on PND 4 to evaluate systemic exposure (See Chan, 2019).

The major changes observed in this study are summarized in Table 20. There were no treatment-related clinical observations in the parental animals or offspring either generation. Males at 3000 ppm and females at 4000 ppm showed decreased food consumption (statistically significant) on study days 1–5 in both generations when compared to controls. Decreases were not observed thereafter. As the transient decreases were considered to be due to low palatability, they not were considered adverse. Parental male body weights at 3000 ppm when compared to controls, were lowered by around 10% or less throughout the majority of the study in both generations; these slight but consistent decreases were considered to be adverse. Body weight and body weight gain in females were comparable in all groups tested, including controls, during the premating, gestation and lactation periods.

In urinalysis, F1 females at 4000 ppm produced a lower mean urine volume (statistically significant) with a higher mean specific gravity. The most common cause of decreased urine volume and increased urine specific gravity is dehydration. However, there were no changes in other clinical pathology parameters consistent with dehydration (increased serum proteins or red cell mass parameters), and no corresponding changes in urine volume or specific gravity were observed in males. Therefore, the differences in urine volume and specific gravity in F1 females were not considered to be toxicologically significant. There were no treatment-related effects on urinalysis parameters of F1 males at any dose level. There were no treatment-related changes in haematology, coagulation parameters or clinical chemistry in F1 adults except lower glucose in females at 4000 ppm. This change was not considered to be toxicologically significant because it was small (ca 10%) and there were no other related findings, including effects on body weight.

Relative liver weights in males at 3000 ppm were slightly (ca 10%) increased in parental F0 and F1 males. The increases were not observed in females. Incidences of very slight to slight hepatocellular hypertrophy with eosinophilia were observed to be a corresponding finding in both generations. Liver hypertrophy without any indication of hepatotoxicity was considered to be adaptive. In F1 males, relative weights of testes, epididymides and thyroids were slightly increased. There was no corresponding morphological changes. Weight changes in testes and epididymides were not considered to be toxicologically significant as they were increases, not decreases, and there were no other related changes. The increase in thyroid weight was not considered to be adverse as it was only small and there were no corresponding morphological alterations. In F0 females, increases in relative ovary weights (by ca 10%) and absolute and relative uterus weights (by ca 30%) were recorded at 4000 ppm. No corresponding histopathological changes were observed. The increase in uterus weight was considered not to be treatment-related, but to reflect the random stage of estrus at the time of weight measurement. The increase in ovary weights was not considered to be adverse since it was small and no corresponding morphological changes were seen at this dose. Microscopically no treatment-related changes other than liver hypertrophy were observed in either males or females from both generations. There were no treatment-related changes in ovarian follicle counts in females at 4000 ppm, or in sperm parameters in F0 or F1 males.

There were no effects of treatment in either generation, at any dose level, on mating, conception, fertility or gestation indices, post-implantation loss, time of mating, gestation length, pup survival, or pup sex ration. In the observation made on F0 and F1 pups, there were no treatment-related litter observations at any dose level. Litter size, pup body weight and anogenital distance were unchanged by treatment. With respect to puberty onset, no effect was observed in the timing of preputial separation in F1 males. Data from F1 females at 4000 ppm demonstrated a statistically significant approximately four-day delay in vaginal opening, but the delay was not observed in the second generation. No effects on reproductive organs were observed in either generation. Florylpicoxamid had no steroid hormonal or anti-hormonal effects (Verkaart, 2020). Taken alongside other evidence, the inconsistent delay in vaginal opening was not considered to be adverse.

Investigating weanling rats, slight but statistically significant decreases in Hb, Ht and mean corpuscular volume (MCV) were detected in F2 males and females at 4000 ppm. Similar findings on Hb, Ht and MCV were seen in adult rats in other repeat-dose dietary studies at 3000 and 5000 ppm (Heward, 2018; Murphy et al., 2018). Thus, the consistent haematological effects observed at 4000 ppm in F1 weanlings developing into adult males, were considered to be treatment-related. However these changes were not unique offspring effects nor representative of developmental toxicity. In F1 females cholesterol levels were slightly increased at 4000 ppm. The increase was not considered to be adverse due to the absence of related changes in the liver, and also the limited size of increase. At the highest dose terminal body weights in F1 males and females on PND 22 were slightly lower than for controls (by 12% in males, 8% in females). On PND 22, absolute weights of thymus in F1 females and spleen were slightly lowered (by ca 20%) in F1 males and females at 4000 ppm compared with controls. Relative weights of these organs were not changed, nor were there any histopathological findings in related organs. Taken together, the decreases in both sexes were considered to effects secondary to the body weight changes, and not considered to be adverse.

*Table 20. Summary of results from the main reproductive toxicity study of florylpicoxamid in rats*

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | First generation | | | | Second generation | | | |
| | 0 | 300 | 1000 | 3000/ 4000 | 0 | 300 | 1000 | 3000/ 4000 |
| **Body weight (g); mean ± standard deviation** | | | | | | | | |
| ***Adult males*** | | | | | | | | |
| *N* | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 24 |
| Day 1 | 189.1 ±9.6 | 188 ±10.7 | 186.9 ±9.8 | 187 ±9.3 | 114.8 ±17.9 | 113.2 ±14.4 | 119.4 ±14.1 | 111.1 ±11.6 |
| Day 29 | 399.6 ±36.9 | 383.7 ±27.6 | 382.1 ±25.6 | 374.9* ±24.6 | 361.4 ±35.6 | 355.2 ±32.9 | 362.9 ±30.7 | 342.1 ±25.3 |
| Day 93 | 576.9 ±66.2 | 541.9 ±56.9 | 551 ±53.3 | 524.2* ±40.6 | 604.7 ±44.6 | 584 ±66 | 601.9 ±57 | 559.8* ±39.8 |
| Day 121 | 628.2 ±75.7 | 591.4 ±66.5 | 595 ±64.1 | 568.1* ±49.1 | 650.9 ±50.3 | 630.3 ±70.2 | 651.2 ±70.5 | 599.1* ±45 |
| Day 135 | NE | NE | NE | NE | 665.4 ±53.1 | 642.9 ±73.4 | 666.8 ±76.3 | 612.9* ±52.6 |
| ***Adult females*** | | | | | | | | |
| *N* | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 |
| Day 1 | 141.6 ±9.0 | 142.0 ±9.9 | 142.2 ±8.1 | 141.0 ±9.8 | 112.3 ±14.5 | 106.7 ±110.6 | 110.6 ±10.7 | 102.6 ±17.4 |
| Day 29 | 215.5 ±18.6 | 215.6 ±18.4 | 217.1 ±20.4 | 211.0 ±16.2 | 225.1 ±18.7 | 227.0 ±23.8 | 223.1 ±17.5 | 223.1 ±19.2 |
| *N* | 21 | 22 | 20 | 23 | 19 | 22 | 23 | 22 |
| GD 21 | 431.1 ±42.1 | 422.0 ±32.8 | 431.3 ±19.9 | 415.7 ±26.9 | 458.5 ±39.0 | 468.4 ±42.5 | 453.4 ±37.2 | 463.3 ±37.2 |
| **Food consumption (g/rat per day); mean ± standard deviation** | | | | | | | | |
| ***Adult males*** | | | | | | | | |
| *N* | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 |
| Days 1–5 | 22.9 ±1.7 | 21.6* ±1.3 | 20.6* ±1.4 | 19.1* ±1.3 | 18.2 ±1.9 | 17.9 ±1.8 | 18 ±1.8 | 16* ±2 |
| Days 5–8 | 24.1 ±1.7 | 23.0 ±1.5 | 22.3* ±1.6 | 23.2 ±1.9 | 19.9 ±2.5 | 19.5 ±1.9 | 20.4 ±2.0 | 18.7 ±2.5 |
| Days 29–36 | 2.67 ±3.2 | 2.42* ±2.4 | 24.6* ±2.3 | 2.46* ±2.4 | 29.3 ±2.1 | 28.3 ±3.0 | 28.7 ±2.8 | 28.3 ±2.7 |
| Days 93.95–100 | 28.6 ±3.4 | 25.7* ±2.9 | 25.7* ±2.8 | 25.5* ±2.5 | 27.3 ±2.3 | 25.66 ±2.2 | 26.9 ±4.9 | 26.3 ±2.3 |
| Days 114–121 | 27.3 ±3.0 | 24.6* ±2.6 | 25.4* ±2.5 | 24.9* ±2.5 | 26.5 ±1.9 | 25.3 ±2.6 | 27.0 ±3.5 | 25.6 ±2.6 |
| ***Adult females*** | | | | | | | | |
| *N* | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 |
| Days 1–5 | 16.7 ±5.2 | 15.5 ±1.2 | 15.4 ±1.2 | **13.8**[#] ±1.1 | 16.7 ±1.5 | 16.1 ±1.5 | 16 ±1.4 | 14.4* ±1.8 |
| Days 5–8 | 15.6 ±1.6 | 15.4 ±1.3 | 15.4 ±1.5 | 15.7 ±1.0 | 17.0 ±1.4 | 17.1 ±1.5 | 16.7 ±1.4 | 16.2 ±1.8 |
| Days 29–36 | 16.3 ±1.9 | 16.6 ±1.6 | 16.6 ±2.9 | 16.5 ±2.5 | 18.5 ±1.5 | 18.3 ±1.9 | 17.9 ±1.6 | 18.0 ±2.3 |

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | First generation | | | | Second generation | | | |
| | 0 | 300 | 1000 | 3000/ 4000 | 0 | 300 | 1000 | 3000/ 4000 |
| *N* | 21 | 23 | 20 | 23 | 21 | 22 | 23 | 22 |
| GDs 14–21 | 23.2 ±2.6 | 22.3 ±2.9 | 22.9 ±2.6 | 22.0 ±1.9 | 22.6 ±3.0 | 23.3 ±2.8 | 22.0 ±2.6 | 24.0 ±3.5 |
| *N* | 22 | 23 | 20 | 23 | 21 | 21 | 23 | 22 |
| LDs 19–21 | 77.0 ±8.7 | 78.0 ±11.1 | 76.1 ±12.3 | 78.0 ±8.6 | 76.2 ±13.3 | 76.3 ±12.2 | 73.4 ±11.8 | 76.9 ±10.9 |
| **Haematology; mean ± standard deviation** | | | | | | | | |
| *N* | | | | | 10 | 10 | 10 | 10 |
| Glucose, males (mg/dL) | NE | NE | NE | NE | 117 ±13 | 126 ±10 | 125 ±12 | 119 ±13 |
| Glucose, females (mg/dL) | NE | NE | NE | NE | 119 ±10 | 119 ±10 | 112 ±5 | 104 ±14* |
| **Organ weights; mean ± standard deviation** | | | | | | | | |
| ***Males*** | | | | | | | | |
| *N* | 25 | 25 | 25 | 25 | 24 | 24 | 23 | 24 |
| Final body weight (g) | 600.8 ±75.1 | 568.6 ±66.6 | 574.3 ±62.8 | 545.9* ±49.1 | 638.2 ±53.8 | 614.0 ±71.3 | 636.5 ±73.6 | 583.2* ±51.3 |
| Liver weight, relative[a] (%) | 2.581 ±0.175 | 2.551 ±0.172 | 2.617 ±0.188 | 2.898* ±0.447 | 2.568 ±0.150 | 2.446 ±0.202 | 2.640 ±0.195 | 2.707* ±0.168 |
| Thyroid weight, relative[a] (%) | 0.0045 ±0.0010 | 0.0043 ±0.0007 | 0.0046 ±0.0006 | 0.0048 ±0.0008 | 0.0042 ±0.0005 | 0.0042 ±0.0009 | 0.0046 ±0.0007 | 0.0047* ±0.0006 |
| ***Females (after lactation day 21)*** | | | | | | | | |
| *N* | 22 | 23 | 20 | 23 | 21 | 21 | 23 | 22 |
| Final body weight (g) | 282.0 ±23.9 | 289.8 ±24.1 | 288.8 ±23.1 | 274.6 ±13.9 | 311.4 ±22.6 | 321.8 ±27.4 | 310.4 ±22.6 | 310.6 ±19.2 |
| Liver weight, relative[a] (%) | 2.898 ±0.245 | 2.879 ±0.170 | 2.981 ±0.184 | 3.274* ±0.219 | 2.906 ±0.265 | 2.901 ±0.258 | 2.960 ±0.300 | 3.133* ±0.312 |
| Thyroid weight, relative[a] (%) | 0.0063 ±0.0010 | 0.0066 ±0.0010 | 0.0066 ±0.0015 | 0.0072 ±0.0014 | 0.0064 ±0.0009 | 0.0064 ±0.0011 | 0.0063 ±0.0011 | 0.0068 ±0.0013 |
| **Histopathology; number affected/number observed** | | | | | | | | |
| Liver: hypertrophy, increased eosinophilia, hepatocyte, centrilobular/midzonal | | | | | | | | |
| Males | 1/25(a) | 2.25 | 12/25 | 25/25 | 0/25 | 1/25 | 9.25 | 24/25 |
| Females | 1/25 | 1/25 | 8/25 | 24/25 | 0/25 | 0/25 | 13/25 | 22/25 |
| **Puberty onset** | | | | | | | | |
| ***Vaginal opening; mean ± standard deviation*** | | | | | | | | |
| *N* | 25 | 25 | 25 | 23 | 20 | 19 | 22 | 22 |
| Days | 32.2 ±2.2 | 32.6 ±1.4 | 33.4 ±2.6 | 36.0* ±3.9 | 33.2 ±15.2 | 33.8 ±4.0 | 33.0 ±2.9 | 33.4 ±1.9 |
| Body weight (g) | 117.9 ±13.7 | 116.6 ±13.0 | 122.4 19.2 ± | 129.8 ±19.0 | 124.0 ±15.2 | 121.3 ±11.8 | 121.0 ±16.9 | 126.2 ±14.6 |
| First estrus[b] | NE | NE | NE | NE | 1.5 ±2.1 | 1.9 ±2.3 | 2.7 ±2.6 | 2.1 ±2.5 |

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | First generation | | | | Second generation | | | |
| | 0 | 300 | 1000 | 3000/ 4000 | 0 | 300 | 1000 | 3000/ 4000 |
| **Haematology (weanlings); mean ± standard deviation** | | | | | | | | |
| ***Weanling males*** | | | | | | | | |
| *N* | | | | | 10 | 10 | 10 | 10 |
| Haemoglobin (g/dL) | NE | NE | NE | NE | 8.5 ±0.9 | 8.1 ±0.5 | 8.7 ±0.7 | 7.3* ±0.8 |
| Haematocrit (%) | NE | NE | NE | NE | 29.3 ±2.1 | 28.7 ±1.7 | 30.2 ±2.1 | 26.2* ±1.9 |
| MCV(fL) | NE | NE | NE | NE | 64.8 ±4.3 | 61.1 ±4.5 | 65.3 ±5.2 | 57.8* ±5.3 |
| ***Weanling females*** | | | | | | | | |
| *N* | | | | | 10 | 10 | 10 | 10 |
| Haemoglobin (g/dL) | NE | NE | NE | NE | 8.7 ±1.0 | 8.4 ±0.9 | 8.5 ±0.6 | 7.5* ±0.7 |
| Haematocrit (%) | NE | NE | NE | NE | 29.5 ±2.4 | 29.5 ±2.6 | 29.6 ±1.7 | 26.7* ±1.8 |
| MCV (fL) | NE | NE | NE | NE | 65.3 ±4.5 | 61.8 ±5.2 | 63.3 ±4.6 | 58.4* ±4.8 |
| **Organ weights (weanlings); mean ± standard deviation** | | | | | | | | |
| ***Weanling males*** | | | | | | | | |
| *N* | 22 | 23 | 20 | 23 | 21 | 21 | 23 | 22 |
| Body weight (g) | 59.3 ±5.7 | 58.1 ±7.1 | 58.8 ±9.7 | 52.2* ±7.0 | 60.9 ±11.9 | 60.2 ±11.7 | 60.4 ±9.5 | 58.2 ±5.0 |
| Spleen weight, absolute (g) | 0.255 ±0.056 | 0.267 ±0.059 | 0.271 ± 0.063 | 0.210* ±0.048 | 0.280 ±0.099 | 0.263 ±0.095 | 0.274 ±0.076 | 0.240 ±0.047 |
| Thymus weight, absolute (g) | 0.241 ±0.045 | 0.241 ±0.045 | 0.248 ±0.033 | 0.202* ±0.037 | 0.239 ±0.066 | 0.235 ±0.065 | 0.254 ±0.063 | 0.214 ±0.041 |
| ***Weanling females*** | | | | | | | | |
| *N* | 22 | 23 | 20 | 23 | 21 | 21 | 23 | 22 |
| Body weight (g) | 55.7 ±6.8 | 55.1 ±7.0 | 55.9 ±7.6 | 51.5 ±5.2 | 57.6 ±10.6 | 56.4 ±12.2 | 56.9 ±7.7 | 55.3 ±5.4 |
| Spleen weight, absolute (g) | 0.257 ±0.059 | 0.264 ±0.058 | 0.247 ±0.076 | 0.209* ±0.042 | 0.278 ±0.086 | 0.251 ±0.079 | 0.247 ±0.065 | 0.223 ±0.042 |
| Thymus weight, absolute (g) | 0.235 ±0.050 | 0.252 ±0.043 | 0.248 ±0.041 | 0.210 ±0.029 | 0.247 ±0.058 | 0.233 ±0.072 | 0.249 ±0.053 | 0.288 ±0.053 |

*N*: Number examined; NE: Not examined; GD: Gestation day; LD: Lactation day; Source: Passage, 2019

[a] Weight relative to body weight as percentage (organ weight × 100/body weight);

[b] Interval (day) between vaginal opening and fist estrus;

Statistically different from control mean by: * Dunnett's test; [#]alpha value; both at $p < 0.05$

The NOAEL for parental toxicity was 1000 ppm (equal to 58.4 mg/kg bw per day) based on lowered body weights in males at 3000 ppm (equal to 179 mg/kg bw per day) in the F0 and F1 adult males. The NOAEL for reproductive toxicity was 3000 ppm (equal to 179 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 1000 ppm (equal to 58.4 mg/kg bw per day) based on decreased body weights and decreases in red blood cell parameters in male and female F1 weanlings at 3000 ppm (equal to 179 mg/kg bw per day) (Passage, 2019).

**(b) Developmental toxicity**

***Rat***

To examine maternal and/or embryonal/fetal toxicities, florylpicoxamid (purity 94.9%; Lot no. EXP-16-BH2635) was given to groups of five time-mated female Crl:CD(SD) rats via the diet at concentrations of 0 or 3000 ppm on gestation days (GDs) 6–21 corresponding to time-weighted average doses of 0 or 212 mg/kg bw per day. The high dose of 3000 ppm was selected based on previously conducted reproductive screening and 28-day subchronic studies. In-life parameters evaluated for all groups included clinical observations, body weight, body weight gain, and food consumption. On GD 21 all surviving rats were euthanized and examined for gross pathological alterations. Blood was collected for toxicokinetic evaluations from dams (GD 20 and 21) and their respective litters (GD 21 at necropsy). Liver weights, kidney weights, and gravid uterine weights were recorded, along with the number of corpora lutea, implantations, resorptions, and live/dead fetuses. All viable fetuses were weighed, given an external examination, visceral examination, and their gonads examined. Approximately half of the fetuses in each litter were randomly selected for craniofacial examination (except fetuses selected for toxicogenomics). All fetuses (except fetuses selected for toxicogenomics) were processed for skeletal evaluations.

There were initial decreases in body weight gain (GDs 6–9) with concomitant decreases in food consumption for animals at 3000 ppm. These decreases were deemed secondary to reduced palatability of the fortified diet mix containing test material. There were no effects throughout the study period on the body weight of animals at 3000 ppm. By GD 12 food consumption was similar to that of controls. There were no effects on clinical observations, organ weights, gross pathology, histopathological observations, gravid uterine weights, number of corpora lutea, implantations, resorptions, or number of live fetuses. There were no effects noted from external, visceral, craniofacial or skeletal examinations, nor effects on fetal body weights. Under the conditions of this study, the NOAELs for maternal toxicity and embryo/fetal toxicity were 3000 ppm, the highest dose tested. (Passage, 2020)

The main rat development study was conducted to evaluate the maternal and developmental toxicity of florylpicoxamid (purity 98.1%; Lot No. EXP-17-BN7923) in Crl:CD(SD) rats following repeated dietary administration. Groups of 24 time-mated female rats were administered florylpicoxamid in the diet at concentrations of 0, 300, 1000 or 4000 ppm on GDs 6–21 (equal to 0, 21.3, 73.1 and 271 mg/kg bw per day). In-life maternal study parameters included clinical observations, body weight, body weight gains and food consumption. On GD 21 all dams were euthanized and examined for gross pathological alterations. Liver, kidneys and gravid uterine weights were recorded, along with the number of corpora lutea, uterine implantations, resorptions and live/dead fetuses. All fetuses were weighed, sexed and examined for external alterations. Approximately half the number of the fetuses were examined for visceral alterations while skeletal examinations were conducted on the remaining ones. Blood concentrations of florylpicoxamid and its metabolites were measured in four dams per group and their respective litters at necropsy. A summary of the results of this study is shown in Table 21.

Maternal toxicity was limited to dams at 4000 ppm and consisted of treatment-related decreases in body weight, body weight gain and food consumption during the treatment period. The marked decrease in food consumption seen at the start of treatment (by ca 40%) was considered due to lowered palatability. The difference in food intake compared to controls had moderated to ca 10% at the end of treatment. The consistent decrease in body weight was slight (ca 5%) throughout the study. The Meeting considered the slight but continuous effects on food consumption and body weight to be adverse, although this had not been the opinion of the study author. At necropsy there were no gross pathological changes observed in the dams, or no changes in kidney or liver weights in dams.

There were no treatment-related effects on pregnancy, resorption rate, litter size, number of corpora lutea or implantations, percentage preimplantation loss, percentage post implantation loss, fetal sex ratios, fetal body weights or gravid uterine weights at any dose level.

There were no statistically significant differences in the incidence of any fetal alteration in any of the treated groups compared to controls, as seen in external, craniofacial and visceral examinations. The incidence of supernumerary hepatic lobes, a variation, was observed as an alteration with low frequency at 4000 ppm, but higher than in controls. This variation also occurred in the control group. There was no treatment-related effect on fetal liver texture or colouration at 4000 ppm. In the reproductive toxicity

study of Passage (2019), where the dietary high dose was the same as in this study, the changes related to abnormal lobes in the liver were not seen in pups in the F1 or F2 generations at 4000 ppm. Taken together, the higher incidence of supernumerary hepatic lobes was not considered to be treatment-related.

***Table 21. The summary of development toxicity study in rats treated with florylpicoxamid***

| | Dose (ppm) | | | |
|---|---|---|---|---|
| | **0** | **300** | **1000** | **4000** |
| **Number of rats** | **23** | **24** | **23** | **23** |
| **Food consumption (g/rat per day); mean ± standard deviation** | | | | |
| GD 5–6 | 20.7 ± 1.8 | 20.4 ± 2.3 | 21.2 ± 2.4 | 19.8 ± 2.7 |
| GD 6–7 | 20.1 ± 2.7 | 18.7 ± 2.8 | 18.7 ± 3.0 | 12.5* ± 3.1 |
| GD 10–11 | 22.2 ± 2.4 | 20.7 ± 2.7 | 22.0 ± 3.5 | 19.1* ± 2.5 |
| GD 12–15 | 25.1 ± 3.7 | 22.7 ± 3.2 | 24.8 ± 3.4 | 20.7 ± 2.3 |
| GD 18–21 | 25.1 ± 3.7 | 22.7 ± 3.2 | 24.8 ± 4.1 | 22.8 ± 2.8 |
| **Body weight (g); mean ± standard deviation** | | | | |
| GD 6 | 254.1 ± 11.2 | 251.8 ± 14.1 | 255.2 ± 12.6 | 250.1 ± 11.4 |
| GD 7 | 257.5 ± 12.1 | 253.3 ± 12.5 | 257.2 ± 13.3 | 246.7* ± 10.1 |
| GD 10 | 274.6 ± 13.7 | 271.6 ± 16.2 | 275.8 ± 15.7 | 264.1* ± 10.9 |
| GD 15 | 306.2 ± 14.6 | 302.6 ± 16.2 | 309.6 ± 18.7 | 298.4 ± 13.7 |
| GD 21 | 301.5 ± 15.3 | 295.1 ± 17.4 | 300.2 ± 32.4 | 286.7# ± 19.1 |
| **Supernumerary hepatic lobe; incidence/number examined** | | | | |
| Fetus | 2/143 | 3/156 | 2/147 | 5/149 |
| Litter | 2/23 | 2/24 | 2/23 | 5/23 |

GD: Gestation day; Source: Passage, 2018a

Statistically different from control mean by: * Dunnett's test; #alpha value; both at $p < 0.05$

Under the conditions of this study, the NOAEL for maternal toxicity was 1000 ppm (equal to 73.1 mg/kg bw per day) based on decreases in body weight and food consumption at 4000 ppm (equal to 271 mg/kg bw per day). The NOAEL for embryo/fetal toxicity was 4000 ppm (equal to 271 mg/kg bw per day) the highest dose tested (Passage, 2018a).

***Rabbit***

Palatability of florylpicoxamid was investigated in nonpregnant rabbits. Groups of three female New Zealand White rabbits were fed diets supplying 1250 ppm on treatment days (TDs) 7–15, 2000 ppm on TDs 4–11, 2700 ppm on TDs 1–8, or 4000 ppm on TDs 4–6 of florylpicoxamid for up to eight consecutive days. These levels corresponded to time-weighted average doses of 33.3, 60.4, 75.0 and 37.2 mg/kg bw per day, respectively. Parameters evaluated were daily cage-side observations, clinical observations, body weight and body weight gain, and food consumption.

Mean food consumption in the 4000 ppm group was decreased by ca 75% during each of the two days of test material exposure. Decreased faeces were also noted during this period. Food consumption immediately returned to pre-exposure levels when this group was given control diet. Mean food consumption in the 1250, 2000 and 2700 ppm groups was reduced by ca 40% during the initial three days of test material exposure. One animal in each of the 1250, 2000 and 2700 ppm groups was returned to control diet due to consuming less than 50 g of food for three consecutive days after receiving diets containing florylpicoxamid. Food consumption of the remaining animals in the 1250, 2000 and 2700 ppm groups almost returned to pre-exposure levels by the end of the exposure period. Body weight and body weight gain were variable, but similar across all groups throughout the study.

Under the conditions of this study, the 4000 ppm diet was considered unpalatable to rabbits, as shown by a clear decrease in food consumption and decreased faeces. (Johnson & Marshall, 2016)

In a preliminary developmental toxicity study, groups of five female premated New Zealand White rabbits were given florylpicoxamid (purity 96.3%; Lot no. EXP-16-BH2635, TSN312284) in the diet at concentrations of 0, 350, 750, 1250, 2000 or 2700 ppm (equal to 0, 12.4, 28.1, 43.7, 49.0 and 50.5 mg/kg bw per day, respectively) from GD 7 to GD 28. In-life parameters evaluated for all groups included clinical observations, body weight, body weight gain and food consumption. On GD 28 all surviving rabbits were euthanized and examined for gross pathological alterations. All fetuses were weighed. Liver, kidney and gravid uterine weights were recorded, along with the number of corpora lutea, implantations, resorptions, and live/dead fetuses. Concentrations of florylpicoxamid-ester and the metabolites were analyzed in blood samples collected from does at three time points prior to necropsy and from does and their respective litters at necropsy.

Animals at 2000 and 2700 ppm exceeded the maximum tolerated dose (MTD) level for a developmental toxicity study as evidenced by excessive decreases in food consumption (by 40% and 50% compared to controls), and minimal body weight gain (or body weight loss) compared to control from GD 7 to GD 10. Clinical observations of perineal faecal soiling and/or soft or decreased faeces were made for two and three rabbits at 2000 and 2500 ppm, respectively. All animals at 2000 and 2700 ppm were euthanized on GD 10 in accordance with the protocol. No further examinations were performed. At 1250 ppm two of five rabbits were removed, #3492 due to abortion on GD27 and #3493 because of watery faeces on GD 18. Both animals also displayed body weight loss and decreased food consumption along with observations of soft/decreased faeces and perineal faecal soiling. No treatment-related clinical signs were seen at 350 or 750 ppm.

Mean body weights at 350, 750 and 1250 ppm were comparable with controls throughout the study. Body weight gains in these groups decreased by 76%–85% compared to controls, but only at GDs 7–10 and without a dose–response relationship. These body weight gains recovered at GDs 10–13. The transient and quickly recovered decreases in body weight gain seen in all treated groups were considered to be due to low palatability. However, the slight but continuous decrease in food consumption at 1250 ppm seen after the transient change, was considered to be adverse. The slight decrease in food consumption at 350 ppm was not toxicologically significant due to lack of a dose–effect relationship. There were no treatment-related gross pathological observations and no treatment-related effects on liver or kidney weights in any dose group when compared to controls. There were no treatment-related effects on gravid uterine weights, pregnancy rates, number of corpora lutea, number of implantations, pre- or post-implantation loss, resorptions, or litter size in animals given florylpicoxamid. No effects were observed on fetal body weight or from external fetal examinations.

The excessive decrease in food consumption, body weight loss, clinical signs and abortions observed at 1250 ppm were considered treatment-related adverse effects. The NOAEL for maternal toxicity was 750 ppm and the NOAEL for embryo/fetal toxicity was 1250 ppm (Passage, 2018b).

In a main developmental toxicity study, groups of 24 female, time-mated, New Zealand White rabbits were given florylpicoxamid (purity 96.3%; Lot no. EXP-16-BH2635, TSN312284) at dietary concentrations of 0, 125, 250 or 750 ppm (equal to 0, 4.73, 9.58 and 25.9 mg/kg bw per day) from GD 7 to GD 28. Animals were monitored twice daily for mortality and moribundity and daily for clinical signs of toxicity. Body weight and food consumption were measured daily from GD 4 to GD 28. A blood sample was taken for toxicokinetics on GD 27, and all surviving animals were subjected to gross necropsy. The uteri of females lacking visible implantations were stained with a 10% aqueous solution of sodium sulfide (method based on Kopf, Lorenz & Salewski, 1964) and examined for evidence of early resorptions in order to verify pregnancy status. All fetuses were examined for body weight, sex and external and visceral malformations. Half the number of each group was stained and examined for craniofacial malformations, and all fetuses were stained and examined for skeletal malformations. The findings from this study are summarized in Table 22.

Two animals at 750 ppm aborted prior to their scheduled necropsy date. The fetuses of animals #293 and #301 were found in the cage or cage pan on the mornings of GD 23 and GD 28, respectively. Although the incidence was low, the abortions were considered to be a treatment-related adverse effect because of consistency with the finding at 1250 ppm in the preliminary study. As the number of rabbits examined per group in the preliminary study was small, the possibility that abortion was induced at 750 ppm was not excluded. Animal #312 was euthanized on GD 20. The cage of this rabbit showed blood on GDs 16–20, and the rabbit produced soft faeces on GDs 17–20, and watery faeces on GD 20.

Another animal was removed from study on GD 12 due to decreased food consumption that resulted in inadequate test material intake.

In clinical observations, two (#302 and #303) at 750 ppm and one animal (#230) at 0 ppm produced soft faeces. The soft faeces at 750 ppm was therefore not considered treatment-related as the same finding appeared in a control animal, and at 2/24 the incidence was low (Passage, 2018b). Perineal faecal soiling, which was observed at 1250 ppm and higher in the preliminary study, was not detected in this study. There were no treatment-related observations noted in animals at 125 or 250 ppm.

Body weights in all treated groups were similar to controls. The body weight gain and food consumption at 750 ppm decreased from GD 7. Any decrease in body weight was quickly recovered and the decrease in food consumption was recovered by GD 14–15. The transient and smoothly recovered decreases at 750 ppm were considered to be due to low palatability. There were no effects on body weight, body weight gain or food consumption at 125 or 250 ppm.

At necropsy, animal #312 (750 ppm group) which had been euthanized on GD 20, showed foul smelling, moderately thick liquid contents in the caecum and small intestines. The litter was found to be totally resorbed, appearing as dark red soft ovoids. This finding was considered to be related to the blood in the cage, seen in clinical observations of this rabbit. The incidence of resorption was not comparable among control and treated groups. In addition, these findings were not observed in any rabbits at the same dose in this group or at 750 or 1250 ppm in the preliminary study. Therefore, the changes observed in this one animal were not considered to be treatment-related. There were no treatment-related gross pathological observations in rabbits dosed at 125 or 250 ppm and there were no treatment-related differences in maternal kidney or liver weights for any of the treated groups when compared to their respective controls.

From reproductive and fetal observations at necropsy, there were no treatment-related effects on numbers of litters totally resorbed, litters with viable fetuses, corpora lutea, implantations, pre- or post-implantation losses, resorptions, viable fetuses, fetal weights, gravid uterus weights.

Examining the fetuses, there were no treatment-related differences in the incidence of fetal malformations in any treated group. Visceral examination revealed the incidence of para-ovarian cyst at 750 ppm to be higher than for the control group, although the finding was observed in all groups including controls and differences were not statistically significant. Para-ovarian cysts are a spontaneous anomaly arising from the mesonephric or paramesonephric duct (Acland, 1995; Schlafer & Miller, 2007). Such cysts are small and clear, reside external to the ovary and oviduct and do not involve any parenchymal tissue. This type of cyst is reported to be common in adult rabbits (Bradley et al, 2021), and was observed in dams in the preliminary developmental toxicity study of florylpicoxamid (Passage, 2018b). Incidences of para-ovarian cyst were 2/5, 0/5, 2/5 and 1/5 at 0, 350, 750 and 1250 ppm, respectively. Overall, the occurrence of para-ovarian cyst was not considered to be treatment-related. There were no other treatment-related or statistically-identified visceral alterations in any dose group. Treatment-related fetal observations were limited to an increased incidence of one skeletal variation, crooked hyoid, at 750 ppm.

From skeletal examinations it was observed that the incidence of crooked hyoid was higher at 750 ppm than in controls, both on a fetus and a litter basis. The difference from controls was not statistically significant, but it was above the upper bound of the historical control range of this finding from eight studies conducted between 2013 and 2019 (see Table 23). The increase was not excluded as possibly being treatment-related. However, the hyoid is known to ossify late in gestation and thus if there is a delay in the ossification, the surrounding musculature could progressively increase mechanical pressure leading to a bent appearance, but is known that eventually the bones ossify and the angulation resolves (DeSesso & Scialli, 2018). In human adults, anatomical variations of the hyoid-larynx complex that originate in embryology are reported to be very high (4%–30% of the general population). In humans, inward curving and fattening of the greater horns (a minor variation) has been reported to occur so often that it cannot be considered an anatomical variants, and a curving upwards or downwards of the greater horns was also reported, but does not have any clinical implications (de Bakker et al, 2019). Considering all available information, variation of the hyoid bone was considered to be a non-adverse. No other treatment-related changes were observed.

The major toxicities observed in dams and fetuses are summarized in Table 22.

*Table 22. The summary of the rabbit development toxicity study of florylpicoxamid*

| | Dietary concentration (ppm) | | | |
|---|---|---|---|---|
| | **0** | **125** | **250** | **750** |
| **Number of dams examined** | **24** | **24** | **24** | **24** |
| **Clinical signs (number of rabbits)** | | | | |
| Within normal limits | 18 | 21 | 18 | 15 |
| ***Miscellaneous*** | | | | |
| Blood in cage | 0 | 1[a] | 1[a] | 3[b] |
| ***Faeces*** | | | | |
| Abnormal quantity, decreased | 1 | 2 | 0 | 1 |
| Abnormal consistency, soft | 1[c] | 0 | 0 | 2[c] |
| Abnormal consistency, soft and watery | 0 | 0 | 0 | 1[d] |
| ***Fate of pregnancy*** | | | | |
| Abortion, unscheduled | 0 | 0 | 0 | 2[e] |
| Moribund, unscheduled | 0 | 0 | 0 | 1[f] |
| Other, unscheduled | 0 | 0 | 0 | 1[g] |
| Scheduled necropsy | 24 | 24 | 24 | 20 |
| **Body weight gain (g); mean ± standard deviation** | | | | |
| GDs 7–10 | 33.6 ± 29.6 | 38.1 ± 32.1 | 34.9 ± 32.7 | 3.4 ± 58.9 |
| GDs 10–13 | 81.2 ± 29.7 | 65.7 ± 43.0 | 66.1 ± 36.1 | 55.1 ± 37.6 |
| GDs 13–16 | 107.4 ± 43.0 | 97.5 ± 59.5 | 102.5 ± 49.1 | 109.4 ± 41.5 |
| GDs 16–20 | 23.8 ± 34.9 | 22.6 ± 40.0 | 20.7 ± 32.4 | 16.6 ± 35.5 |
| GDs 24–28 | 69.1 ± 28.2 | 58.3 ± 45.8 | 68.2 ± 35.2 | 59.8 ± 52.5 |
| **Food consumption (g/animal per day); mean ± standard deviation** | | | | |
| GDs 7–8 | 125.1 ± 0.3 | 122.6 ± 11.9 | 124.4 ± 1.7 | 99.5 ± 32.3* |
| GDs 11–12 | 125.0 ± 0.2 | 120.8 ± 14.6 | 123.1 ± 1.7 | 103.4 ± 28.9* |
| GDs 14–15 | 124.9 ± 0.5 | 116.4 ± 26.8 | 121.9 ± 11.1 | 106.6 ± 29.0 |
| GDs 18–19 | 123.8 ± 4.4 | 125.1 ± 0.2 | 124.0 ± 4.0 | 113.6 ± 26.6 |
| GDs 26–27 | 124.6 ± 1.3 | 120.7 ± 10.6 | 123.7 ± 6.0 | 117.3 ± 13.3 |
| **Visceral examinations in fetuses; incidence/examined** | | | | |
| Para-ovarian cyst[h]: fetuses, [litters] | 1/109, [1/24] | 2/108, [2/24] | 2/91, [2/23] | 4/89, [3/20] |
| **Skeletal examinations in fetuses; incidence/examined** | | | | |
| Crooked hyoid: fetuses, [litters] | 1/107, [1/24] | 1/107, [1/24] | 0/100, [0/23] | 4/89, [4/20] |

[a] Caused by machinery injury; GD: Gestation day; Source: Passage, 2018c

[b] Blood in two animals caused by machinery injury; blood from remaining animal (#301) related to abortion ;

[c] Animal #230 (0 ppm) on GD 6, #302 (750 ppm) during GDs 18–19, #303 on GD27;

[d] Animal #312 during GDs 17–20 (soft faeces) and on GD 20 (watery faeces);

[e] Animal #293 showed no remarkable observations during GDs 2–22, and absorbed on GD 23; animal #301showed no remarkable observations during GDs 1–27, and absorbed on GD 28 with blood in cage;

[f] Animal #312 showed no remarkable observation during GDs 1–15; this animal showed miscellaneous blood in cage on GDs 16–20 and was euthanized when moribund on GD 20;

[g] Animal #310 was removed due to decreased food consumption that resulted in inadequate test material intake;

[h] Historical control of para-ovarian cyst during five studies 2013–2018 were 1/93 (1.1%), 2/107 (1.9%) , 0/90 (0%), 1/104 (1.0%) and 4/98 (4.1%) as the number of fetuses, and 1/22 (4.5%), 2/24 (8.3%), 0/21 (0%0, 1/23 (4.3%) and 4/23 (17.4%) per litter, respectively;

* Statistically different from control mean by Wilcoxon's test, alpha value = 0.05

*Table 23. Historical control data for crooked hyoid in rabbit developmental toxicity studies*

| Study number | Year | Route | Crooked hyoid; affected/examined [%][a] | |
|---|---|---|---|---|
| | | | Fetuses | Litters |
| 1 | 2013 | Dietary | 0/96 [0.0%] | 0/22 [0.0%] |
| 2 | 2014 | Dietary | 1/108 [0.9%] | 1/24 [4.2%] |
| 3 | 2017 | Gavage | 0/95 [0.0%] | 0/21 [0.0%] |
| 4 | 2018 | Gavage | 0/102 [0.0%] | 0/23 [0.0%] |
| 5 | 2018 | Gavage | **2/92 [2.2%]** | **2/23 [8.7%]** |
| 6 | 2018 | Dietary | 0/102 [0.0%] | 0/24 [0.0%] |
| 7 | 2019 | Dietary | 0/90 [0.0%] | 0/23 [0.0%] |
| 8 | 2019 | Gavage | 2/95 [2.1%] | **2/23 [8.7%]** |

[a] Historical control from studies in NZW rabbits reported between 2013 and 2019; Source: Passage, 2018c

**Bold** type indicates the highest observed value for the end-point.

In conclusion, the NOAEL for maternal toxicity was 250 ppm (equal to 9.58 mg/kg bw per day) based on increased incidence of abortions. The NOAEL for embryo/fetal toxicity was 750 ppm (equal to 25.9 mg/kg bw per day), the highest dose tested (Passage, 2018c; Chan, 2019).

## 2.6 Special studies

### (a) Neurotoxicity

A weight of evidence approach on neurotoxic potential of florylpicoxamid was adopted instead of an acute neurotoxicity study. Using this approach the existing data on neurotoxicity of florylpicoxamid were reviewed.

In acute oral and dermal toxicity studies the $LD_{50}$ values were greater than the limit dose of 2000 mg/kg bw (Verma, 2015; Dalal, 2021a). Even via inhalation, the $LC_{50}$ was greater than the limit concentration of 5.48 mg/ (Patel, 2016b). With the lack of any treatment-related mortalities, clinical signs or pathological changes up to the limit dose or concentrations tested there was no evidence to suggest florylpicoxamid possesses any acute neurotoxic potential.

Neurotoxic potential was evaluated in the 13-week dietary rat study with florylpicoxamid dietary exposure levels of 300, 1000 or 3000 ppm (Mukerji, 2019; described above in Section 2.2a). There were no treatment-related finding from the FOB which included cage-side, hand-held and open-field observations and measurements of rectal temperature, fore- and hind-limb grip performance, landing foot splay, and motor activity findings. Nor were there histopathological changes in central or peripheral nervous tissues after 90 days of treatment up to the highest dose tested. Based on this data, it was concluded that florylpicoxamid was not neurotoxic after 90-days of repeated dietary exposure.

No evidence of neurotoxicity was seen when florylpicoxamid was administered for 90-day days to mice (Mukerji, 2018) or dogs (Heward, 2019). There was no evidence of neuropathology or abnormalities observed in the development of the fetal nervous system in the developmental and reproductive studies conducted with florylpicoxamid in rats and rabbits (Passage, 2018a, c).

The neurotoxic potential of florylpicoxamid was also assessed on the basis of the information available on fungicides with a similar MOA. Florylpicoxamid belongs to complex III inhibitor fungicides which inhibit ubiquinone reductase. There are two Fungicide Resistance Action Group (FRAC) codes associated with ubiquinone reductase inhibition; 11 (Qo inhibitors) and 21 (Qi inhibitors). Representative molecules from both FRAC code groups were included in the read-across employed here. Toxicology reviews by USEPA and EFSA indicated that compounds in this class of fungicides, fenpicoxamid, famoxadone, picoxystrobin, fluoxastrobin, fenamidone, trifloxystrobin, kresoxim-methyl, amisulbrom, azoxystrobin, mandestrobin and pyraclostrobin, lack acute and sub-chronic neurotoxicity potential up to the limit dose or highest dose levels tested. This further supports the lack of neurotoxic potential for florylpicoxamid, a member to this class of fungicides.

In conclusion, there is no evidence of neurotoxicity in the existing data package via acute or repeat-dose exposures with florylpicoxamid. In addition, there is no evidence of neurotoxicity for compounds in this same fungicidal class, similar by virtue of their chemistry. This further supports the conclusion that there is no potential for neurotoxicity associated with exposure to florylpicoxamid (Papineni & Henriquez, 2019).

**(b) Studies on the endocrine disrupting potential of florylpicoxamid**

In order to assess a potential of florylpicoxamid to interfere with endocrine-mediated biological processes in vertebrates, one study, an in vitro estrogen receptor transactivation assay in HeLa cells, was conducted. This study investigated the ability of florylpicoxamid (purity 97.1%, Lot no. EXP-17-BN-7913_A[TSN313145]) to establish activation or blocking of the human estrogen receptor α (ER) using the human hERα-HeLa-9903 cell line. The hERα-HeLa-9903 cell line is a cell-based reporter assay, expressing human ERα and an ERα-inducible luciferase reporter gene, that confers estrogen-dependent luminescence. Two valid independent experiments were performed for the ER agonist assay and ER antagonist assay. Solubility analysis was conducted before the experiments were performed to identify a suitable top concentration for the test item, for use in a cytotoxicity assay under agonist and antagonistic conditions. As cytotoxicity was observed for florylpicoxamid at 10 µM before agonist and antagonist experiments, 1 µM florylpicoxamid was selected as the top concentration for both experiments. The final exposure concentrations ranged from 1 pM to 1 µM in the ER agonist assay, and from 10 pM to 1 µM in the ER antagonist assay.

After evaluation all acceptability parameters of an ER agonist assay were met or accepted. Therefore, both the following ER agonist experiments were considered valid. In two experiments florylpicoxamid did not show any estrogenic agonist response up to a concentration of 1 µM: the $RPC_{max}$ (maximum level of response, as a percentage of that induced by 1 nM) was 2.4% and 0.8% in the two experiments. As such, florylpicoxamid was considered negative in the ER agonist assay. In the ER antagonist assay, all assay acceptability parameters were met and the control items were correctly classified as positive (tamoxifen) or negative (flutamide). Therefore both ER antagonist experiments were considered valid. A log $IC_{30}$ (the concentration at which activity is inhibited to 30% of the positive control value) could not be determined for florylpicoxamid up to a concentration of 1 µM. As such, florylpicoxamid was considered negative in the ER antagonist assay.

In conclusion, florylpicoxamid did not show any estrogenic agonist or antagonist activity up to 1 µM; florylpicoxamid was negative in the stably transfected human estrogen receptor-α transactivation assay using the hERα-HeLa-9903 cell line. (Verkaart, 2020)

In addition, florylpicoxamid underwent in vivo toxicology that covered a broad spectrum of end-points, including those of relevance to the endocrine system. This testing, as well as the study cited above, was been reviewed and evaluated as part of a weight of evidence assessment. Based on the data available, florylpicoxamid had no effect on the endocrine system.

**(c) Immunotoxicity**

A dedicated immunotoxicity study was not conducted, as the immunotoxicity end-point had been integrated into the 90-day study report. There were no effects of florylpicoxamid on immunotoxicity end-points, including the IgM response to sheep red blood cells in the standard 90-day dietary study with integrated immunotoxicity (Mukerji, 2019). In addition, effects on haematology, blood biochemistry, organs weights, histopathology of immunotoxicity target tissue/organs were not observed in the various repeat-dose studies in rats, mice or dogs. The Meeting concluded that florylpicoxamid was not immunotoxic.

### (d) Studies on metabolites

The experimental studies on metabolites of florylpicoxamid are summarized in Table 24.

***Table 24. Toxicology studies on metabolites of florylpicoxamid***

| Acute toxicity | | | | |
|---|---|---|---|---|
| **Route/ method** | **Species; strain (sex)** | **Test substance Purity, lot number** | **End-point** | |
| Oral/gavage | Rat;Wistar (F) | **X12485647** 98%, SS17000602-2 (TSN402954) | $LD_{50}$: 1000 mg/kg bw | Dalal, 2021a |
| Dermal | Rat; Wistar (RccHan:WIST) (F) | **X12485647** 98%, S17000602-2 (TSN402954) | $LD_{50}$: >2000 mg/kg bw | Dalal, 2021b |
| Inhalation/ nose only (4-hour) | Rat; Wistar (RccHan:WIST) (M/F) | **X12485647** 98%, SS17000602-2 (TSN402954)) | $LC_{50}$: cut-off value 0.125 mg/L air | Verma, 2021 |
| Topical/ in vitro | EpiDerm Reconstituted human epidermis (NA) | **X12485647** 98%, SS17000602-2 (TSN402954) | Irritant (mean relative tissue viability = 0.37%) | Costin, Filbert-Findley, & Abraham, 2021a |
| Topical/ in vitro | EpiDerm Reconstituted human epidermis (NA) | **X696476** 100%, ENBK-172341-024 (TSN402909) | Not irritant | Costin, Filbert-Findley, & Abraham, 2021b |
| **In vitro genotoxicity tests** | | | | |
| **Test** | **Test system** | **Test substance Purity, lot number Test conditions** | **End-point** | **Reference** |
| In vitro bacterial reverse mutation | *Salmonella typhimurium* and *Escherichia coli* | **X12563767** 99.95%; YL0-170673-020 (TSN400378) : ***Toxicity test***: 0, 33.3, 66.7, 100, 333, 667, 1000, 3333, 5000 µg/plate; ± S9 ***Mutation test***: 0, 333, 667, 1000, 3333, 5000 µg/plate; ± S9 | Not mutagenic | Myhre, 2019b |
| In vitro mammalian cell micronucleus | Human peripheral blood lymphocytes | **X12563767** 100%, XZ7- 169400-64(TSN314853) ***Experiment 1*** 4 hours: 0, 6.25, 12.5, 25, 50, 100, and 200 µg/mL; ± S9 24 hours, S9: 0, 12.5, 25, 50, 100, 200 and 300 µg/mL; without S9 ***Experiment 2:*** 4 hours: 0, 7.5, 15, 30, 60, 100 and 200 µg/mL; with S9 24 hours without S9: 0, 15, 30, 60, 100 and 200 µg/mL; without S9 | Neither clastogenic nor aneugenic | Xie, 2019b |

| | | | | |
|---|---|---|---|---|
| In vitro bacterial reverse mutation[a] | *Salmonella typhimurium* | **X696476**<br>100%, YHI-143590 TSN307152<br>***Cytotoxicity test***: 5000, 2500, 1250, 625, 312.5, 156.25, 78.13, 39.06, 19.53, and 9.77 µg/plate; ± S9<br>***Mutagenicity test, trial I***: 156.25, 312.5, 625, 1250, 2500 and 5000 µg/plate; ± S9<br>***Mutagenicity test, trial II***: 51.2, 128, 320, 800, 2000 and 5000 µg/plate; ± S9 | Not mutagenic | Mishra, 2018 |
| In vitro mammalian cell micronucleus | Human peripheral blood lymphocytes | **X696476** (100%; YH1-143590-18-3)<br>***Experiment 1:***<br>4 hours, with / without S9: 105.71, 211.41, 422.83, 845.65 and 1691.3 µg/mL of culture media; ± S9<br>***Experiment 2:***<br>24 hours, without S9: 105.71, 211.41, 422.83, 845.65 and 1691.3 µg/mL of culture media | Neither clastogenic nor aneugenic | Mishra, 2019 |

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*;

[a] Later submission than other study reports

### *Acute toxicity studies on metabolites*

*Acute oral toxicity of X12485647 in rats*

Wistar rats were given a single oral dose of metabolite X12485647 at 2000 and 300 mg/kg bw (three females per group). Two rats died following treatment at 2000 mg/kg bw. At 300 mg/kg bw, body weight was lost during days 0–1, and one rat was dead following treatment on day 1.Tremors were observed in rats treated at 2000 mg/kg bw, while no clinical signs were observed in rats treated at 300 mg/kg bw. The rats found dead revealed reddish discolouration in the lung in one rat and multiple white foci in the stomach in two rats. Based on mortality results, the oral $LD_{50}$ cut-off value in females was considered to be 1000 mg/kg bw (Dalal, 2021a).

*Acute dermal toxicity of X12485647 in rats*

Wistar rats (RccHan:WIST) were dermally exposed to metabolite X12485647 for 24 hours, with approximately 10% of their body surface area treated at the limit dose of 2000 mg/kg bw. No effects on mortality, clinical signs, the dermal site treated, body weights or gross pathology were observed. The dermal $LD_{50}$ for females was considered to be greater than 2000 mg/kg bw (Dalal, 2021b).

*Acute inhalation toxicity of X12485647 in rats*

Male and female Wistar rats (RccHan:WIST) were exposed by inhalation, nose only, to metabolite X12485647 for four hours at concentrations of 0.52 and 0.051 mg/L air, in a dust aerosol.

All rats in the high dose group were found dead post exposure by the end of day 1. Two male and one female rat died by the end of the exposure period and another male and two females died on day 1 post exposure. Abdominal breathing was observed in all animals during exposure. In the high-dose group, a body weight decrease was observed for all animals at death compared to day 1. In the low-dose group there was a weight decrease on day 1, but this was recovered by day 3. Necropsy revealed a reddish discolouration in the lungs in one male and two females from the high-dose group. The $LC_{50}$ cut-off value was 0.125 mg/L air (Verma, 2021).

*Skin irritation test on X12485647 using the Epiderm™ skin model*

In an in vitro EpiDerm skin irritation assay, a test chemical is considered to possess skin irritation potential if the relative cell viability is equal to 50% or less, when compared to a negative control.

The mean relative cell viability of X12485647 treated tissue was 0.37% compared to negative controls, therefore far below the 50% threshold. X12485647 was predicted to be a skin irritant (Costin, Filbert-Findley & Abraham, 2021a).

*Skin irritation test on X696476 using the EpiDerm™ skin model*

In an in vitro EpiDerm skin irritation assay, a test chemical is considered to possess skin irritation potential if the relative cell viability is equal to 50% or less, when compared to a negative control.

The mean relative cell viability of X696476-treated tissue was 97.74% compared to the negative controls, that is well above the 50% threshold. Metabolite X696476 was predicted not to be a skin irritant. (Costin, Filbert-Findley & Abraham, 2021b)

***Genotoxicity of metabolites***

*Bacterial reverse mutation assay with X12563767*

The potential of the X12563767 to induce reverse mutations in *Salmonella typhimurium* (strains TA1537, TA1535, TA98 and TA100) and *Escherichia coli* (strain WP2 *uvr*A) was evaluated in a bacterial reverse mutation test. Metabolite X12563767 was tested in two independent trials, with and without S9 metabolic activation. After 52–70 hours of incubation at 37 ± 2°C, any revertant colonies were scored.

X12563767 did not induce any significant increase in the number of revertants in either trial, with or without S9 mix, in any of the five tester strains. It was concluded that X12563767 was not mutagenic in the bacterial reverse mutation assay using *S. typhimurium* and *E. coli*. (Myhre, 2019b)

*In vitro mammalian cell micronucleus assay with X12563767*

In a mammalian cell micronucleus assay, cultured human peripheral blood lymphocytes (HPBLs) were exposed to X12563767 at different concentrations, both in the absence and presence of an Aroclor induced S9 activation system. The test substance was formulated in DMSO and formed a clear solution at 500 mg/mL, the highest stock concentration tested.

A preliminary toxicity test was performed to establish the dose range to be used for the micronucleus assay. In this study X12563767 was tested with and without metabolic activation. Human peripheral blood lymphocyte cultures were exposed to X12563767 at doses of 0.2, 0.6, 2, 6, 20, 60, 200, 600 and 2000 µg/mL. The maximum dose tested approximated to the limit dose for this assay. Visible precipitate was observed at doses of 200, 600 and 2000 µg/mL at the beginning of treatment. At the end of the treatment period, visible precipitate was observed at doses of 60, 200, 600 and 2000 µg/mL in the nonactivated and activated four-hour exposure groups, and at doses of 200, 600 and 2000 µg/mL in the nonactivated 24-hour exposure group. Cytotoxicity (defined as 50% cytokinesis-blocked proliferation index relative to a vehicle control) was not observed at any dose in the nonactivated 4- and 24-hour exposure groups. Cytotoxicity was observed at 2000 µg/mL in the S9-activated four-hour exposure group. Therefore, dose levels for the initial micronucleus assay were selected based on the precipitation profile observed at the end of treatment in this preliminary toxicity assay.

In the initial micronucleus assay, visible precipitate was observed at doses 100 and 200 µg/mL in the nonactivated four-hour exposure group, at 200 µg/mL in the S9-activated four-hour exposure group, and at doses 100, 200 and 300 µg/mL in the nonactivated 24-hour exposure group. Cytotoxicity was not observed at any concentrations in any of the three groups. However, due to a discrepancy in the precipitate profile between the preliminary toxicity assay and initial micronucleus assay, the micronucleus assay for the S9-activated, four-hour exposure group was repeated. The nonactivated, 24-hour group was also repeated as in the initial study DMSO content exceeded 1% and a concurrent solvent control had not been included.

In the repeat micronucleus assay, visible precipitate was observed at 200 µg/mL in the activated four-hour exposure group, also at doses of 100 and 200 µg/mL in the nonactivated, 24-hour exposure group. Cytotoxicity was not observed at any dose in either exposure group.

The doses selected for evaluation of micronuclei were 25, 50 and 100 µg/mL for nonactivated, four hour exposures, 30, 60and 200 µg/mL for activated four hour exposures, and 30, 60 and 100 µg/mL for nonactivated, 24 hour exposures.

No significant or dose-dependent increases in micronucleus induction were observed at any dose in treatment groups with or without S9, where applying Fisher's exact and Cochran–Armitage tests, *p* was greater than 0.05. All positive and vehicle control values were within acceptable ranges, and all criteria for a valid assay were met.

It was concluded that X12563767 was negative in the in vitro mammalian cell micronucleus assay in HPBLs (Xie, 2019b).

*Bacterial reverse mutation assay with X696476, using Salmonella typhimurium*

The potential of the X696476 to induce reverse mutations in *Salmonella typhimurium* (strains TA1537, TA1535, TA98, TA100 and TA102) was evaluated in a bacterial reverse mutation test. Metabolite X696476 was tested in a preliminary cytotoxicity test and in two independent trials, with and without metabolic activation.

The result of both trials showed that X696476 did not induce any significant increase in the number of revertants, with or without S9 mix, in any of the five tester strains. It was concluded that X696476 was not mutagenic in the bacterial reverse mutation assay using *Salmonella typhimurium*. (Mishra, 2018)

*In vitro mammalian cell micronucleus assay with X696476 in HPBLs*

In a mammalian cell micronucleus test, human peripheral blood lymphocytes, cultured in vitro, were exposed to X696476 at different concentrations in the absence and presence of metabolic activation, consisting of 2% (v/v) S9 mix; the metabolite was tested in two independent experiments. In main study human peripheral blood lymphocyte cultures were exposed, duplicates at each dose level, to X696476 at five dose levels (selected from a cytotoxicity test) between 105.71 and 1691.3 µg/mL of culture medium.

Metabolite X696476 did not induce a statistically significant or biologically relevant increase in the number of binucleate cells with micronuclei in the absence or presence of metabolic activation in either of the two independently conducted experiments. It was concluded that X696476 did not show potential to induce micronuclei in isolated HPBLs either in the presence or absence of metabolic activation, under the experimental conditions described, and thus showed no potential for clastogenicity or aneugenicity (Mishra, 2019).

***In silico analysis of metabolites for genotoxic potential***

The metabolites examined were: X12485649, X12568155, X12629973, X12641685, X12584261, oxamic acid, X12485647, X12516991, X12493055 (and its sulfate conjugate), X696476, X12714411, X12764243 (and its glucose conjugate), X12530089 and X12562911

The genotoxic potential of florylpicoxamid (XDE-659) and its metabolites was evaluated in silico using the software packages Derek Nexus (v 6.2.1), Nexus (v 2.5.2), OASIS Times (v 2.29.1), and OECD QSAR Toolbox (v 4.5).

Organic functional groups can be used to classify substances to allow potential read-across in association with OECD QSAR Toolbox. The grouping was based partly on functional groups, and partly on general structural similarity. Florylpicoxamid and its metabolites were categorized as Cramer Class III by OECD QSAR Toolbox. Results of the genotoxicity prediction resulting from in silico analysis using Derek Nexus and OASIS Times and genotoxicity profilers using OECD QSAR Toolbox are summarized in Tables 25 and 26, respectively.

The majority of the substances evaluated, including the parent compound, were not predicted by Derek Nexus or by OASIS Times to be mutagenic. Several of the substances, however, were predicted to have mutagenic metabolites due to the predicted possible formation of a dicarbonyl functional group. As this prediction was also made for metabolite X696476, which had been shown negative in an Ames assay, this prediction can be disregarded. In conclusion, neither florylpicoxamid nor its metabolites are likely to be mutagenic in an Ames assay.

With regard to chromosomal aberration, both the parent and several of its metabolites were predicted to be clastogenic (with S9) by OASIS Times, based on the predicted formation of substituted phenols, as well as prediction of an acetate ester from the parent. As florylpicoxamid had been shown

negative in an in vitro chromosome aberrations assay and in an in vitro micronucleus assay, the prediction for the parent compound can be disregarded. The predicted substituted phenols were the same for both florylpicoxamid and the other substances evaluated. However, this metabolic transformation was not observed in the metabolism studies with florylpicoxamid, so predictions of genotoxicity based on the expected formation of a substituted phenol group can also be disregarded. In conclusion, neither florylpicoxamid nor its metabolites are likely to be positive in the in vitro chromosome aberrations assay.

Similarly, florylpicoxamid and its metabolites were predicted by OASIS Times to be positive in an in vitro mammalian mutagenicity assay in the presence of S9, based primarily on the formation of substituted phenols or dicarbonyl structures. Based on the similarity of the predicted metabolites from florylpicoxamid and those from the queried substances, these predictions can be disregarded. The known metabolite X696476 was predicted to form an α,β-unsaturated carbonyl structure, which is associated with genotoxicity. However, this metabolite reaction was not predicted by activated models of either Ames mutagenicity or in vitro chromosome aberrations, or by the in vivo micronucleus model, thus its relevance is questionable at best. Furthermore, formation of the unsaturated carbonyl would require demethylation of the 4-methoxypyridine ring, which metabolism studies indicate is only a very minor reaction in vivo when florylpicoxamid is administered, and is thus not expected to occur. Therefore, it is unlikely that X696476 would prove mutagenic in mammalian cells in vitro. Neither florylpicoxamid nor its metabolites, it was concluded, are likely to be positive in an in vitro mammalian mutagenicity assay.

Both florylpicoxamid and several metabolites were predicted to be positive in the in vivo mouse micronucleus assay, due to predicted metabolites containing an acetate ester (parent only) or substituted phenol moieties (both parent and its known metabolites). As all of the predicted substituted phenol metabolites were the same, and as such a reaction is not seen either in vivo or in vitro, these predictions can be disregarded. In conclusion, neither florylpicoxamid nor its metabolites are likely to be positive in an in vivo mouse micronucleus assay.

In summary, neither florylpicoxamid nor the metabolites which were evaluated are likely to be genotoxic either in vitro or in vivo (Shipp, 2023).

The following OECD QSAR Toolbox mechanistic profilers were examined to assess relevant molecular initiating events for genotoxicity:

- DNA binding by OASIS and OECD;
- DNA alerts for Ames or for chromosomal aberrations (CA) and micronucleus (MNT) by OASIS;
- protein binding for chromosomal aberration by OASIS;
- alerts for in vitro mutagenicity (Ames test) and in vivo clastogenicity (micronucleus) by ISS.

All of the queried substances only triggered the H-acceptor alert for an in vivo micronucleus assay. This specific structural alert has very low positive predictivity (Benigni, Bossa & Worth, 2010) and when it is the only alert triggered it can be disregarded.

***Stereoisomers (X12518891, X12504939 and X12506173)***

The study author reported that the metabolite coded X12518891 was the (*R*,*L*) stereoisomer of florylpicoxamid (*S*,*L*), whilst metabolite X12485649 (*S*,*L*) had the stereoisomers X12504939 (*S*,*D*) and X12506173 (*R*,*L*). Since in silico predictive software is stereoisomer-agnostic, predictions have not been made for the stereoisomers, and predictions for their reference substances can be applied (Shipp, 2023).

***Crop metabolites XDE-659 M2, XDE-659 M3, XDE-659 M4, XDE-659 M5***

These metabolites were present in wheat forage, hay and straw and only at low levels, but not in rats. They were not structurally identified in the wheat metabolism study. No toxicological information for these metabolites has been obtained. The sponsor replied to the request for toxicology information as follows:

"Based on retention times, these metabolites are more polar than parent florylpicoxamid (XDE-659) and X12485649 (eluting earlier by UHPLC), therefore, these metabolites are likely to be more readily excreted by animals and not accumulate in tissues. Therefore, exposure to these metabolites via animal matrices is expected to be very low. This low exposure scenario is further demonstrated by calculating a metabolite ratio from the wheat metabolism study. Furthermore, it is noted that florylpicoxamid does not contain any functional groups that typically give rise to genotoxic alerts (Benigni and Bossa, 2006) , therefore, XDE-659-M2, XDE-659-M3, XDE-659-M4, XDE-659-M5 are unlikely to have any genotoxic potential."

## 3. Microbiological aspects

There was no information available in the public domain and no experimental data were submitted that addressed the possible impact of florylpicoxamid residues on the human intestinal microbiome.

## 4. Observations in humans

The sponsor provided information on observation in humans as shown below.

### (a) Medical surveillance on manufacturing plant personnel

In a hazard-specific medical surveillance programme for employees where biomonitoring for florylpicoxamid or its metabolites has been identified, no specific biomonitoring problem had been described. Viewing all medical assessment programme data, and searches of personal medical records, for employees involved in the manufacture or handling of florylpicoxamid, there were no reports or findings of adverse health effects that had been deemed attributable to florylpicoxamid exposure.

### (b) Data collected on humans

No clinical cases of poisoning incidents have been found in the open literature, based on the data collected by the sponsor.

# Comments

## Biochemical aspects

When radiolabelled florylpicoxamid was administered orally to mice, rats or rabbits, radioactivity was rapidly absorbed. For a single dose of [*pyridinyl-2-*$^{14}$C]florylpicoxamid at 100 mg/kg bw, the maximum plasma concentration ($C_{max}$) occurred 2–3 hours after administration in rats and 0.5 hours in rabbits. In mice, the blood $C_{max}$ occurred between 30 minutes and six hours after dosing. Alpha and beta plasma/blood elimination half-lives ($t_{½}$) for radiolabelled florylpicoxamid were 3–12 hours and 22–44 hours respectively, with no significant differences in $t_{½}$ when positions of radiolabel or different species were compared. Radiolabelled metabolite X12485649, the first metabolite of florylpicoxamid formed by deacetylation, showed a similar toxicokinetic profile to the parent when administered to rats. Florylpicoxamid was highly metabolized and quickly eliminated in all species (Himmelstein, 2020).

Following oral administration of [*pyridinyl-2-*$^{14}$C]florylpicoxamid at 100 mg/kg bw, total urinary elimination over 168 hours accounted for 19%–22%, 18%–33% and 75% of administered dose (AD) in mice, rats and rabbits respectively. Faecal elimination of [*pyridinyl-2-*$^{14}$C]florylpicoxamid accounted for 61%–82%, 69%–73% and 17% of AD in rats, mice and rabbits respectively. Biliary elimination in bile duct-cannulated rats treated with 25 or 250 mg/kg bw of [*pyridinyl-2-*$^{14}$C] florylpicoxamid or [*phenyl-UL-*$^{14}$C]florylpicoxamid, was up to 21% of AD. Absorption rates in mice, rats and rabbits were approximately 21%, 34% and 77% respectively.

When radiolabelled florylpicoxamid was administered orally to rats, values for $C_{max}$ and the area under the concentration–time curve (AUC) in plasma were approximately 2-fold to 3-fold (for the $C_{max}$) and 5-fold to 6-fold (for the AUC) greater at 250 mg/kg bw than at 25 mg/kg bw. The results indicated saturation of absorption with increasing dose from 25 to 250 mg/kg bw (Himmelstein 2019a, b).

Tissue distribution of florylpicoxamid was widespread, but levels in tissues were low. Tissue:plasma ratios indicated very low potential for accumulation. The highest tissue:plasma ratios

were in the gastrointestinal tract and liver. Female rats tended to have lower retained tissue levels than did males (Himmelstein 2019a, b).

Up to five metabolites were identified in rat urine. The most abundant identified urinary metabolite in all species was X12485473 after administration of radiolabelled florylpicoxamid or X12485649. The overall metabolism was consistent with hydrolysis, hydroxylation, and glucuronide conjugation reactions (Himmelstein 2020).

The in vitro metabolism of radiolabelled florylpicoxamid was compared in human, rat, mouse, dog and rabbit hepatocytes, and in another study in liver microsomes from mouse, rat, rabbit, dog, and human donors. No unique human metabolites were identified in the hepatocyte samples when compared to the rat, mouse, dog and rabbit preparations. Chiral analysis was carried out to investigate possible interconversion of stereoisomeric florylpicoxamid and the florylpicoxamid metabolite X12485649 (deacetylated) and their isomers; no interconversion of stereoisomers was observed (Chan, 2020a).

The key initial metabolic step in the metabolism of florylpicoxamid involved deacetylation to give X12485649. Subsequent reactions involved, aromatic oxidation, aliphatic and aromatic hydroxylation, ester bridge formation and amide cleavage, *O*-demethylation, and phase II glucuronide conjugation. Metabolites identified in bile and plasma were generally consistent with those previously identified in faeces and urine, and confirmed the role of first-pass hepatic metabolism contributing to biliary elimination and systemic uptake as key processes in the extensive metabolism and elimination of florylpicoxamid (Himmelstein, 2019b).

Toxicokinetic evaluation of florylpicoxamid associated with continuous daily dietary intake indicated rapid absorption, hydrolysis of parent florylpicoxamid to X12485649 and further metabolism to X12485473 and X12641325. Quantitation of X12485473 and X12641325 in blood, urine, and/or milk confirmed these analytes were the major metabolites in animals exposed to florylpicoxamid. Blood analysis as part of reproductive and development toxicity studies indicated no apparent direct transfer of florylpicoxamid or its metabolites (X12485649, X12584261, and X12530093) from dams to fetuses via the blood (Chan, 2019;, 2020b, c).

## Toxicological data

The acute oral median lethal dose ($LD_{50}$) for florylpicoxamid in rats was greater than 2000 mg/kg bw (Verma, 2015) and the dermal $LD_{50}$ was greater than 2000 mg/kg bw (Patel, 2016a). The inhalation median lethal concentration ($LC_{50}$) for florylpicoxamid was greater than 5.48 mg/L (Patel, 2016b). Florylpicoxamid was not irritating to the skin (Patel, 2016c), but produced a slightly irritating effect on rabbit eyes (Patel, 2016d). Florylpicoxamid was shown not to be sensitizing in a local lymph node assay (LLNA) test in mice (Patel, 2016e).

In repeat-dose oral toxicity studies with florylpicoxamid in mice, rats and dogs the main targets of toxicity were decreased food consumption, body weight and body weight gain. Florylpicoxamid showed CAR/PXR activation leading to liver hypertrophy.

In a 13-week dietary toxicity study in which mice were given florylpicoxamid at dietary concentrations of 0, 150, 500 or 1500 ppm (equal to 0, 19.1, 59.7 and 192 mg/kg bw per day for males, 0, 21.9, 68.3 and 201 mg/kg bw per day for females), the NOAEL was 500 ppm (equal to 59.7 mg/kg bw per day) based on slight decreases in body weight, body weight gain and food consumption in females at 1500 ppm (equal to 201 mg/kg bw per day) (Mukerji, 2018).

In a 28-day dietary toxicity study in which rats were given florylpicoxamid at dietary concentrations of 0, 300, 1000 or 3000 ppm (equal to 0, 24.9, 84.0 and 230 mg/kg bw per day for males, 0, 26.1, 85.2 and 243 mg/kg per day for females), the NOAEL was 1000 ppm (equal to 84.0 mg/kg bw per day) based on decreases in food consumption, suppressed body weight gains and effects on haematological parameters in males at 3000 ppm (equal to 230 mg/kg bw per day) (Murphy et al., 2018).

In a separate 28-day dietary toxicity study, florylpicoxamid was administered to a different strain of rat from the first study, at dietary concentrations of 0, 1000, 3000, 4000 or 5000 ppm (equal to 0, 69.9, 206, 278 and 345 mg/kg bw per day for males, 0, 75.8, 247, 320 and 355 mg/kg bw per day for females) The NOAEL was 3000 ppm (equal to 206 mg/kg bw per day) based on marginal hepatocellular hypertrophy and thyroid follicular cell hypertrophy in males at 4000 ppm (equal to 278 mg/kg bw

per day) (Heward, 2018).

In a thirteen-week dietary toxicity study, rats were given florylpicoxamid at dietary concentrations of 0, 300, 1000 or 3000 ppm (equal to 0, 18.2, 59.4 and 177 mg/kg bw per day for males, 0, 18.8, 63.0 and 185 mg/kg bw per day for females). The NOAEL was 1000 ppm (equal to 59.4 mg/kg bw per day) based on slight decreases in body weight and suppressed body weight gain, and lowered haematological parameters in males at 3000 ppm (equal to 177 mg/kg bw per day). Florylpicoxamid induced metabolic enzymes in the liver in both sexes via CAR/PXR (Mukerji, 2019).

In a 90-day oral toxicity study, dogs were administered florylpicoxamid in capsules at doses of 0, 60, 150 or 400 mg/kg bw per day. The NOAEL was 60 mg/kg bw per day based on lower body weights, decreased body weight gains and decreased food consumption in males at 150 mg/kg bw per day (Heward, 2017).

In an 18-month carcinogenicity study in mice, florylpicoxamid was given to mice at dietary concentrations of 0, 150, 500 or 1500 ppm (equal to 0, 16.4, 55.3 and 172 mg/kg per day for males, 0, 20.4, 72.2 and 230 mg/kg bw per day, but only 45 weeks at this highest dose for females). All female mice receiving 1500 ppm were euthanized at 46 weeks due to high mortality. No further investigation was conducted with this group. The NOAEL for chronic toxicity in mice was 500 ppm (equal to 55.3 mg/kg bw per day) based on lower body weight in males at 1500 ppm (equal to 172 mg/kg bw per day). The NOAEL for carcinogenicity in male mice was 1500 ppm (equal to 172 mg/kg bw per day), the highest dose tested. In females, the high dose of 1500 ppm caused excess mortality that prevented proper evaluation of carcinogenicity. No increased cancer incidence was found at 500 ppm (equal to 72.2 mg/kg bw per day) (Albretson, 2019a).

In a two-year combined toxicity and carcinogenicity study in rats, florylpicoxamid was administered at dietary concentrations for males of 0, 300, 1000 or 3000 ppm (equal to 0, 11.9, 39.7 and 123 mg/kg bw per day), and for females of 0, 300, 1000 or 4000 ppm (equal to 0, 14.0, 46.9 and 200 mg/kg bw per day). The NOAEL for chronic toxicity was 1000 ppm (equal to 46.9 mg/kg bw per day) based on low body weight in females at 4000 ppm (equal to 200 mg/kg bw per day). The NOAEL for carcinogenicity was 3000 ppm (equal to 123 mg/kg bw per day), the highest dose tested (Albretsen, 2019b).

The Meeting concluded that florylpicoxamid is not carcinogenic in male mice or rats.

Florylpicoxamid was tested for genotoxicity in an adequate range of studies. No evidence of genotoxicity was found.

The Meeting concluded that florylpicoxamid is unlikely to be genotoxic.

In view of the lack of genotoxicity and the absence of carcinogenicity in male mice and rats, the Meeting concluded that florylpicoxamid is unlikely to pose a carcinogenic risk to humans.

In a two-generation reproductive toxicity study, rats were administrated florylpicoxamid at dietary concentrations of 0, 300, 1000 or 3000 ppm (males only) or a top dose of 4000 ppm in the case of females. Corresponding time-weighted average doses of florylpicoxamid were 0, 17.5, 58.4 and 179 mg/kg bw per day for males of the first-generation parents (F0), and 0, 19.3, 65.3 and 255 mg/kg bw per day during gestation. The NOAEL for parental toxicity was 1000 ppm (equal to 58.4 mg/kg bw per day) based on lowered body weight in males at 3000 ppm (equal to 179 mg/kg bw per day) in the F0 and F1 adult males. The NOAEL for reproductive toxicity (based on males only) was 3000 ppm (equal to 179 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 1000 ppm (equal to 58.4 mg/kg bw per day) based on decreased body weight and decreases in red blood cell parameters in male and female F2 weanlings at 3000 ppm (equal to 179 mg/kg bw per day) (Passage, 2019).

In a rat developmental study, florylpicoxamid was given to rats at dietary levels of 0, 300, 1000, or 4000 ppm (equal to 0, 21.3, 73.1 and 271 mg/kg bw per day), from gestation day (GD) 6 to GD 21. The NOAEL for maternal toxicity was 1000 ppm (equal to 73.1 mg/kg bw per day), based on decreases in body weight and food consumption at 4000 ppm (equal to 271 mg/kg bw per day). The NOAEL for embryo/fetal toxicity was 4000 ppm (equal to 271 mg/kg bw per day), the highest dose tested (Passage, 2018a).

In a developmental toxicity study, female rabbits were given florylpicoxamid at dietary concentrations of 0, 125, 250 or 750 ppm (equal to 0, 4.73, 9.58 and 25.9 mg/kg bw per day) from GD 7 to GD 28. The Meeting considered the incidence of crooked hyoid which occurred at a frequency slightly above the historical control data not to be adverse because it is known that eventually the bones ossify and the angulation resolves after the birth. The NOAEL for maternal toxicity was 250 ppm (equal to 9.58 mg/kg bw per day) based on abortions. The NOAEL for embryo/fetal toxicity was 750 ppm (equal to 25.9 mg/kg bw per day), the highest dose tested (Chan, 2019; Passage, 2018c).

The Meeting concluded that florylpicoxamid is not teratogenic.

In a thirteen-week dietary toxicity study in rats that included specific investigations of neurotoxicity and immunotoxicity, no evidence of neurotoxicity or immunotoxicity was observed up to 3000 ppm (equal to 177 mg/kg bw per day), the highest dose tested (Mukerji, 2019).

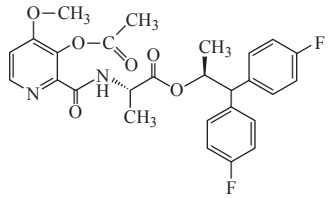
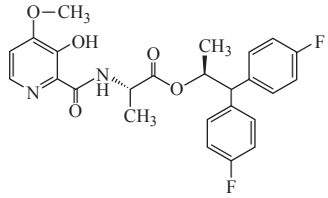
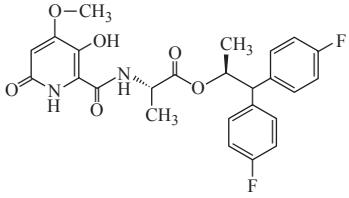
The Meeting concluded that florylpicoxamid is not neurotoxic.

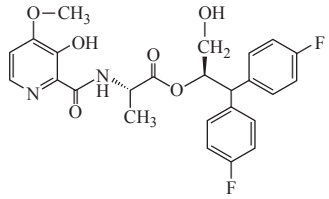
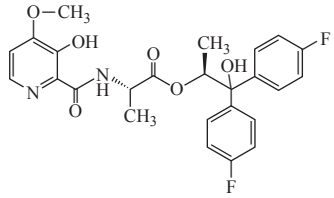
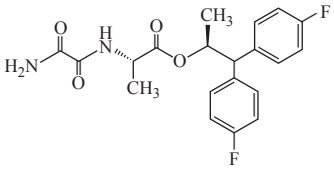
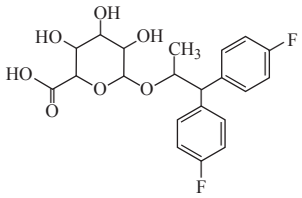
The Meeting concluded that florylpicoxamid is not immunotoxic.

Florylpicoxamid did not show any estrogenic agonist or antagonist activity in vitro (Verkaart, 2020).

## Toxicological data on metabolites and/or degradates

***Table 25. Summary of toxicological characterization of plant/livestock metabolites***

| **Compound, codes and structure** | **Major rat metabolite** (> 10% AD) | **Genotoxicity assessment** (data, QSAR, read-across) | **General toxicity** | **Toxicological reference values** |
|---|---|---|---|---|
| **Florylpicoxamid** (X12485659; XDE-659;)  | Parent | Not genotoxic (data) | Full dataset | ADI: 0–0.1 mg/kg bw per day<br>ARfD: Unnecessary |
| **X12485649**  | The first metabolite of the parent; X12485649-glucuronide was observed at >10% of AD in rat bile. | Not genotoxic in QSAR analysis | Covered by parent | Parent ADI |
| **X12629973**  | No | Not genotoxic in QSAR analysis | No information | TTC Cramer class III value: 1.5 µg/kg bw/day |

| Compound, codes and structure | Major rat metabolite (> 10% AD) | Genotoxicity assessment (data, QSAR, read-across) | General toxicity | Toxicological reference values |
|---|---|---|---|---|
| **X12641685**  | No | Not genotoxic in QSAR analysis | No information | TTC Cramer class III value: 1.5 µg/kg bw/day |
| **X12584261**  | No | Not genotoxic in QSAR analysis | No information | TTC Cramer class III value: 1.5 µg/kg bw/day |
| **X12563767**  | No | Not genotoxic (data) | No information | TTC Cramer class III value: 1.5 µg/kg bw/day |
| **X12641325**  | >10% of AD in rat urine and bile | Not genotoxic as covered by parent | Covered by parent | Parent ADI |

QSAR Quantitative structure–activity relationship; AD Administered dose; ARfD Acute reference dose
TTC Threshold of toxicological significance; ADI Acceptable daily intake

## Microbiological aspects

There was no information available in the public domain and no experimental data were submitted that addressed the possible impact of florylpicoxamid residues on the human intestinal microbiome.

## Human data

A compilation of all the medical assessment program data revealed no reports or findings of adverse health effects deemed attributable to exposure to the product. Additionally, no reports of any adverse health effects deemed attributable to exposure to the product were found in searches of the personal medical records of the employees involved.

No clinical cases of poisoning incidents have been detected from the open literature, based on the data collected by the sponsor.

The Meeting concluded that the existing database on florylpicoxamid was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

## Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.1 mg/kg bw for florylpicoxamid based on the NOAEL of 9.58 mg/kg bw per day in the developmental toxicity study in rabbits, and applying a safety factor of 100. This ADI also applies to metabolites X12485649 and X12641325.

The Meeting concluded that it was not necessary to establish an acute reference dose (ARfD) for florylpicoxamid in view of its low acute oral toxicity and the absence of developmental toxicity likely to be elicited by a single dose or any other toxicological effects likely to be elicited by a single dose. The early suppression of body weight gain, observed in the rabbit developmental toxicity study, was considered not to be a suitable basis for an ARfD because the effect was likely caused by lower palatability.

***Levels relevant to risk assessment of florylpicoxamid***

| Species | Study | Effect | NOAEL | LOAEL |
|---|---|---|---|---|
| Mouse | 78-week study of toxicity and carcinogenicity [a] | Toxicity | 500 ppm, equal to 55.3 mg/kg bw per day | 1500 ppm, equal to 172 mg/kg bw per day |
| | | Carcinogenicity | 1500 ppm, equal to 172 mg/kg bw per day [g] | - |
| | 13-Week toxicity study [a] | Toxicity | 500 ppm, equal to 68.3 mg/kg bw per day | 1500 ppm, equal to 201 mg/kg bw per day |
| Rat | 13-Week toxicity study [a] | Toxicity | 1000 ppm, equal to 59.4 mg/kg bw per day | 3000 ppm, equal to 177 mg/kg bw per day |
| | | Neurotoxicity | 3000 ppm, equal to 177 mg/kg bw per day [c] | - |
| | | Immunotoxicity | 3000 ppm, equal to 177 mg/kg bw per day [c] | - |
| | Two-year studies of toxicity and carcinogenicity [a, d] | Toxicity | 1000 ppm, equal to 46.9 mg/kg bw per day | 4000 ppm, equal to 200 mg/kg bw per day |
| | | Carcinogenicity | 3000 ppm, equal to 123 mg/kg bw per day [c] | - |
| | Two-generation study of reproductive toxicity [a] | Reproductive toxicity | 3000 ppm, equal to 179 mg/kg bw per day [c] | - |
| | | Parental toxicity | 1000 ppm, equal to 58.4 mg/kg bw per day | 3000 ppm, equal to 179 mg/kg bw per day |
| | | Offspring toxicity | 1000 ppm, equal to 58.4 mg/kg bw per day | 3000 ppm, equal to 179 mg/kg bw per day |
| | Developmental toxicity study [a] | Maternal toxicity | 1000 ppm, equal to 73.1 mg/kg bw per day | 4000 ppm, equal to 271 mg/kg bw per day |
| | | Embryo/fetal toxicity | 4000 ppm, equal to 271 mg/kg bw per day [c] | - |
| Rabbit | Developmental toxicity study [a] | Maternal toxicity | 250 ppm, equal to 9.58 mg/kg bw per day | 750 ppm, equal to 25.9 mg/kg bw per day |
| | | Embryo/fetal toxicity | 750 ppm, equal to 25.9 mg/kg bw per day [c] | - |
| Dog | 13-week studies of toxicity [b] | Toxicity | 60 mg/kg bw per day | 150 mg/kg bw per day |

[a] Dietary administration; [b] Gavage administration; [c] Highest dose tested

[d] Two or more studies combined; [e] Lowest dose tested; [f] Capsule administration

[g] The value was obtained from males; no available information on toxicity or carcinogenicity in females at 1500 ppm due to early termination. The NOAEL for carcinogenicity in males was 172 mg/kg bw per day, the highest dose tested.

*Acceptable daily intake (ADI) applies to florylpicoxamid, metabolites X12485649 and X12641325 expressed as florylpicoxamid*

0–0.1 mg/kg bw

*Acute reference dose (ARfD)*

Unnecessary

## Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other observational studies of human exposure

***Critical end-points for setting guidance values for exposure to florylpicoxamid***

| | |
|---|---|
| **Absorption, distribution, excretion and metabolism in mammals** | |
| Rate and extent of oral absorption | Rapid: 21% (mice), 34% (rats), 77% (rabbits) |
| Dermal absorption | No data |
| Distribution | Widespread |
| Potential for accumulation | No evidence of accumulation |
| Rate and extent of excretion | Rapid elimination via faeces, urine or bile |
| Metabolism in animals | Deacetylation, aromatic oxidation, aliphatic and aromatic hydroxylation, ester bridge formation or amide cleavages, *O*-demethylation, and phase II glucuronide conjugation |
| Toxicologically significant compounds in animals and plants | Florylpicoxamid, X12485649, X12641325, X12641685, M12563767, X12584261 and X12629973 |
| **Acute toxicity** | |
| Rat, $LD_{50}$, oral | >2000 mg/kg bw |
| Rat, $LD_{50}$, dermal | >2000 mg/kg bw |
| Rat, $LC_{50}$, inhalation | 5.48 mg/L |
| Rabbit, dermal irritation | Not irritating. |
| Rabbit, ocular irritation | Slightly irritating |
| Mouse, dermal sensitization | Not sensitizing (LLNA) |
| ***Short-term studies of toxicity*** | |
| Target/critical effect | Body weight, body wt gain, food consumption (rat, dog) |
| Lowest relevant oral NOAEL | 59.4 mg/kg bw per day (rat)<br>60 mg/kg bw per day (dog) |
| Lowest relevant dermal NOAEL | No data. |
| Lowest relevant inhalation NOAEC | No data. |
| **Long-term studies of toxicity and carcinogenicity** | |
| Target/critical effect | Body weight |
| Lowest relevant NOAEL | 46.9 mg/kg bw per day (rat) |
| Carcinogenicity | Not carcinogenic [a] |
| **Genotoxicity** | Unlikely to be genotoxic [a] |
| **Reproductive toxicity** | |
| Target/critical effect | Body weight/red blood cells |
| Lowest relevant parental NOAEL | 58.4 mg/kg bw per day (rat) |
| Lowest relevant offspring NOAEL | 58.4 mg/kg bw per day (rat) |
| Lowest relevant reproductive NOAEL | 179 mg/kg bw per day, highest dose tested (rat) |

| | |
|---|---|
| **Developmental toxicity** | |
| Target/critical effect | Abortion |
| Lowest relevant maternal NOAEL | 9.58 mg/kg bw per day (rabbit) |
| Lowest relevant embryo/fetal NOAEL | 25.9 mg/kg bw per day, highest dose tested (rabbit) |
| **Neurotoxicity** | |
| Acute neurotoxicity NOAEL | No evidence from routine studies |
| Subchronic neurotoxicity NOAEL | 177 mg/kg bw per day, highest dose tested (rat) |
| Developmental neurotoxicity NOAEL | No data |
| **Other toxicological studies** | |
| Immunotoxicity | 177 mg/kg bw per day, highest dose tested (rat) |
| **Studies on metabolites** | |
| ***X12485649*** | Acute oral $LD_{50}$: 1000 mg/kg bw (rat)<br>Acute dermal $LD_{50}$: >2000 mg/kg bw<br>Acute inhalation $LC_{50}$: 0.125 mg/L air<br>Mildly irritating |
| ***X696476*** | Not irritating (EpiDerm reconstituted human epidermis)<br>Not clastogenic (micronucleus in vitro) |
| ***X12563767*** | Not mutagenic (in vitro bacterial reverse mutation test)<br>Not clastogenic (in vitro mammalian micronucleus test) |
| **Microbiological aspects** | No data submitted |
| **Human data** | No reports of any adverse health effects |

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

## Summary

| | Value | Study | Safety factor |
|---|---|---|---|
| ADI | 0–0.1 mg/kg bw [a] | Developmental toxicity study in rabbits | 100 |
| ARfD | Unnecessary | | |

[a] Applies to florylpicoxamid, X12485649 and X12641325, expressed as florylpicoxamid

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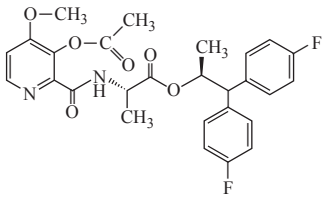
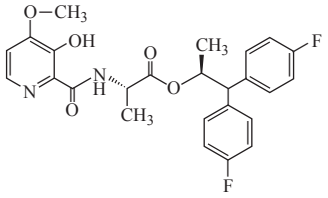
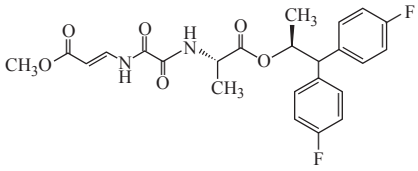
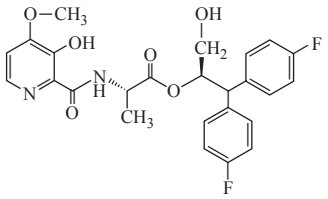
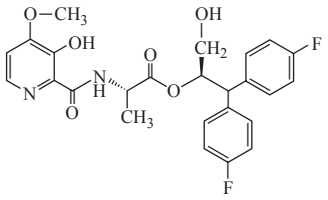
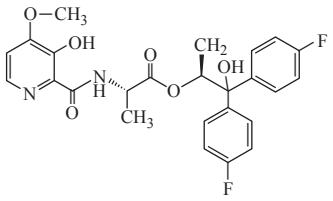
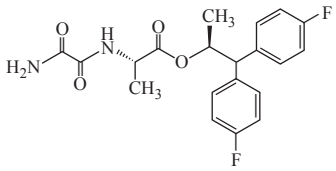
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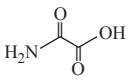
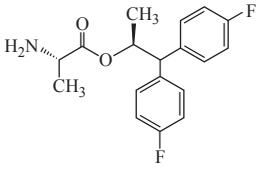
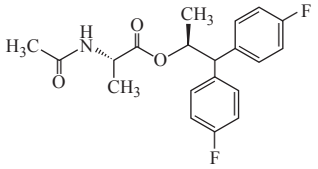
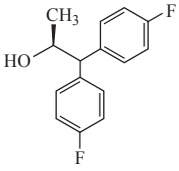
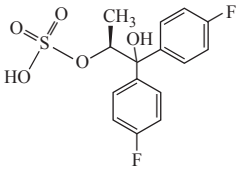
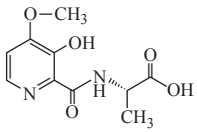
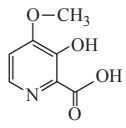
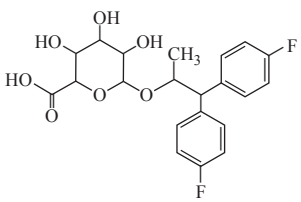
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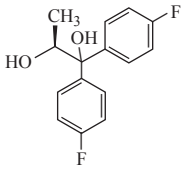
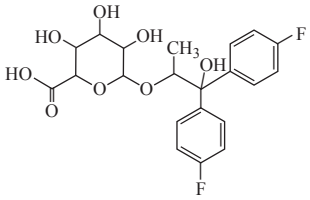
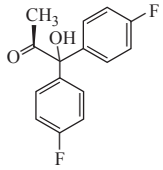
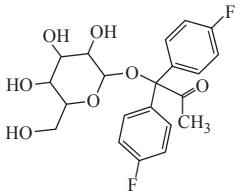
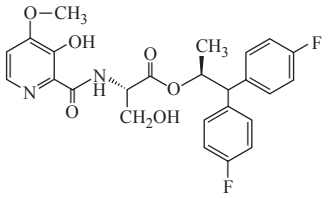
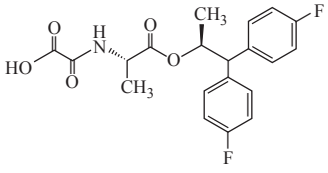
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# Annex 1
# List of relevant florylpicoxamid metabolites

*Ann. 1, Table 1. relevant florylpicoxamid metabolites*

| Name & abbreviation | Chemical structure | Major rat metabolite? (greater than 10% of AD /General toxicity) | Genotoxicity assessment (data, QSAR, read-across) |
|---|---|---|---|
| **Florylpicoxamid** (XDE-659; X12485659) |  | Parent/<br>Full dataset | Not genotoxic (data) |
| **X12485649** |  | The first metabolite of the parent; X12485649-glucuronide was observed at > 10% of AD in rat bile/<br>No information | Not genotoxic in QSAR analysis |
| **X12568155** |  | Not found/<br>No information | Not genotoxic in QSAR analysis |
| **X12629973** |  | No/<br>No information | Not genotoxic in QSAR analysis |
| **X12641685** |  | No/<br>No information | Not genotoxic in QSAR analysis |
| **X12584261** |  | No/<br>No information | Not genotoxic in QSAR analysis |
| **X12563767** |  | No/<br>No information | Not genotoxic (data) |

| **Name & abbreviation** | **Chemical structure** | **Major rat metabolite?** (greater than 10% of AD /General toxicity) | **Genotoxicity assessment** (data, QSAR, read-across) |
|---|---|---|---|
| **Oxamic acid** |  | No/<br>No information | Not genotoxic in QSAR analysis |
| **X12485647** |  | No/<br>Oral $LD_{50}$: 1000 mg/kg bw;<br>Dermal $LD_{50}$: > 2000 mg/kg bw;<br>$LC_{50}$: 0.125 mg/L air;<br>EpiDerm, irritant | Not genotoxic in QSAR analysis |
| **X12516991** |  | No/<br>No information | Not genotoxic in QSAR analysis |
| **X12485631** |  | >10% AD in rat bile after glucuronidase treatment/<br>No information | Covered by the parent toxicity |
| **X12493055-sulfate conjugate** |  | Not found/<br>No information | Not genotoxic in QSAR analysis |
| **X12485473** |  | > 10% of AD in rat urine; high concentration in mouse, rat and rabbit blood/<br>No information | Covered by the parent toxicity |
| **X696476** |  | No/<br>EpiDerm, Not irritant | Not genotoxic (data);<br>Not genotoxic in QSAR analysis |
| **X12641325** |  | > 10% of AD in rat urine and bile;<br>high concentration in rat and rabbit blood/<br>No information | Covered by the parent toxicity |

| Name & abbreviation | Chemical structure | Major rat metabolite? (greater than 10% of AD /General toxicity) | Genotoxicity assessment (data, QSAR, read-across) |
|---|---|---|---|
| **X12493055** |  | No/<br>No information | Not genotoxic in QSAR analysis |
| **X12714411** |  | No/<br>No information | Not genotoxic in QSAR analysis |
| **X12764243** |  | Not found/<br>No information | Not genotoxic in QSAR analysis |
| **Glucose conjugate of X12764243 (Metabolite B)** |  | Not found/<br>No information | Not genotoxic in QSAR analysis |
| **X12530089** |  | Not found/<br>No information | Not genotoxic in QSAR analysis |
| **X12562911** |  | Not found/<br>No information | Not genotoxic in QSAR analysis |
| **X12518891** | A stereoisomer of florylpicoxamid | | |
| **X12504939**<br>**X12506173** | } Stereoisomers of X12485649 | | |

AD: Administered dose; QSAR: Quantitative structure–activity relationship;

In rat ADME:

No: Not a major rat metabolite; Not found: Not a rat metabolite;

In general toxicity:

No information: No toxicity study conducted; blank in genotoxicity assessment; no genotoxicity test including in silico analysis conducted.

# Annex 2
# Detailed toxicokinetic data from selected studies

## 1. Toxicokinetic analysis of florylpicoxamid and its metabolites in feeding studies (excluding long-term studies)

The major numerical data on kinetics of metabolites shown in Table 9 (p 215) are given in detail below.

### 1.1 Developmental toxicity probe in female Crl:CD(SD) rats

The analysis results show that florylpicoxamid and metabolite X12530093 were below the LOD in the blood samples from rats at 3000 ppm. The most abundant metabolite quantified in rats and fetuses was X12485473 followed by X12641325. The dose-normalized AUC responses for female rats had a ranking order of X12485473 (0.0107 h × kg × mg/kg/mg) ≈ X12641325 (0.00911 h × kg × mg/kg/mg) > X12485649 (0.00156 h × kg × mg/kg/mg) >> X12584261 (0.000141 (h × kg × mg/kg/mg). In general, systemic exposure in female rats was highest for X12485473 followed by X12641325, then X12485649, and the lowest for X12584261.

Blood concentrations of X12641325 in fetuses (1.08 mg/kg) were higher than rats (0.182 mg/kg) at terminal sacrifice. Blood concentrations of X12485473 in fetuses (0.341 mg/kg) were slightly lower than rats (0.382 mg/kg). X12485649 was detected in 4 rats at terminal sacrifice at 3000 ppm (0.0142 to 0.0415 mg/kg) but was only detected in 2 fetuses (0.0167 to 0.0221 mg/kg). X12584261 was detected in one rat and was below the LOD in all fetuses. The mean blood concentrations in rat:fetus ratios had a ranking order of X12485473 (1.12:1) >> X12641325 (0.17:1).

***Ann. 2, Table 1. Toxicokinetics parameters of florylpicoxamid and its metabolites from female rats in developmental toxicity probe in female rats***

| | | Dietary concentration | | | |
|---|---|---|---|---|---|
| | | 0 ppm | | 3000 ppm | |
| **Analyte** | **Parameter** | **Mean** | **SD** | **Mean** | **SD** |
| | Mean TMI, GDs 18–21, (mg/kg bw per day) | 0 | 0 | 922 | 68.9 |
| Florylpicoxamid | $AUC_{24}$(mg × hour/kg) | NC | - | NC | - |
| | $AUC_{24}$/TMI | NC | - | NC | - |
| X12485649 | $AUC_{24}$ (mg × hour/kg) | NC | - | 1.36 | 1.12 |
| | $AUC_{24}$/TMI | NC | - | 0.00156 | 0.00145 |
| X12485473 | $AUC_{24}$ (mg × hour/kg) | NC | - | 9.80 | 2.09 |
| | $AUC_{24}$/TMI | NC | - | 0.0107 | 0.00253 |
| X12584261 | $AUC_{24}$ (mg × hour/kg) | NC | - | 0.114 | 0.255 |
| | $AUC_{24}$/TMI | NC | - | 0.000141 | 0.000316 |
| X12641325 | $AUC_{24}$ (mg × hour/kg) | NC | - | 8.34 | 3.16 |
| | $AUC_{24}$/TMI | NC | - | 0.00911 | 0.00351 |
| X12530093 | $AUC_{24}$ (mg × hour/kg) | NC | - | NC | - |
| | $AUC_{24}$/TMI | NC | - | NC | - |

TMI: Test material intake; SD: Standard deviation; GD: Gestation day Source: Chan, 2019

NC: Toxicokinetic parameters were not calculated due to the blood concentration values being set to 0 mg/kg;

$AUC_{24}$: Area under the concentration–time curve in the first 24 hours following administration

## 1.2 Dietary developmental toxicity study in Crl:CD(SD) rats

Blood samples were analyzed to quantify the concentration of the parent test substance, florylpicoxamid, and metabolites (X12485649, X12485473, X12584261, X12641325, and X12530093). The analysis results show that the parent test substance, florylpicoxamid and X12530093 were below the LOD in the blood samples of rats and pups in all dietary concentration groups. The most abundant metabolite quantified in rats and pups was X12641325, followed by X12485473. Blood concentrations of X12641325 and X12485473 in pups were 0.264–3.83 mg/kg and 0.0422–0.501 mg/kg, respectively compared with blood concentrations in rats, which were 0.0865–0.662 mg/kg and 0.0636–1.00 mg/kg, respectively. Blood concentrations of X12641325 and X12485473 in rats and pups were relatively dose-dependent in that the concentrations of each analyte increased in a relatively proportional manner with increasing doses. Metabolite X12485649 was detected in adult rats at all dietary concentrations (0.0153–0.143 mg/kg) but was only detected in pups from the 1000 and 4000 ppm dietary dose groups (0.00815–0.0295 mg/kg). Metabolite X12584261 in the 300 ppm dose groups was below the LOD in adult rats but was detected in increasing amounts (0.00600–0.0398 mg/kg) in the 1000 and 4000 ppm groups. Metabolite X12584261 was below the LOD in pups dosed at 300 and 1000 ppm, and was detected only in 4000 ppm group pups, registering a mean blood concentration of 0.0147 mg/kg. Blood concentrations of X12485649 and X12584261 appeared to be similar in rats and pups. The mean ratio in blood concentrations for rat: pup, ratios ranked as follows:

| | | | | | | |
|---|---|---|---|---|---|---|
| X12485649 | >> | X12584261 | > | X12485473 | >> | X12641325 |
| (4.85:1 to 6.25:1) | | (2.71:1) | | (1.51:1 to 2.00:1) | | (0.173:1 to 0.628:1) |

***Ann. 2, Table 2. Toxicokinetics parameters of florylpicoxamid and its metabolites from female rats and pups in dietary developmental toxicity study in Crl:CD(SD) Rats***

| | Dietary concentration | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 ppm | | 300 ppm | | 1000 ppm | | 4000 ppm | |
| **Analyte** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** |
| Mean TMI, GDs18–21, (mg/kg bw per day) | 0 | 0 | 19.3 | 1.82 | 59.4 | 12.2 | 264 | 9.09 |
| **Adult rats** | | | | | | | | |
| Florylpicoxamid (mg/kg) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| X12485649 (mg/kg) | 0 | 0 | 0.0153 | 0.0190 | 0.0509 | 0.0522 | 0.143 | 0.0530 |
| X12485473 (mg/kg) | 0 | 0 | 0.0636 | 0.0126 | 0.234 | 0.0346 | 1.00 | 0.116 |
| X12584261 (mg/kg) | 0 | 0 | 0 | 0 | 0.006 | 0.0120 | 0.0398 | 0.0174 |
| X12641325 (mg/kg) | 0 | 0 | 0.0865 | 0.0139 | 0.279 | 0277 | 0.662 | 0.138 |
| X12530093 (mg/kg) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Pups** | | | | | | | | |
| Florylpicoxamid (mg/kg) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| X12485649 (mg/kg) | 0 | 0 | 0 | 0 | 0.00815 | 0.0163 | 0.0295 | 0.0352 |
| X12485473 (mg/kg) | 0 | 0 | 0.0422 | 0.0326 | 0.119 | 0.0854 | 0501 | 0.268 |
| X12584261 (mg/kg) | 0 | 0 | 0 | 0 | 0 | 0 | 0.0147 | 0.0178 |
| X12641325 (mg/kg) | 0 | 0 | 0.264 | 0.244 | 0.444 | 0.279 | 3.83 | 3.26 |
| X12530093 (mg/kg) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

TMI: Test material intake; SD: Standard deviation; GD: Gestation day Source: Chan, 2019

## 1.3 Palatability probe study in rabbits

In female New Zealand White rabbits, florylpicoxamid, X12485649, X12584261 and X12530093 for 350 and 750 ppm dose groups were below the LOQ; in the 1250 ppm dietary group, amongst all time points, there were less than three animals with measurable values. Therefore, $AUC_{24}$ values were not calculated for these analytes according to their respective dietary concentration groups. Metabolites X12485473 and X12641325 increased from 5.35 to 18.2 hour × mg/kg (a 3.4-fold increase) and from

7.25 to 20.1 hour × mg/kg (a 2.8-fold increase), as the test material intake rose from 12.6 to 46.2 mg/kg bw per day (a 3.7-fold increase), suggesting a less than dose proportional increase in $AUC_{24}$ between the low (350 ppm) and high (1250 ppm) dietary exposure concentrations. The dose-normalized $AUC_{24}$ responses for X12485473 and X12641325 appeared to show a marginal decrease with increasing dose. The general pattern of decreasing dose-normalized $AUC_{24}$ values suggest a non-dose proportional response due to saturation of metabolite formation. The magnitude of systemic exposure was slightly higher for X12641325 in female rabbits.

At terminal sacrifice on GD 28, florylpicoxamid, X12584261 and X12530093 in the 350, 750, and 1250 ppm dose groups, and X12485649 in the 750 ppm group were below the LOQ in blood samples from dams and fetuses. This finding indicated no apparent direct transfer of florylpicoxamid or these metabolites to the fetuses from maternal blood. Blood concentrations of X12485473 and X12641325 in rabbits ranged from 0.198–0.480 mg/kg and 0.316–0.559 mg/kg for the 350 and 750 ppm dietary groups respectively. Blood concentrations in fetuses ranged from 0.0542–0.124 mg/kg and 0.0680–0.143 mg/kg for the 350 and 750 ppm dose groups respectively. The mean blood concentrations for X12485473 and X12641325 were not calculated at 1250 ppm due to less than 3 animals having measured values.

***Ann. 2, Table 3. Toxicokinetics parameters of florylpicoxamid and its metabolites in a palatability probe study in rabbits***

| | | Dietary concentration (ppm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 350 | | 750 | | 1250 | |
| **Analyte** | | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** |
| **Dams** | | | | | | | |
| | Mean TMI, GDs 26–28, (mg/kg bw per day) | 12.6 | 1.18 | 27.1 | 0.868 | 46.2 | 1.95 |
| Florylpicoxamid | $AUC_{24}$ (mg × hour/kg) | NC | - | NC | - | NC | - |
| | $AUC_{24}$/TMI | NC | - | NC | - | NC | - |
| | Expected $AUC_{24}$ (h*mg/kg)[a] | <LOQ | - | <LOQ | - | <LOQ | - |
| X12485649 | $AUC_{24}$ (mg × hour/kg) | ISS | ISS | NC | - | NC | - |
| | $AUC_{24}$/TMI | ISS | ISS | NC | - | NC | - |
| | Expected $AUC_{24}$ (mg × hour/kg)[a] | <LOQ | - | <LOQ | - | <LOQ | - |
| X12485473 | $AUC_{24}$ (mg × hour/kg) | 5.35 | 1.76 | 11.3 | 2.91 | 18.2 | 1.55 |
| | $AUC_{24}$/TMI | 0.426 | 0.141 | 0.420 | 0.121 | 0.395 | 0.0293 |
| | Expected $AUC_{24}$ (mg × hour/kg)[a] | 5.35 | - | 11.5 | - | 19.6 | - |
| X12584261 | $AUC_{24}$ (mg × hour/kg) | NC | - | NC | - | NC | - |
| | $AUC_{24}$/TMI | NC | - | NC | - | NC | - |
| | Expected $AUC_{24}$ (mg × hour/kg)[a] | <LOQ | - | <LOQ | - | <LOQ | - |
| X12641325 | $AUC_{24}$ (mg × hour/kg) | 7.25 | 2.28 | 13.6 | 4.17 | 20.1 | 2.05 |
| | $AUC_{24}$/TMI | 0.576 | 0.179 | 0.506 | 0.171 | 0.434 | 0.0267 |
| | Expected $AUC_{24}$ (mg × hour/kg)[a] | 7.25 | - | 15.6 | - | 26.6 | - |
| X12530093 | $AUC_{24}$ (mg × hour/kg) | NC | - | NC | - | NC | - |
| | $AUC_{24}$/TMI | NC | - | NC | - | NC | - |
| | Expected $AUC_{24}$ (mg × hour/kg)[a] | <LOQ | - | <LOQ | - | <LOQ | - |

TMI: Test material intake; SD: Standard deviation; GD: Gestation day Source: Chan, 2019

NC: Toxicokinetic parameters were not calculated due to the blood concentration values being set to 0 mg/kg;

<LOQ: Not estimated due to the blood concentration values in individual animal below LOQ (0.0134 mg/kg)

$AUC_{24}$: Area under the concentration–time curve in the first 24 hours following administration;

ISS: Inadequate sample size; mean and SD were not calculated due to less than three animals giving measurable values;

[a] The measured $AUC_{24}$ at the lowest TMI level was extrapolated through the origin to the higher TMI levels;

*Ann. 2, Table 4. Mean blood concentrations at terminal sacrifice of dams and fetuses in dietary developmental toxicity probe study in rabbits*

| | | Dietary concentrations (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | | 350 | | 750 | | 1250 | |
| Analyte | | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| | Mean TMI, GDs 26–28, (mg/kg bw per day) | 0 | 0 | 12.6 | 1.18 | 27.1 | 0.868 | 46.2 | 1.95 |
| Florylpicoxamid | Terminal[a] (mg/kg) | 0 | 0 | 0 | 0 | 0 | 0 | ISS | ISS |
| | Fetus[a] (mg/kg) | 0 | 0 | 0 | 0 | 0 | 0 | ISS | ISS |
| X12485649 | Terminal[a] (mg/kg) | 0 | 0 | 0.00450 | 0.0090 | 0 | 0 | ISS | ISS |
| | Fetus[a] (mg/kg) | 0 | 0 | 0 | 0 | 0 | 0 | ISS | ISS |
| X12485473 | Terminal[a] (mg/kg) | 0 | 0 | 0.198 | 0.0999 | 0.480 | 0.175 | ISS | ISS |
| | Fetus[a] (mg/kg) | 0 | 0 | 0.0542 | 0.0303 | 0.124 | 0.0437 | ISS | ISS |
| X12584261 | Terminal[a] (mg/kg) | 0 | 0 | 0 | 0 | 0 | 0 | ISS | ISS |
| | Fetus[a] (mg/kg) | 0 | 0 | 0 | 0 | 0 | 0 | ISS | ISS |
| X12641325 | Terminal[a] (mg/kg) | 0 | 0 | 0.316 | 0.158 | 0.559 | 0.240 | ISS | ISS |
| | Fetus[a] (mg/kg) | 0 | 0 | 0.0680 | 0.0299 | 0.143 | 0.0456 | ISS | ISS |
| X12530093 | Terminal[a] (mg/kg) | 0 | 0 | 0 | 0 | 0 | 0 | ISS | ISS |
| | Fetus[a] (mg/kg) | 0 | 0 | 0 | 0 | 0 | 0 | ISS | ISS |

TMI: Test material intake; SD: Standard deviation; GD: Gestation day; Source: Chan, 2019

ISS: Inadequate sample size; mean and SD were not calculated due to less than three animals giving measurable values;

[a] For calculation of mean and SD, blood concentration value from individual animals reported as below the LOQ (0.0134 mg/kg) was set to 0 mg/kg.

### 1.4 Dietary developmental toxicity study in rabbits

In dams, the dose-normalized $AUC_{24}$ values for X12485473 and X12641325 appeared to show a marginal decrease with increasing dose. The general pattern of decreasing dose-normalized $AUC_{24}$ values suggests a response that was not dose-proportional due to saturation of metabolite formation. Systemic exposure was higher for X12641325 than for X12485473. Florylpicoxamid, X12485649, X12584261 and X12530093 were below the limit of detection (LOD) for groups dosed at 125, 250 or 750 ppm.

At terminal sacrifice on GD 28, florylpicoxamid, X12485649, X12584261 and X12530093 for the 125, 250, and 750 ppm dose groups were below the LOD in blood samples from both adult rabbits and fetuses. Metabolite X12485473 was detected in adult rabbits across all dietary concentrations. X12485473 was not quantifiable in fetuses at 125 ppm and was present in two blood samples (at 0.0322 and 0.0316 mg/kg in animals 265 and 273) from animals dosed at 250 ppm. Metabolite X12485473 was consistently quantifiable at 750 ppm. The mean blood concentrations of X12485473 in adult rabbits were 0.0851 mg/kg and 0.102 mg/kg for 125 and 250 ppm groups respectively. The blood concentration of X12485473 was 0.0160 mg/kg in fetuses dosed at 250 ppm. This finding indicated no apparent direct transfer of florylpicoxamid or metabolites X12485649, X12584261 or X12530093 to the fetuses via blood from the mothers. Metabolite X12641325 was quantifiable in adult rabbits and fetuses across the dietary concentration range tested. The mean blood concentrations of X12641325 were 0.114, 0.132 and 0.486 mg/kg for 125, 250 and 750 ppm dose groups, respectively. Metabolite X12641325 concentrations in fetuses were 0.0176, 0.0371 and 0.217 mg/kg for the 125, 250 and 750 ppm dose groups, respectively. The mean blood concentrations of X12485473 and X12641325 in adult rabbits were 2–6-fold higher than the mean blood concentrations in the corresponding fetuses.

***Ann. 2, Table 5. Toxicokinetic parameters for florylpicoxamid (mean ± standard deviations shown) and its metabolites on GD 27 in pregnant rabbits fed florylpicoxamid from GD 6 to GD 27***

| | | Dietary concentration (ppm) | | |
|---|---|---|---|---|
| **Analyte** | **Parameter** | **125** | **250** | **750** |
| Florylpicoxamid (XDE-659) | Mean TMI,(mg/kg bw per day) | 4.48 | 9.14 | 26.8 |
| | $AUC_{24}$, (mg × hour/kg) | NC | NC | NC |
| | $AUC_{24}$/TMI | NC | NC | NC |
| X12485649 | $AUC_{24}$, (mg × hour/kg) | 0 | NC | NC |
| | $AUC_{24}$/TMI | 0 | NC | NC |
| X12485473 | $AUC_{24}$, (mg × hour/kg) | 2.42 ± 0.508 | 4.29 ± 0.990 | 10.8 ± 4.10 |
| | $AUC_{24}$/TMI | 0.541 ± 0.114 | 0.468 ± 0.0989 | 0.407 ± 0.114 |
| X12584261 | $AUC_{24}$, (mg × hour/kg) | NC | NC | NC |
| | $AUC_{24}$/TMI | NC | NC | NC |
| X12641325 | $AUC_{24}$, (mg × hour/kg) | 3.41 ± 0.646 | 5.77 ± 1.54 | 13.3 ± 6.17 |
| | $AUC_{24}$/TMI | 0.762 ± 0.149 | 0.629 ± 0.156 | 0.496 ± 0.186 |
| X12530093 | $AUC_{24}$, (mg × hour/kg) | NC | NC | NC |
| | $AUC_{24}$/TMI | NC | NC | NC |

TMI: Test material intake; GD: Gestation day Source: Chan, 2019

$AUC_{24}$: Area under the concentration–time curve in the first 24 hours following administration;

NC: Toxicokinetic parameters were not calculated due to the blood concentration values being set to 0 mg/kg

***Ann. 2, Table 6. Blood concentrations of florylpicoxamid (mean ± standard deviations shown) and its metabolites in dams and fetuses through dietary administration to dams from a development toxicity study in rabbits***

| | | Dietary concentrations (ppm) | | |
|---|---|---|---|---|
| **Compartment** | **Analyte** | **125** | **250** | **750** |
| Dams | Florylpicoxamid | 0 | 0 | 0 |
| | X12485649 | 0 | 0 | 0 |
| | X12485473 | 0.0851 ± 0.0410 | 0.102 ± 0.0321 | 0.429 ± 0.156 |
| | X12584261 | 0 | 0 | 0 |
| | X12641325 | 0.114 ± 0.0579 | 0.132 ± 0.0440 | 0.486 ± 0.190 |
| | X12530093 | 0 | 0 | 0 |
| Fetuses | Florylpicoxamid | 0 | 0 | 0 |
| | X12485649 | 0 | 0 | 0 |
| | X12485473 | 0 | 0.0160 ± 0.0184 | 0.153 ± 0.163 |
| | X12584261 | 0 | 0 | 0 |
| | X12641325 | 0.0176 ± 0.0125 | 0.0371 ± 0.0148 | 0.217 ± 0.223 |
| | X12530093 | 0 | 0 | 0 |

Source: Chan, 2019

### 1.5 Dietary reproduction/developmental toxicity screening test in Crl:CD(SD) rats

In male rats, florylpicoxamid concentrations in blood were below the LOQ at the low (300 ppm) and middle (1000 ppm) dose levels and only occasionally detected at the highest (3000 ppm) feeding concentration. These data were insufficient to calculate $AUC_{24}$ values for florylpicoxamid. Although the concentration of X12485649 was below the LOQ for all samples at 300 ppm, it was consistently quantifiable at 1000 and 3000 ppm and the mean dose-normalized $AUC_{24}$ values appeared to show a marginal increase with increasing dose. The dose-normalized responses for X12485473, X12584261 and X12641325 also increased in a similar manner. Like X12485649, X12584261 was not quantifiable in blood from 300 ppm dose groups, but was thereafter at 1000 and 3000 ppm. Metabolite X12530093 was not detected in any of the samples. The general pattern of increasing dose-normalized $AUC_{24}$ values suggested a response that was not dose-proportional, due to saturation of metabolite elimination. For male rats, systemic exposure was highest for X12641325 followed by X12485473 then X12485649, and lowest for X12584261.

The dose-normalized $AUC_{24}$ response for X12485649 in female rats was similar to that of male rats. Female rats had noticeably higher dose-normalized $AUC_{24}$ values for X12485473 at all three dietary concentrations when compared with male rats, and systemic exposure appeared to decline at 1000 ppm and 3000 ppm relative to 300 ppm. Levels of X12584261 were much lower in female than male rats. The dose-normalized $AUC_{24}$ values for X12641325 in female rats increased slightly between the 300 ppm and 1000 ppm dose groups, and appeared to decline slightly in 3000 ppm group. Although the standard deviations were quite large, the response for X12485473 suggests a tendency towards saturation of formation, while X12641325 showed an initial saturation of elimination, followed by saturation of formation. In general, for female rats during premating, the magnitude of systemic exposure was similar for X12485473 and X12641325, lower for X12485649 and even lower for X12584261.

Analysis of blood and milk samples in female rats on LD 4 resulted in mean milk:blood ratios in dams with a ranking order of:

| | | | | | | |
|---|---|---|---|---|---|---|
| X12485649 | > | X12584261 | >> | X12485473 | >> | X12641325 |
| (11:1–14:1) | | (9.3:1) | | (0.74:1–1.6:1) | | (0.22:1–0.24:1). |

No florylpicoxamid was observed in milk. The presence of these metabolites in milk suggests the potential for transfer to pups. The analysis of pup blood showed limited transfer of X12485473 and X12641325. Metabolite X12485473 was only observed at the highest (3000 ppm) dietary concentration at mean blood concentrations of 0.00363 and 0.00768 mg/kg in male and female pups, respectively. Metabolite X12641325 was quantifiable in dose groups at 1000 ppm and 3000 ppm at mean blood concentrations ranging from 0.0181–0.0765 mg/kg in male pups and 0.0215–0.0818 mg/kg in female pups. These concentrations in pup blood were 1–2 orders of magnitude lower than the corresponding mean blood concentrations in the dams.

***Ann. 2, Table 7. Toxicokinetic parameters of florylpicoxamid and its metabolites in adult rats from a dietary reproduction/developmental toxicity screening test in rats; mean ± SD shown***

| | | Dietary concentrations (ppm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Males | | | Females | | |
| **Analyte** | **Parameter** | **300** | **1000** | **3000** | **300** | **1000** | **3000** |
| Mean TMI, test days 7–14 (mg/kg bw per day) | | 23.0 ± 1.54 | 77.7 ± 5.16 | 234 ± 5.87 | 22.9 0 ± 0 .834 | 76.4 ± 4.35 | 236 ± 17.0 |
| Florylpicoxamid | $AUC_{24}$, (mg × hour/kg)[a] | NC | NC | 0.115 ± 0.231 | NC | NC | 0.0425 ± 0.0850 |
| | $AUC_{24}$/TMI | NC | NC | 0.000488 ± 0.000975 | NC | NC | 0.000165 ± 0.000330 |
| | Expected $AUC_{24}$ (mg × hour/kg)[a] | <LOQ | NC | 0.115 | <LOQ | <LOQ | 0.425 |

| | | Dietary concentrations (ppm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Males | | | Females | | |
| **Analyte** | **Parameter** | **300** | **1000** | **3000** | **300** | **1000** | **3000** |
| X12485649 | $AUC_{24}$, (mg × hour/kg) | NC | 0.263 ± 0.381 | 1.14 ± 0.784 | NC | 0.130 ± 0.151 | 1.43 ± 0.895 |
| | $AUC_{24}$/TMI | NC | 0.00339 ± 0.00486 | 0.00485 ± 0.00329 | NC | 0.00166 ± 0.00191 | 0.00591 ± 0.00336 |
| | Expected $AUC_{24}$ (mg × hour/kg)[a] | <LOQ | 0.263 | 0.792 | <LOQ | 0.130 | 0.402 |
| X12485473 | $AUC_{24}$, (mg × hour/kg) | 0.176 ± 0.121 | 1.07 ± 0.245 | 4.47 ± 3.15 | 1.09 ± 0.274 | 2.81 ± 0.349 | 8.67 ± 1.97 |
| | $AUC_{24}$/TMI | 0.00445 ± 0.00890 | 0.0139 ± 0.000364 | 0.0190 ± 0.0132 | 0.0479 ± 0.0132 | 0.0367 ± 0.00411 | 0.0373 ± 0.0103 |
| | Expected $AUC_{24}$ (mg × hour/kg)[a] | 0.176 | 0.595 | 1.79 | 1.09 | 3.64 | 11.2 |
| X12584261 | $AUC_{24}$, (mg × hour/kg) | NC | 0.0785 ± 0.121 | 0.422 ± 0.258 | NC | NC | 0.0783 ± 0.157 |
| | $AUC_{24}$/TMI | NC | 0.00104 ± 0.00162 | 0.00181 ± 0.00109 | NC | NC | 0.000328 ± 0.000655 |
| | Expected $AUC_{24}$ (mg × hour/kg)[a] | <LOQ | 0.07885 | 0.236 | <LOQ | <LOQ | 0.0783 |
| X12641325 | $AUC_{24}$, (mg × hour/kg) | 0.108 ± 0.216 | 1.52 ± 0.835 | 6.75 ± 2.67 | 0.824 ± 0.466 | 4.10 ± 2.22 | 9.94 ± 4.28 |
| | $AUC_{24}$/TMI | 0.00445 ± 0.00890 | 0.0193 ± 0.00964 | 0.0287 ± 0.0109 | 0.0359 ± 0.0206 | 0.0527 ± 0.0254 | 0.0426 ± 0.0194 |
| | Expected $AUC_{24}$ (mg × hour/kg)[a] | 0.108 | 0.365 | 1.10 | 0.824 | 2.75 | 8.49 |
| X12530093 | $AUC_{24}$, (mg × hour/kg) | NC | NC | NC | NC | NC | NC |
| | $AUC_{24}$/TMI | NC | NC | NC | NC | NC | NC |
| | Expected $AUC_{24}$ (mg × hour/kg)[a] | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |

TMI: Test material intake; SD: Standard deviation; Source: Chan, 2019
$AUC_{24}$: Area under the concentration–time curve in the first 24 hours following administration;
NC: Toxicokinetic parameters were not calculated due to the blood concentration values being set to 0 mg/kg;
<LOQ: Not estimated due to the blood concentration values in individual animal below LOQ (0.00445 mg/kg)
[a] The measured $AUC_{24}$ at the lowest TMI level was extrapolated through the origin to the higher TMI levels;

***Ann. 2, Table 8. Milk and/or blood concentrations of florylpicoxamid and its metabolites in dams and pups in the reproduction/developmental toxicity screening test in rats***

| | | Dietary concentration (ppm) | | | |
|---|---|---|---|---|---|
| **Analytes** | **Parameter** | **0** | **300** | **1000** | **3000** |
| **Mean milk and blood concentrations in dams** | | | | | |
| Florylpicoxamid | Milk (mg/kg) | 0 | 0.173 ± 0.300 | 0 | 0.00470 ± 0.00940 |
| | Blood (mg/kg) | 0 | 0 | 0.0110 ± 0.0220 | 0 |
| | MB ratio | NC | NC | NC | NC |
| X12485649 | Milk (mg/kg) | 0 | 0.350 ± 0.168 | 0.949 ± 0.384 | 2.34 ± 0.156 |
| | Blood (mg/kg) | 0 | 00397 ± 0.0176 | 0.105 ± 0.0653 | 0.169 ± 0.0197 |
| | MB ratio | NC | 11.3 ± 9.43 | 12.4 ± 10.5 | 14.1 ± 2.76 |

| | | Dietary concentration (ppm) | | | |
|---|---|---|---|---|---|
| **Analytes** | **Parameter** | **0** | **300** | **1000** | **3000** |
| X12485473 | Milk (mg/kg) | 0 | 0.0727 ± 0.0314 | 0.395 ± 0.237 | 1.41 ± 0.242 |
| | Blood (mg/kg) | 0 | 0.133 ± 00713 | 0.381 ± 0.159 | 0.903 ± 0.111 |
| | MB ratio | NC | 0.739 ± 0.535 | 1.13 ± 0.718 | 1.55 ± 0.0997 |
| X12584261 | Milk (mg/kg) | 0 | 0.0214 ± 0.0200 | 0.0566 ± 0.0196 | 0.286 ± 0.0539 |
| | Blood (mg/kg) | 0 | 0 | 0 | 0.0310 ± 0.00753 |
| | MB ratio | NC | NC | NC | 9.33 ± 0.766 |
| X12641325 | Milk (mg/kg) | 0 | 0.00853 ± 0.0165 | 0.0531 ± 0.0232 | 0.181 ± 0.0731 |
| | Blood (mg/kg) | 0 | 0.0966 ± 0.0828 | 0.268 ± 0.0966 | 0.784 ± 0.402 |
| | MB ratio | NC | ISS | 0.221 ± 0.124 | 0.242 ± 0.0417 |
| X12530093 | Milk (mg/kg) | 0 | 0 | 0 | 0.00443 ± 0.00895 |
| | Blood (mg/kg) | 0 | 0 | 0 | 0 |
| | MB ratio | NC | NC | NC | NC |
| **Mean blood concentrations in male and female pups (SD calculated from 3 animals)** | | | | | |
| Mean TMI LD1–4 (mg/kg bw/day) | | 0 | 33.5 ± 3.14 | 115 ± 3.85 | 350 ± 26.5 |
| Florylpicoxamid | Male (mg/kg) | 0 | 0 | 0 | 0 |
| | Females (mg/kg) | 0 | 0 | 0 | 0 |
| X12485649 | Male (mg/kg) | 0 | 0 | 0 | 0 |
| | Females (mg/kg) | 0 | 0 | 0 | 0 |
| X12485473 | Male (mg/kg) | 0 | 0 | 0 | 0.00363 ± 0.00725 |
| | Females (mg/kg) | 0 | 0 | 0 | 0.00768 ± 0.00896 |
| X12584261 | Male (mg/kg) | 0 | 0 | 0 | 0 |
| | Females (mg/kg) | 0 | 0 | 0 | 0 |
| X12641325 | Male (mg/kg) | 0 | 0 | 0.0181 ± 0.00212 | 0.0765 ± 0.0194 |
| | Females (mg/kg) | 0 | 0.00368 ± 0.00735 | 0.0215 ± 0.00464 | 0.0818 ± 0.00579 |
| X12530093 | Male (mg/kg) | 0 | 0 | 0 | 0 |
| | Females (mg/kg) | 0 | 0 | 0 | 0 |

TMI: Test material intake; SD: Standard deviation; Source: Chan, 2019

MB: Milk:blood concentration ratio; for calculation of mean and SD, milk or blood concentration value from individual animal reported as < LOD (0.00445 mg/kg) was set to 0 mg/kg;

NC: Ratio were not calculated due to milk and blood concentration values being set to 0 mg/kg;

ISS: Inadequate sample size; not calculated due to less than three animals having measurable concentration values

### 1.6 90-Day dietary toxicity study with a 28-day recovery period in rats with an assessment of immunotoxicity, neurotoxicity, in vivo genetic toxicity and toxicokinetics

In male F344/DuCrl rats, florylpicoxamid concentrations in blood were below the LOQ for groups dosed at 300 ppm, and sporadically detected at 1000 and 3000 ppm. The mean dose-normalized $AUC_{24}$ values for florylpicoxamid appeared to show a marginal decrease with increasing dose due to saturation of absorption. Although X12485649 and X12584261 were below the LOQ for all samples from rats dosed at 300 ppm, they were consistently quantifiable in the 1000 and 3000 ppm dose group samples. The dose-normalized responses for X12485649 and X12584261 appeared to show a marginal increase with increasing dose. The response for X12485473 showed an initial saturation of elimination, followed by a less pronounced saturation of formation. Metabolite X12530093 was not detected in any of the samples. The general pattern of increasing dose-normalized $AUC_{24}$ values suggested a response that was not dose-proportional, due to saturation of metabolite elimination. For male rats, systemic exposure was highest for X12485473 followed by X12485649, then X12584261, and lowest for florylpicoxamid. Systemic exposures to X12485649, X12584261 and florylpicoxamid were about 5-fold to 27-fold lower than for X12485473.

In female from this study, florylpicoxamid concentrations in blood were below the LOQ for groups dosed at 300 ppm and sporadically detected at 1000 ppm and 3000 ppm. The mean dose-normalized $AUC_{24}$ values for florylpicoxamid appeared to show a marginal decrease with increasing dose due to saturation of absorption. Although X12485649 in rats dosed at 300 ppm and X12584261 in those at 300 and 1000 ppm were below the LOQ for all samples, amounts of X12485649 were consistently quantifiable in rats dosed at 1000 ppm, whilst X12485649 and X12584261 were measurable in those dosed at 3000 ppm. The dose-normalized $AUC_{24}$ response for X12485649 appeared to decrease at 3000 ppm, suggesting saturation of formation. Female rats had noticeably higher dose-normalized $AUC_{24}$ values for X12485473 at all three dietary concentrations compared with male rats, and the systemic exposure appeared to increase at 1000 ppm and decline at 3000 ppm, suggesting an initial saturation of elimination followed by saturation of formation. The level of metabolite X12584261 was slightly lower in female than male rats. Metabolite X12530093 was not detected in any of the samples. In general, the magnitude of systemic exposure in female rats was highest for X12485473 followed by X12485649, then X12584261, and lowest for florylpicoxamid. Systemic exposure to X12485649, X12584261 and florylpicoxamid was about 4-fold to 400-fold lower than for X12485473. The elimination half-life of X12485473 in females dosed at 3000 ppm was 7.66 (± 2.26) hours.

The majority of the dose in urine (calculated as the mole percentage of daily test material intake) was recovered as X12485473, and ranged from 5.45% to 6.80% in males and 17.5% to 18.1% in females. The percentage of the daily dose ingested recovered in urine for each analyte remained relatively constant with increasing dose. Overall, the percentage of the daily dose ingested that was recovered in urine as florylpicoxamid, X12485649, X12584261, X12530093 and X12485473 combined, was 5.49%, 6.52%, and 6.86% in males and 17.82%, 17.53%, and 18.12% in females at dose levels of 300, 1000 and 3000 ppm, respectively.

***Ann. 2, Table 9. Toxicokinetic parameters of florylpicoxamid and its metabolites in a 90-day dietary toxicity study in rats; (mean ± standard deviations shown)***

| | | Dietary concentrations (ppm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Males | | | Females | | |
| **Analyte** | **Parameter** | **300** | **1000** | **3000** | **300** | **1000** | **3000** |
| Mean TMI test day 85–91 (mg/kg bw per day) | | 13.9 ± 0.416 | 45.0 ± 2.30 | 138 ± 12.6 | 13.8 ± 0.709 | 50.0 ± 1.25 | 144 ± 10.1 |
| Florylpicoxamid | $AUC_{24}$, (mg × hour/kg) | NC | 0.0438 ± 0.107 | 0.123 ± 0.226 | NC | 0.0345 ± 0.0845 | 0.0142 ± 0.0347 |
| | $AUC_{24}$/TMI | NC | 0.000910 ± 0.00223 | 0.000865 ± 0.00163 | NC | 0.000692 ± 0.00169 | 0.0000932 ± 0.000228 |
| | Elimination half-life, (hours) | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| X12485649 | $AUC_{24}$, (mg × hour/kg) | NC | 0.097 ± 0.106 | 0.378 ± 0. 454 | NC | 0.466 ± 0.0369 | 0.773 ± 0.106 |
| | $AUC_{24}$/TMI | NC | 0.00216 ± 0.00238 | 0.00275 ± 0.00328 | NC | 0.00933 ± 0.000784 | 0.00541 ± 0.000884 |
| | Elimination half-life, (hours) | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| X12584261 | $AUC_{24}$, (mg × hour/kg) | NC | 0.0248 ± 0.0608 | 0.416 ± 0.141 | NC | NC | 0.322 ± 0.172 |
| | $AUC_{24}$/TMI | NC | 0.000568 ± 0.00139 | 0.00299 ± 0.000918 | NC | NC | 0.00219 ± 0.00112 |
| | Elimination half-life, (hours) | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| X12530093 | $AUC_{24}$, (mg × h/kg) | NC | NC | NC | NC | NC | NC |
| | $AUC_{24}$/TMI | NC | NC | NC | NC | NC | NC |
| | Elimination half-life, (hours) | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |

| | | Dietary concentrations (ppm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Males | | | Females | | |
| Analyte | Parameter | 300 | 1000 | 3000 | 300 | 1000 | 3000 |
| X12485473 | $AUC_{24}$, (mg × hour/kg) | 0.0337 ± 0.0825 | 0.693 ± 0.237 | 2.12 ± 1.21 | 0.449 ± 0.167 | 2.16 ± 0.333 | 5.34 ± 1.04 |
| | $AUC_{24}$/TMI | 0.00243 ± 0.00596 | 0.0154 ± 0.00523 | 0.0152 ± 0.00852 | 0.0324 ± 0.0113 | 0.0431 ± 0.00592 | 0.0373 ± 0.00734 |
| | Elimination half-life, (hours) | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | 7.66 ± 2.26 |

TMI: Test material intake; SD: Standard deviation; Source: Chan, 2019

$AUC_{24}$: Area under the concentration–time curve in the first 24 hours following administration;
NC: Toxicokinetic parameters were not calculated due to the blood concentration values being set to 0 mg/kg;
<LOQ: Not estimated due to the blood concentration values in individual animals below LOQ (0.0134 mg/kg)

## 1.7 90 Day dietary toxicity study in mice

In male Crl:CD1 (ICR) mice, florylpicoxamid and X12530093 concentrations in blood were below the LOQ at all time points across the diet concentrations tested. As a result the $AUC_{24}$ values were not calculated for these analytes. Metabolite X12485649 levels were below LOQ in mice fed at 150 ppm, but was consistently quantifiable at the 500 and 1500 ppm dose levels at 13:00 on test day 90. Metabolite X12485649 was sporadically detected at the time of sacrifice at 08:00 on test day 91 across all doses. These data were insufficient to estimate an elimination half-life forX12485649. Metabolites X12584261 and X12485473 were sporadically detected at 08:00 on test day 91 in 150 ppm samples, similarly to X12485649. Metabolite X12584261 was detected in 1500 ppm samples only at 13:00 on test day 90, but was present in three and two samples at 08:00 on test day 91 (the time of sacrifice ) in the 500 ppm and 1500 ppm samples. Metabolite X12485473 was quantifiable only in the 1500 ppm dose group at 13:00 on test day 90, but was present in two samples and below LOQ for all other samples at 08:00 on test day 91 (the time of sacrifice) in the 500 and 1500 ppm samples, similar to what was seen for X12584261. Due to less than three animals having measurable blood concentrations of X12584261 and X12485473, the elimination half-lives were not estimated.

In females the mean blood concentrations of florylpicoxamid and X12530093 were similar to those in male mice. Levels of metabolites X12485649 and X12485473 were below the LOQ for the dose group at 150 ppm. Females had noticeably higher dose-normalized $AUC_{24}$ values for X12485649 and X12485473 compared with males, and systemic exposure appeared to decline for mice fed at 500 ppm and 1500 ppm. The responses for X12485649 and X12485473 suggested a tendency toward saturation of formation. Metabolite X12584261 was only detected in the 1500 ppm samples. For females, systemic exposure was highest for X12485649 followed by X12485473, and lowest for X12584261. Due to less than three animals having measurable blood concentrations of X12584261 and X12485473, these elimination half-lives were not estimated.

Urine volumes appeared consistent between sexes and levels of florylpicoxamid intake. Metabolite X12530093 was below the LOQ in all urine samples at all doses. Metabolite X12485473 was detected as the major metabolite in mice. Concentrations of X12485473 ranged from 3.61–43.7 mg/kg in male mice and 3.59–56.8 mg/kg in females for samples from 150, 500 and 1500 ppm groups. A small amount of florylpicoxamid, X12485649 and X12584261were detected in urine.

***Ann. 2, Table 10. Toxicokinetics parameters of florylpicoxamid and its metabolites in 90-day dietary toxicity study in mice; (mean ± standard deviations shown)***

| | | Dietary concentrations (ppm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Males | | | Females | | |
| **Analyte** | **Parameter** | **150** | **500** | **1500** | **150** | **500** | **1500** |
| Mean TMI, test days 85–91 (mg/kg bw per day) | | 15.1 ± 2.22 | 53.7 ± 1.84 | 173 ± 32.7 | 16.9 ± 1.23 | 50.4 ± 3.54 | 167 ± 18.3 |
| Florylpicoxamid | $AUC_{24}$, (mg × hour/kg) | NC | NC | NC | NC | NC | 0.0142 ± 0.0347 |
| | $AUC_{24}$/TMI | NC | NC | NC | NC | NC | 0.0000932 ± 0.000228 |
| | Elimination half-life, (hours) | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| X12485649 | $AUC_{24}$, (mg × hour/kg) | NC | 0.461 ± 0.258 | 1.34 ± 0.316 | 0640 ± 0.165 | 1.47 ± 0. 19 | 0.773 ± 0.106 |
| | $AUC_{24}$/TMI | NC | 0.00846 ± 0.00466 | 0.00796 ± 0.00219 | 0.0127 ± 0.00312 | 0.00911 ± 0.00130 | 0.00541 ± 0.000884 |
| | Elimination half-life, (hours) | <LOQ | <LOQ | <LOQ | ISS | ISS | <LOQ |
| X12584261 | $AUC_{24}$, (mg × hour/kg) | NC | NC | NC | NC | NC | 0.533 ± 0.0848 |
| | $AUC_{24}$/TMI | NC | NC | NC | NC | NC | 0.000330 ± 0.000535 |
| | Elimination half-life, (hours) | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| X12530093 | $AUC_{24}$, (mg × h/kg) | NC | NC | NC | NC | NC | NC |
| | $AUC_{24}$/TMI | NC | NC | NC | NC | NC | NC |
| | Elimination half-life, (hours) | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| X12485473 | $AUC_{24}$, (mg × hour/kg) | NC | 0.0631 ± 0.0851 | 0.716 ± 0.230 | NC | 0.494 ± 0.181 | 1.59 ± 0.493 |
| | $AUC_{24}$/TMI | NC | 0.00121 ± 0.00164 | 0.00440 ± 0.00178 | NC | 0.00974 ± 0.00347 | 0.00983 ± 0.00290 |
| | Elimination half-life, (hours) | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | ISS |

TMI: Test material intake; SD: Standard deviation; Source: Chan, 2019

$AUC_{24}$: Area under the concentration–time curve in the first 24 hours following administration;

NC: Toxicokinetic parameters were not calculated due to the blood concentration values being set to 0 mg/kg;
<LOQ: Not estimated due to the blood concentration values in individual animals below LOQ (0.0134 mg/kg) at sacrifice;
ISS: Inadequate sample size; not calculated due to less than three animals having measurable concentration values;

## 1.8 Two-generation dietary reproduction toxicity study in Crl:CD(SD) rats

To evaluate the daily systemic exposure during the premating phase of the study $AUC_{24}$ values were calculated. Additionally, adult female blood and milk samples on lactation LD 4 and pup blood on PND 4 were collected and analyzed.

### First generation

In F0 males, florylpicoxamid concentrations in blood were below the LOD for the 300 ppm dose group and only occasionally detected at 1000 ppm. These data were insufficient for calculation of florylpicoxamid $AUC_{24}$. Florylpicoxamid was detected in the 3000 ppm group samples, and the resulting mean $AUC_{24}$ value was 4.10 mg × hour/kg. Although X12485649 was only occasionally detected in blood from the 300 and 1000 ppm males, it was consistently quantifiable in 3000 ppm group samples. The

dose-normalized responses for X12485473 and X12641325 showed an initial saturation of formation, followed by saturation of elimination. Like X12485649, X12584261 was not quantifiable in blood from the 300 ppm group, but was measurable in 1000 and 3000 ppm samples. Metabolite X12530093 was not detected in any of the samples. For F0 male rats, systemic exposure was highest for X12485473 followed by X12641325, then X12485649, and lowest for X12584261.

In F0 females, the dose-normalized responses for X12485649 and X12485473 showed a similar pattern, suggesting an initial saturation of elimination followed by a combination of saturation of elimination and formation. Females had slightly lower dose-normalized $AUC_{24}$ values for X12485473 at dose levels of 300 and 4000 ppm, compared with males at 300 and 3000 ppm. In a similar way to X12485649, dose-normalized $AUC_{24}$ values for X12485473 in females appeared increase from low to mid dose level and then decline at 4000 ppm. In females X12584261 was only detected only in 4000 ppm samples. Metabolite X12641325 levels appeared to increase sub-proportionally, suggesting saturation of elimination. In general, for F0 females during premating, the magnitude of systemic exposure was similar for X12485473 and X12641325, and lower for X12485649, with X12584261 exposure lowest of all.

Analysis of blood and milk samples in F0 females on LD 4 resulted in mean milk:blood concentration ratios in dams with a ranking order of X12485649 > X12584261 >> X12485473 > X12641325. No parent florylpicoxamid or X12530093 was detected in milk. The presence of metabolites in milk suggested a potential for transfer to pups. Analysis of pup blood showed limited transfer of X12485473 and X12641325. Metabolite X12485473 was only measured in pup blood at 4000 ppm. These concentrations in pup blood were one to two orders of magnitude lower than the corresponding mean blood concentrations in the dams.

**Second generation**

Compared with F0 males, blood concentrations of florylpicoxamid in F1 males were below the LOD in samples from the 300 ppm group, and sporadically detected in 1000 and 3000 ppm samples. These data were insufficient to calculate $AUC_{24}$ values for florylpicoxamid. The dose-normalized responses for X12485649, X12485473 and X12641325 showed an initial saturation of elimination followed by a combination of saturation of elimination and saturation of formation. Although X12584261 was present in only one blood sample (animal 3007) at 300 ppm, it was consistently quantifiable in the 1000 and 3000 ppm samples. Metabolite X12530093 was not detected in any of the F1 samples. For F1 male rats, systemic exposure was highest for X12641325 followed by X12485473, then X12485649 and lowest for X12584261.

In F1 females, the dose-normalized values for X12485649 and X12485473 showed a similar response pattern to dose level, suggesting an initial saturation of formation, followed by saturation of elimination. Female rats had slightly higher dose-normalized $AUC_{24}$ values of X12485473 in 300 ppm and 4000 ppm samples compared with samples from male rats dosed at 300 and 3000 ppm. In a similar pattern to X12485649, dose-normalized $AUC_{24}$ values for X12485473 and X12641325 appeared to decrease from 300 to 1000 ppm with values at 4000 ppm higher than at 300pp. Metabolite X12584261 was not detected in any of the samples. In general, for F1 female rats during premating, the magnitude of systemic exposure was similar for X12485473 and X12641325, and lower for X12485649.

Analysis of blood and milk samples in F1 females on LD 4 revealed mean milk:blood concentration ratios in dams with a ranking order: X12485649 >> X12584261 >> X12485473 > X12641325. The milk:blood concentration ratios for X12584261 were only calculated from the 4000 ppm values, due to less than three animals yielding measurable values at other dose levels. No florylpicoxamid or X12530093 were observed in milk. The presence of metabolites in milk suggested a potential for transfer to the pups. The analysis of pup blood revealed limited transfer of X12485473 and X12641325. Concentrations of metabolites in pup blood were one to two orders of magnitude lower than the corresponding mean blood concentrations in the dams.

***Ann. 2, Table 11. Toxicokinetic parameters of florylpicoxamid and selected metabolites in adult F0 and F1 generation rats; (mean ± standard deviations shown)***

| | | Dietary concentration (ppm)[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | F0 generation | | | F1 generation | | |
| **Analyte** | **Parameter** | **300** | **1000** | **3000/4000** | **300** | **1000** | **3000** |
| | | | | **Males** | | | |
| Florylpicox-amid | $AUC_{24}$, (mg × hour/kg) | NC | ISS | 4.10 ± 5.51 | NC | ISS | ISS |
| | $AUC_{24}$/TMI | NC | ISS | 0.0262 ± 0.0347 | NC | ISS | ISS |
| X12485649 | $AUC_{24}$, (mg × hour/kg) | ISS | ISS | 4.23 ± 2.19 | 0.0754 ± 0.07831 | 2.73 ± 4.17 | 1.54 ± 0.604 |
| | $AUC_{24}$/TMI | ISS | ISS | 0.0276 ± 0.0137 | 0.00457 ± 0.00511 | 0.0477 ± 0.0716 | 0.00870 ± 0.00336 |
| X12485473 | $AUC_{24}$, (mg × hour/kg) | 0.872 ± 1.08 | 1.46 ± 0.979 | 9.75 ± 6.94 | 0.452 ± 0.431 | 7.52 ± 10.3 | 6.91 ± 1.63 |
| | $AUC_{24}$/TMI | 0.0582 ± 0.0729 | 0.0289 ± 0.0197 | 0.0647 ± 0.0451 | 0.0270 ± 0.0262 | 0.131 ± 0.176 | 0.0391 ± 0.00818 |
| X12584261 | $AUC_{24}$, (mg × hour/kg) | NC | 0.247 ± 0.0353 | 0.655 ± 0.374 | ISS | 0.667 ± 0.0734 | 0.745 ± 0.387 |
| | $AUC_{24}$/TMI | NC | 0.00479 ± 0.000707 | 0.00430 ± 0.00225 | ISS | 0.0130 ± 0.00114 | 0.00419 ± 0.00211 |
| X12641325 | $AUC_{24}$, (mg × hour/kg) | 0.917 ± 0.317 | 1.41 ± 0.167 | 4.23 ± 2.87 | 0.808 ± 0.658 | 7.34 ± 4.00 | 8.00 ± 6.79 |
| | $AUC_{24}$/TMI | 0.0617 ± 0.0233 | 0.0274 ± 0.00439 | 0.0280 ± 0.0186 | 0.0495 ± 0.0403 | 0.131 ± 0.0671 | 0.0452 ± 0.0381 |
| X12530093 | $AUC_{24}$, (mg × hour/kg) | NC | NC | NC | NC | NC | NC |
| | $AUC_{24}$/TMI | NC | NC | NC | NC | NC | NC |
| | | | | **Females** | | | |
| Florylpicox-amid | $AUC_{24}$, (mg × hour/kg) | NC | ISS | ISS | ISS | ISS | 0.608 ± 0.822 |
| | $AUC_{24}$/TMI (mg × hour/kg) | NC | ISS | ISS | ISS | ISS | 0.00256 ± 0.00351 |
| X12485649 | $AUC_{24}$, (mg × hour/kg) | 0.129 ± 0.184 | 0.601 ± 0.251 | 2.18 ± 0.873 | 0.692 ± 0.450 | ISS | 2.93 ± 2.65 |
| | $AUC_{24}$/TMI | 0.00658 ± 0.00932 | 0.0104 ± 0.00495 | 0.0100 ± 0.00451 | 0.0376 ± 0.0257 | ISS | 0.0122 ± 0.0114 |
| X12485473 | $AUC_{24}$, (mg × hour/kg) | 0.563 ± 0.310 | 2.73 ± 0.740 | 9.53 ± 2.04 | 1.66 ± 0.921 | 4.96 ± 1.55 | 21.6 ± 4.34 |
| | $AUC_{24}$/TMI | 0.0301 ± 0.0151 | 0.0469 ± 0.0133 | 0.0428 ± 0.00950 | 0.0902 ± 0.0525 | 0.0774 ± 0.0267 | 0.0892 ± 0.0217 |
| X12584261 | $AUC_{24}$, (mg × hour/kg) | NC | NC | 0.292 ± 0.279 | NC | NC | ISS |
| | $AUC_{24}$/TMI | NC | NC | 0.00137 ± 0.00136 | NC | NC | ISS |

| | | Dietary concentration (ppm)[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | F0 generation | | | F1 generation | | |
| **Analyte** | **Parameter** | **300** | **1000** | **3000/4000** | **300** | **1000** | **3000** |
| X12641325 | $AUC_{24}$, (mg × hour/kg) | 0.536 ± 0.417 | 2.31 ± 0.949 | 9.92 ± 5.41 | 1.30 ± 0.790 | 2.37 ± 0.584 | 18.3 ± 4.60 |
| | $AUC_{24}$/TMI | 0.0303 ± 0.0267 | 0.0394 ± 0.0156 | 0.0444 ± 0.0242 | 0.0708 ± 0.0456 | 0.0364 ± 0.00649 | 0.0756 ± 0.0220 |
| X12530093 | $AUC_{24}$, (mg × hour/kg) | NC | NC | NC | NC | NC | NC |
| | $AUC_{24}$/TMI | NC | NC | NC | NC | NC | NC |

TMI: Test material intake; Source: Chan, 2019

$AUC_{24}$: Area under the concentration–time curve in the first 24 hours following administration;

NC: Ratio were not calculated as milk and/or blood concentrations were set to 0 mg/kg;

ISS: Inadequate sample size; not calculated due to less than three animals having measurable concentration of the analyte;

[a] 3000ppm for males, 4000 ppm for females

***Ann. 2, Table 12. Milk and blood concentrations of florylpicoxamid and its metabolites in F0 and F1 dams on LD 4; (mean ± standard deviations shown)***

| | | Dietary concentration (ppm)[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | F0 dams | | | F1 dams | | |
| **Analyte** | **Compartment** | **300** | **1000** | **4000** | **300** | **1000** | **4000** |
| Florylpicox-amid | Milk | 0 | 0 | 0 | 0 | 0 | 0 |
| | Blood | 0.00530 ± 0.0106 | 0 | 0.947 ± 1.12 | 0 | 0 | 0.221 ± 0.239 |
| | MB ratio[a] | NC | NC | NC | NC | NC | NC |
| X12485649 | Milk | 0.694 ± 0.673 | 1.42 ± 0.798 | 2.94 ± 1.22 | 0.890 ± 0.628 | 1.72 ± 1.14 | 3.23 ± 1.50 |
| | Blood | 0.0737 ± 0.0723 | 0.106 ± 0.0588 | 0.681 ± 0.660 | 0.0541 ± 0.0415 | 0.0853 ± .0548 | 0.412 ± 0.131 |
| | MB ratio[a] | 9.44 ± 2.33 | 13.4 ± 1.10 | 8.38 ± 6.20 | 16.8 ± 3.18 | 19.0 ± 3.26 | 9.12 ± 6.04 |
| X12485473 | Milk | 0.169 ± 0.0554 | 0.517 ± 0.155 | 1.236 ± 0.418 | 0.156 ± 0.0400 | 0.458 ± 0.0706 | 1.47 ± 0.304 |
| | Blood | 0.278 ± 0.0792 | 0.915 ± 0.205 | 4.22 ± 2.51 | 0.127 ± 0.0312 | 0.462 ± 0.0954 | 2.15 ± 0.905 |
| | MB ratio[a] | 0.623 ± 0.220 | 0.589 ± 0.205 | 0.391 ± 0.266 | 1.23 ± 0.0171 | 1.00 ± 0.137 | 0.791 ± 0.351 |
| X12584261 | Milk | 0.0391 ± 0.0211 | 0.131 ± 0.107 | 0.597 ± 0.284 | 0.0877 ± 0.0456 | 0.163 ± 0.120 | 0.620 ± 0.281 |
| | Blood | 0 | 0.0144 ±0.0184 | 0.0994 ± .0331 | 0.0440 ± 0.00880 | 0.0130 ± .0151 | 0.0724 ± .0338 |
| | MB ratio[a] | NC | ISS | 6.14 ± 2.01 | ISS | ISS | 8.62 ± 0.933 |

| | | Dietary concentration (ppm)[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | F0 dams | | | F1 dams | | |
| Analyte | Compartment | 300 | 1000 | 4000 | 300 | 1000 | 4000 |
| X12641325 | Milk | 0.0419 ± 0.0376 | 0.106 ± 0.0765 | 0.251 ± 0.0729 | 0.0299 ± 0.0112 | 0.118 ± 0.111 | 0.186 ± 0.0279 |
| | Blood | 0.238 ± 0.250 | 0.587 ± 0.378 | 2.28 ± 1.39 | 0.149 ± 0.0761 | 0.526 ± 0.522 | 1.14 ± 0.275 |
| | MB ratio[a] | 0.193 ± 0.0414 | 0.177 ± 0.0226 | 0.131 ± 0.0648 | 0.211 ± 0.0301 | 0.229 ± 0.0119 | 0.168 ± 0.0267 |
| X12530093 | Milk | 0 | 0 | 0 | 0 | 0 | 0 |
| | Blood | 0 | 0 | 0 | 0 | 0 | 0 |
| | MB ratio[a] | NC | NC | NC | NC | NC | NC |

[a] Milk:blood concentration ratio; LD: Lactation day; Source: Chan 2019

NC: Ratio were not calculated as milk and/or blood concentrations were set to 0 mg/kg;

ISS: Inadequate sample size; not calculated as less than three animals had measurable concentration of the analyte;

***Ann. 2, Table 13. Blood concentrations of florylpicoxamid and its metabolites in F1 and F2 pups on PND 4; (mean ± standard deviations shown)***

| | | Dietary concentration (ppm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | F1 pups | | | F2 pups | | |
| Sex | Analyte | 300 | 1000 | 4000 | 300 | 1000 | 4000 |
| | | | | Males | | | |
| | Florylpicoxamid | 0 | 0 | 0 | 0 | 0 | 0 |
| | X12485649 | 0 | 0 | 0 | 0 | 0 | 0 |
| | X12485473 | 0 | 0.00360 ± 0.00720 | 0.0243 ± 0.00446 | 0 | 0 | 0.00935 ± 0.0112 |
| | X12584261 | 0 | 0 | 0.00545 ± 0.0109 | 0 | 0 | 0 |
| | X12641325 | 0.0134 ± 0.00907 | 0.0352 ± 0.00820 | 0.103 ± 0.0310 | 0 | 0.0273 ± 0.0217 | 0.0887 ± 0.0218 |
| | X12530093 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | | | Females | | | |
| | Florylpicoxamid | 0 | 0 | 0 | 0 | 0 | 0 |
| | X12485649 | 0 | 0 | 0 | 0 | 0 | 0 |
| | X12485473 | 0 | 0 | 0.0215 ± 0.00478 | 0 | 0 | 0.0141 ± 0.00978 |
| | X12584261 | 0 | 0 | 0.00423 ± 0.00845 | 0 | 0 | 0.0141 ± 0.00978 |
| | X12641325 | 0.00485 ± 0.00970 | 0.0367 ± 0.0120 | 0.0920 ± 0.0401 | 0 | 0.0208 ± 0.0143 | 0.0937 ± 0.0109 |
| | X12530093 | 0 | 0 | 0 | 0 | 0 | 0 |

Source: Chan, 2019

## 2. Toxicokinetic analysis of florylpicoxamid and its metabolites in long-term studies, (Chan, 2020b and 2020c)

### 2.1 Study in mice

Florylpicoxamid and its metabolites (X12485649, X12584261, X12485473 and X12641325) were quantified in mouse blood and urine to evaluate systemic exposure and urinary excretion. Blood and urine samples were collected during the 18-month dietary oncogenicity study in which mice consumed diets containing 0, 150, 500 or 1500 ppm of florylpicoxamid. The test material intake data on test days 84–91, 168–175, 308–315, 364–371 and 532–539 for the male and female mice were used to bracket the test day periods when blood and urine collection occurred. In female mice the experiment at 1500 ppm was terminated on week 46. Blood and urine samples were analyzed for florylpicoxamid, X12485649, X12584261, X12485473 and X12641325. To evaluate the daily systemic exposure $AUC_{24}$ values were calculated. Urine samples were collected from male and female mice, and excreted urine weights recorded.

Florylpicoxamid concentrations in blood were below the LOQ in both sexes for all time points on all test days across the dietary concentration range tested. In males, systemic exposure was highest for X12641325 followed by X12485473, then X12485649, and lowest for X12584261. There was no significant differences observed in daily systemic exposure to X12485649, X12584261, X12485473, and X12641325 on test days 92, 176, 316, 372 or 540 across all diet groups. This lack of difference indicated that the steady state of exposure to X12485649, X12485473 and X12641325 had been reached within the dose range tested. For female mice, systemic exposure was highest for X12485473 in the highest dose group, followed by X12485649. The pattern of daily systemic exposure to X12485473 on test days 92, 176 and 316 at 1500 ppm in female mice was similar to that in males, indicating that a steady state had been reached in this dose group.

Overnight urine volumes collected over the 24-hour period across all test days ranged from 0.1 mL to 4.0 mL for male mice and 0.1 mL to 7.5 mL for females mice when measured together as a diet group. Only X12485473 and X12641325 were quantified in the urine of male and female mice. The largest proportion of AD recovered in urine was of X12485473 (calculated as the mole percentage of daily test material intake), and ranged from 1.06% to 2.84% in male mice and 1.45% to 4.65% in females. Overall, the percentage of daily intake recovered in urine as X12485473 and X12641325 combined, at doses of 150, 500 and 1500 ppm, ranged from 1.68%–4.00%, 1.95%–3.74% and 0.087%–3.08% respectively in male mice, and 4.35%–6.20%, 3.66%–4.38% and 0.177%–3.62% respectively in females, when assessed across all test days.

***Ann. 2, Table 14. The AUC and urine concentrations of florylpicoxamid and its metabolites in a long-term study in mice; (mean ± standard deviations shown)***

| | | Dietary concentration (ppm) | | | | | |
|---|---|---|---|---|---|---|---|
| | Test | Males | | | Females | | |
| Analytes | day/s | 150 | 500 | 1500 | 150 | 500 | 1500 |
| Test material intake (mg/kg bw per day): | 84–91 | 15.4 ± 1.33# | 56.2 ± 8.85 | 177 ± 24.8 | 17.9 ± 1.89 | 70.1 ± 5.27 | 181 ± 12.0 |
| | 168–175 | 15.4 ± 1.63 | 53.1 ± 9.56 | 161 ± 20.1 | 17.9 ± 1.12 | 67.7 ± 6.73 | 180 ± 14.5 |
| | 308–315 | 14.3 ± 1.95 | 47.1 ± 2.96 | 147 ± 13.7 | 16.4 ± 1.81 | 60.6 ± 9.48 | 173 ± 21.6 |
| | 364–371 | 14.0 ± 1.56 | 48.4 ± 2.44 | 146 ± 18.6 | 17.4 ± 1.78 | 58.0 ± 9.46 | TERM |
| | 532–539 | 13.9 ± 1.25 | 46.5 ± 1.69 | 150 ± 26.0 | 16.9 ± 1.13 | 53.5 ± 6.27 | TERM |

| Analytes | Test day/s | Dietary concentration (ppm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Males | | | Females | | |
| | | 150 | 500 | 1500 | 150 | 500 | 1500 |
| | | $AUC_{24}$ (ng × hour/mL) | | | | | |
| Florylpicoxamid | All | NC | NC | NC | NC | NC | NC |
| X12485649 | 92 | NC | NC | ISS | NC | NC | 406 ± 445 |
| | 176 | NC | NC | 487 ± 620 | NC | NC | ISS |
| | 316 | NC | NC | 524 ± 450 | NC | NC | 997 ± 545 |
| | 372 | NC | NC | 1430 ± 795 | NC | ISS | TERM |
| | 540 | NC | NC | 918 ± 909 | NC | NC | TERM |
| X12584261 | 92 | NC | NC | NC | NC | NC | NC |
| | 176 | NC | NC | NC | NC | NC | ISS |
| | 316 | NC | NC | ISS | NC | NC | NC |
| | 372 | NC | NC | 269 ± 371 | NC | NC | TERM |
| | 540 | NC | NC | 284 ± 394 | NC | NC | TERM |
| X12485473 | 92 | NC | NC | 760 ± 554 | NC | NC | 1780 ± 841 |
| | 176 | NC | NC | 1370 ± 675 | NC | NC | 1790 ± 596 |
| | 316 | NC | NC | 934 ± 910 | NC | NC | 1870 ± 757 |
| | 372 | NC | ISS | 1680 ± 1090 | NC | ISS | a |
| | 540 | NC | NC | 1640 ± 1070 | NC | ISS | a |
| X12641325 | 92 | 915 ± 1460 | 5590 ± 3820 | ISS | NC | NC | NC |
| | 176 | 1060 ± 1550 | 5800 ± 5160 | 484 ± 937 | NC | NC | NC |
| | 316 | 789 ± 985 | 2810 ± 3110 | ISS | NC | NC | NC |
| | 372 | ISS | 4910 ± 5540 | ISS | NC | NC | TERM |
| | 540 | 1020 ± 1260 | 4870 ± 7260 | 839 ± 1790 | 345 ± 522 | NC | TERM |

| Analytes | Test day/s | Dietary concentration (ppm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Males | | | Females | | |
| | | 150 | 500 | 1500 | 150 | 500 | 1500 |
| | | Urine concentrations (ng/mL) | | | | | |
| Florylpicoxamid | All | 0 | 0 | 0 | 0 | 0 | 0 |
| X12485649 | All | 0 | 0 | 0 | 0 | 0 | 0 |
| X12584261 | All | 0 | 0 | 0 | 0 | 0 | 0 |
| X12485473 | 93 | 8410 ± 1340 | 32500 ± 9460 | ISS | 10600 ± 2910 | 37700 ± 9090 | ISS |
| | 177 | 7970 ± 2690 | 36800 ± 9140 | ISS | 9600 ± 2560 | 36800 ± 14000 | 43200 ± 25000 |
| | 317 | 9210 ± 2440 | 30200 ± 18000 | ISS | 10400 ± 3030 | 39400 ± 14400 | 56000 ± 19700 |
| | 373 | 10300 ± 3190 | 32300 ± 18000 | NC | 8250 ± 2960 | 29100 ± 14100 | TERM |
| | 541 | 6890 ± 2110 | 25300 ± 16000 | 56700 ± 14000 | 8570 ± 4480 | 28200 ± 17100 | TERM |
| X12641325 | 93 | 6890 ± 3290 | 22900 ± 6190 | 13100 ± 21800 | 8780 ± 3570 | 6360 ± 2990 | 6750 ± 2870 |
| | 177 | 6100 ± 2920 | 22800 ± 7890 | 9340 ± 10600 | 7160 ± 2920 | 7600 ± 2540 | 3720 ± 2320 |
| | 317 | 8490 ± 5120 | 13000 ± 13500 | 6910 ± 8170 | 7600 ± 2660 | 6320 ± 2870 | 3370 ± 1700 |
| | 373 | 8460 ± 3290 | 13400 ± 11400 | 8710 ± 9480 | 4720 ± 1620 | 5000 ± 2960 | TERM |
| | 541 | 7910 ± 4630 | 12100 ± 14300 | 6620 ± 5690 | 4360 ± 4110 | 5020 ± 3390 | TERM |

TERM: Experiment terminated for this group; Source: Chan, 2020b

$AUC_{24}$: Area under the concentration–time curve in the first 24 hours following administration;

NC Not calculated due to concentration values in individual animals reported as below the LOQ were set to 0 ng/mL or concentration values reported as "dilution quality control failed "(DQCF), "inconsistent internal standard response" (ILL) or "not reportable results" (NRR) which were set to 0 but excluded from calculation;

ISS: Inadequate sample size; not calculated due to less than three animals having measurable values

## 2.2 Study in rats

Florylpicoxamid and its metabolites (X12485649, X12584261, X12485473 and X12641325) were quantified in rat blood and urine to evaluate systemic exposure and urinary excretion. Blood and urine samples were collected during a two-year dietary chronic toxicity study in which rats consumed diets containing 0, 300, 1000, 3000 or 4000 ppm florylpicoxamid. The test material intake data for test weeks 12–13, 24–25 and 48–49 were used to bracket the test days when blood and urine collection occurred. Blood and urine samples were analyzed for florylpicoxamid, X12485649, X12584261, X12485473 and X12641325. To evaluate the daily systemic exposure $AUC_{24}$ values were calculated. Urine samples were collected from male and female rats and excreted urine volumes recorded.

Florylpicoxamid concentrations in blood were below the LOQ at all time points for all test days in both sexes. In male and female rats, X12485473 or X12641325 was either generally, or sporadically, detected in blood. Florylpicoxamid, X12485649 and X12584261 was not detected or sporadically detected in blood. Systemic exposure in male rats was highest for X12641325, then X12485473, in groups receiving 1000 and 3000 ppm, similarly for both test day 183 and day 363. Systemic exposure in female rats, was highest for X12641325, then X12485473, in rats receiving 1000 ppm, similarly for both test day 184 and day 364.

Overnight urine volumes collected over the 24-hour period and analyte concentrations were used to calculate the percentage of daily intake recovered in the urine. In male rats, florylpicoxamid was not detected, or sporadically detected, in the urine of 300 and 1000 ppm group rats on test days 100, 191 and 359, but it was detected from 3000 ppm males on all test days. Metabolite X12485649 was detected in the urine on all test days. Metabolites X12584261 and X12485473 was not detected or reported in results across all test days group. Metabolite X12641325 was detected in samples from all dose levels on test day 100. Metabolite X12641325 was also detected in the 300 and 1000 ppm diet group samples on test day 191. In female rats, a similar pattern was observed for florylpicoxamid, X12485649 and X12584261. Metabolite X12485649 was detected in the urine on all test days. Metabolite X12584261 was not detected on any test day. Metabolite X12485473 was not reported for any dose group on test days 101 and 192, but was detected on test day 360. Metabolite X12641325 was detected in the 300 ppm dose group on test day 101, but was not reported in 1000 and 4000 ppm group samples. Nor was X12641325 reported in any of the dose groups on test days 192 or 360. In general, urinary excretion of florylpicoxamid and selected metabolites (X12485649, X12485473 and X12641325) was higher in female rats than in male rats on all test days.

***Ann. 2, Table 15. AUC and urine concentrations of florylpicoxamid and its metabolites in a long-term study in rats; (mean ± standard deviations shown)***

| | | **Dietary concentration (ppm)** | | | | | |
|---|---|---|---|---|---|---|---|
| | | **Males** | | | **Females** | | |
| **Analytes** | **Test day/s** | **300** | **1000** | **3000** | **300** | **1000** | **3000** |
| Test material intake (mg/kg bw per day): | 12–13 | 13.1 ± 0.420 | 43.3 ± 2.35 | 136 ± 3.41 | 15.8 ± 2.03 | 53.9 ± 8.14 | 181 ± 62.2 |
| | 24–25 | 11.4 ± 0.331 | 40.0 ± 5.46 | 125 ± 16.9 | 14.4 ± 0.581 | 46.6 ± 5.15 | 205 ± 50.3 |
| | 48–49 | 9.82 ± 0.668 | 34.7 ± 9.10 | 108 ± 5.07 | 12.5 ± 1.16 | 42.2 ± 3.38 | 180 ± 24.4 |
| | | **$AUC_{24}$ (ng × hour/mL)** | | | | | |
| Florylpicoxamid | All | NC | NC | NC | NC | NC | NC |
| X12485649 | 183/184[a] | NC | NC | ISS | NC | ISS | ISS |
| | 363/364[a] | NC | NC | ISS | NC | NC | ISS |
| X12584261 | All | NC | NC | ISS | NC | NC | NC |
| X12485473 | 183/184 | NC | 955 ± 543 | 3620 ± 1890 | 257 ± 332 | 3490 ± 1150 | ISS |
| | 363/364 | NC | 489 ± 470 | 4880 ± 3580 | ISS | 2210 ± 756 | ISS |
| X12641325 | 183/184 | ISS | 4100 ± 4430 | 9150 ± 7750 | NC | 4140 ± 3580 | ISS |
| | 363/364 | ISS | 3900 ± 3520 | 15200 ± 9820 | NC | 1920 ± 1330 | ISS |
| | | **Urine concentrations (ng/mL)** | | | | | |
| Florylpicoxamid | 14 | 7.93 ± 25.1 | 53.4 ± 108 | 947 ± 1010 | ISS | 238 ± 378 | 2420 ± 1740 |
| | 27 | ISS | 116 ± 128 | 347 ± 470 | ISS | 143 ± 212 | 1510 ± 1530 |
| | 51 | 0 | NC | 256 ± 254 | 53.9 ± 77.0 | 125 ± 217 | 1270 ± 520 |

| | | Dietary concentration (ppm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Males | | | Females | | |
| **Analytes** | **Test day/s** | **300** | **1000** | **3000** | **300** | **1000** | **3000** |
| X12485649 | 14 | 100 ± 162 | 369 ± 401 | 1510 ± 2940 | 135 ± 78.7 | 646 ± 575 | 1510 ± 1020 |
| | 27 | 112 ± 164 | 337 ± 297 | 494 ± 436 | 123 ± 130 | 634 ± 459 | 1050 ± 767 |
| | 51 | 44.0 ± 70.8 | 205 ± 174 | 868 ± 924 | 36.2 ± 47.7 | 185 ± 165 | 1050 ± 686 |
| X12584261 | 14 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 27 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 51 | ISS | 0 | ISS | 0 | 0 | 0 |
| X12485473 | 14 | 27100 ± 9220 | 94500 ± 11200 | ISS | ISS | ISS | ISS |
| | 27 | ISS | ISS | ISS | ISS | ISS | ISS |
| | 51 | 18300 ± 6240 | 71600 ± 26600 | 196000 ± 91100 | 25400 ± 9340 | 84300 ± 37400 | 329000 ± 32800 |
| X12641325 | 14 | 5500 ± 1040 | 32700 ± 12200 | 69400 ± 49600 | 16100 ± 5960 | 69800 ± 36400 | 249000 ± 111000 |
| | 27 | 13800 ± 8090 | 40800 ± 10700 | ISS | ISS | ISS | ISS |
| | 51 | NC | ISS | NC | ISS | ISS | ISS |

TMI Mean test material intake (mg/kg bw/day); Source: Chan, 2020c

[a] Test days on 183 and 363 were for males and on 184 and 364 for females, respectively.

NC Not calculated due to concentration values in individual animals reported as below the LOQ were set to 0 ng/mL, or concentration values reported as "not reportable results" (NRR) which were set to 0 but excluded from calculation;

ISS: Inadequate sample size; not calculated due to less than three animals having measurable values

# Iprodione

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## Explanation

Iprodione is the International Organization for Standardization (ISO)-approved common name for 3-(3,5-dichlorophenyl)-2,4-dioxo-*N*-(propan-2-yl)imidazolidine-1-carboxamide (IUPAC), Chemical Abstracts Service number 3674-19-7. It belongs to the class of organic compounds known as phenylhydantoins. It is a broad-spectrum foliage contact fungicide which inhibits DNA and RNA synthesis in the germinating fungal spore as well as inhibiting the enzyme NADH cytochrome c reductase, thereby preventing lipid and membrane synthesis and ultimately mycelial growth.

Iprodione was previously evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1977, 1992 and 1995. In 1977 an acceptable daily intake (ADI) of 0–0.3 mg/kg body weight (bw) was established. In 1992, the ADI was reduced to 0–0.2 mg/kg bw. The Meeting in 1995 withdrew the previous value and established a new ADI of 0–0.06 mg/kg bw. Iprodione was reviewed by the present Meeting under the periodic review programme of the Codex Committee on Pesticide Residues (CCPR).

All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with relevant national or international test guidelines, unless otherwise specified. Several studies previously reviewed by the JMPR were not submitted for the present evaluation. These studies were not critical to the risk assessment or for setting guidance values. Although studies on metabolites submitted to other regulatory bodies were not made available to the Meeting, the Meeting was able to conclude the evaluation. A search of the open literature did not reveal any relevant publications that would have an impact on the evaluation.

# Evaluation for acceptable daily intake

## 1. Biochemical aspects

### 1.1 Absorption, distribution and excretion

#### (a) Oral route

*Study 1*

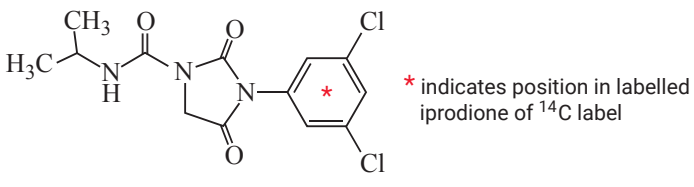
In the absorption, distribution, and metabolism study (not GLP), two male and two female rats (strain not specified) were treated with uniformly [$^{14}$C] phenyl ring labelled iprodione (radiochemical purity 92.4%; see Fig. 1) via a single gavage dose of 100 mg/kg bw, administered in 10% aqueous gum arabic. Urine and faeces were collected separately at 6, 12, 24, 48 and 96 hours after administration. Exhaled carbon dioxide was trapped with potassium hydroxide. Animals were killed after 96 hours, and selected organs and tissues were analyzed for radioactivity. A separate group of 20 rats was treated via single gavage dose of non-radioactive iprodione at 200 mg/kg bw and urine and faeces were collected at selected intervals up to 48 hours after dosing for elucidation of the different metabolites. Radioactivity in major organs and tissues was measured after 96 hours.

The radioactivity in exhaled air was negligible. The total excretion of radioactivity in the urine was ca 61.7%–61.9% of the administered dose (AD) in males and females, respectively in 96 hours. Total excretion of radioactivity in faeces of males and females at 96 hours ranged from 35.8% to 36.2% of AD. Radioactivity present in cage wash was about 1.2%–1.5% of AD. About 1% of AD was found in the organs and carcasses at 96 hours (Laurent et al. 1976).

*Study 2*

Male and female CD rats (five of each sex per group) received a single oral dose of [*phenyl*-$^{14}$C] iprodione (radiochemical purity greater than 99%; batch KWC 1670) at 50 or 900 mg/kg bw, suspended in 0.5% carboxymethyl cellulose (CMC) in distilled water. A separate group of rats (five per sex) received 14 consecutive daily doses of iprodione at 50 mg/kg bw per day, suspended in 0.5% CMC in distilled water followed by a single dose of [*phenyl*-$^{14}$C]iprodione at 50 mg/kg bw. Urine and faeces were collected at various intervals up to 168 hours post dosing, at which time rats were killed. A separate group of five male and five female rats received a single oral gavage dose of [*phenyl*-$^{14}$C]iprodione at 50 or 900 mg/kg bw. Blood (approximately 200–300 µL) was collected at 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 48, 96, 120 and 168 hours after dosing for evaluation of pharmacokinetic parameters.

***Figure 1. Iprodione; position of radiolabel***




Iprodione

Following administration of a single 50 mg/kg bw oral dose to males the mean recovery of radioactivity by 168 hours post dosing was 67.4% ± 6.9% in the urine, 25.0% ± 6.4% in the faeces, 0.2% ± 0.1% in tissues and organs, and 1.1% ± 0.7% in the cage wash. Total recovery of the AD was 93.8% ± 2.0%. Approximately 79% of the AD was excreted in the urine and faeces within 48 hours of administration. The highest concentration of radioactivity was found in the large intestine and contents (0.452 µg equiv./g; see Table 1). Concentrations greater than 0.1 µg equiv./g were found in liver, small intestine, caecum, kidney, salivary gland, lymph nodes and blood. Approximately 0.1% of the AD remained in carcass and skin.

***Table 1. Distribution of radioactivity at 196 hours in male and female rats following administration of single oral dose of 50 or 900 mg/kg bw and repeat dose of 50 mg/kg bw of iprodione; radioactive residue concentration shown in µg equiv./g of tissue/organ***

| | Dose regime | | | | | |
|---|---|---|---|---|---|---|
| | Single oral high dose (900 mg/kg bw) | | Single oral low dose (50 mg/kg bw) | | Repeated oral low dose (50 mg/kg bw/day, 14-days) | |
| **Tissue** | **M** | **F** | **M** | **F** | **M** | **F** |
| Liver | 3.99 | 5 | 0.375 | 0.502 | 0.4 | 0.411 |
| Stomach | 0.402 | 1.3 | 0.01 | 0.154 | 0.043 | 0.08 |
| Small Intestine | 2.16 | 5.34 | 0.177 | 0.28 | 0.226 | 0.366 |
| Large intestine | 5.33 | 6.68 | 0.452 | 0.666 | 0.25 | 1.04 |
| Caecum | 6.72 | 9.9 | 0.429 | 0.694 | 0.418 | 1 |
| Pancreas | 1.57 | 1.54 | 0.057 | 0.145 | 0.184 | 0.161 |
| Spleen | 0.764 | 1.68 | ND | 0.028 | ND | 0.018 |
| Kidney | 2.44 | 2.11 | 0.207 | 0.209 | 0.301 | 0.199 |
| Testis | 0.656 | NA | ND | NA | ND | NA |
| Uterus | NA | 1.2 | NA | 0.136 | NA | 0.128 |
| Lung | 1.57 | 0.694 | 0.054 | 0.076 | 0.035 | 0.075 |
| Heart | 0.823 | 0.638 | 0.009 | 0.051 | ND | 0.049 |
| Thymus | 0.526 | 1.46 | ND | 0.04 | ND | ND |
| Salivary gland | 1.78 | 2.38 | 0.166 | 0.117 | 0.095 | 0.152 |
| Brain | 0.157 | 0.319 | 0.012 | 0.01 | ND | ND |
| Muscle | 0.146 | 0.138 | 0.0251 | 0.012 | ND | 0.016 |
| Carcass | 0.568 | 0.536 | 0.048 | 0.047 | 0.015 | 0.055 |
| Skin | 2.44 | 2.54 | 0.163 | 0.226 | 0.296 | 0.877 |
| Fat | 2.63 | 1.92 | 0.135 | 0.308 | 0.208 | 0.262 |
| Bone | 0.105 | 0.05 | 0.017 | 0.015 | 0.004 | ND |
| Eyes | 0.236 | 0.513 | 0.03 | 0.035 | 0.042 | 0.036 |
| Adrenals | 0.61 | 2.66 | 0.046 | 0.106 | 0.11 | 0.113 |
| Thyroid | ND | 1.75 | ND | ND | ND | ND |
| Lymph nodes | 1.95 | 5.58 | 0.13 | 0.239 | 0.128 | 0.222 |
| Ovary | NA | 1.86 | NA | 0.147 | NA | 0.139 |
| Blood | 1.62 | 0.754 | 0.103 | 0.109 | 0.135 | 0.072 |
| Plasma | 1.91 | 1.47 | 0.051 | 0.061 | 0.121 | 0.064 |

M Males; F Females;

NA:. Not applicable

ND: Not detected

Source: Hallifax, 1989

Following administration of a 50 mg/kg bw single oral dose to females, the mean recovery of radioactivity by 168 hours post dosing was 52.6% ± 11.2% in the urine, 38.8% ± 11.4% in the faeces, 0.2% ± 0.1% in tissues and organs, and 0.9% ± 0.4% in cage wash. Total recovery of the AD was 92.5% ± 2.0% by 168 hours post dosing. Approximately 85% of the AD was excreted in the urine and faeces within the 48 hours following administration. The highest concentration of radioactivity was found in the caecum and its contents (0.694 µg equiv./g). Concentrations greater than 0.1 µg equiv./g were found in liver, stomach, small and large intestine, pancreas, kidney, uterus, salivary gland, adrenals, lymph nodes, ovary, fat and blood. Approximately 0.1% of AD remained in the carcass and skin.

Following administration of a 900 mg/kg bw single oral dose to males, the mean recovery of radioactivity by 168 hours post dosing was 43.0% ± 11.3% in the urine, 55.6% ± 9.9% in the faeces, 0.1% ± 0.1% in tissues and organs, and 0.4% ± 0.3% in cage wash. The total recovery of the AD was 99.3% ± 6.0%. Approximately 91% of AD was excreted in the urine and faeces within the 48 hours following administration. The highest concentration of radioactivity was found in the caecum and its contents (6.72% ± 10.4 µg equiv./g). Concentrations greater than 1.0 µg equiv./g were found in the liver, small and large intestine, pancreas, kidney, lung, salivary gland, lymph nodes, skin, fat and blood.

Following administration of a 900 mg/kg bw single oral dose to females, the mean recovery of radioactivity by 168 hours post dosing was 46.1% ± 8.0% in the urine, 51.6% ± 17.6% in the faeces, 0.1% ± 0.1% in tissues and organs, and 0.8% ± 0.5% in cage wash. The total recovery of the AD was 98.5% ± 10.2%. Approximately 79% of the AD was excreted in the urine and faeces within the 48 hours following administration. The highest concentration of radioactivity was found in the caecum and contents (9.90 ± 11.8 µg equiv./g). Concentrations greater than 1.0 µg equiv./g were found in the liver, stomach, small and large intestine, pancreas, kidney, spleen, salivary gland, adrenals, lymph nodes, uterus, skin, fat, and blood.

Following repeated administration of 50 mg/kg bw per day doses to males (14 unlabelled doses followed by one radiolabelled dose), the mean recovery of radioactivity by 168 hours post dosing was 74.7% ± 6.2% in the urine, 20.4% ± 5.7% in the faeces, 0.4% ± 0.1% in tissues and organs, and 1.1% ± 0.2% in cage wash. The total recovery of the AD was 96.6% ± 1.5%. Approximately 79% of the AD was excreted in the urine and faeces within the 48 hours following administration. The highest concentration of radioactivity was found in the large intestine and its contents (0.418 ± 0.294 µg equiv./g). Concentrations greater than 0.1 µg equiv./g were found in the liver, small and large intestine, caecum, pancreas, kidney, fat, adrenals, lymph nodes and blood. All organs and tissues contained less than 0.05% of the radiochemical dose except for the skin (0.1%).

Following repeated administration of 50 mg/kg bw per day oral doses to females (14 unlabelled doses followed by one radiolabelled dose), the mean recovery of radioactivity by 168 hours post dosing was 65.1% ± 11.8% in the urine, 28.0% ± 11.6% in the faeces, 0.3% ± 0.0% in tissues and organs, and 0.6% ± 0.2% in cage wash. The total recovery of the AD was 94.0% ± 3.5%. Approximately 84% of the AD was excreted in the urine and faeces within the 48 hours after administration. The highest concentration of radioactivity was found in the large intestine and its contents (1.04 ± 0.945 µg equiv./g). Concentrations greater than 1.0 µg equiv./g were found in the liver, small intestine, caecum, pancreas, kidney, uterus, salivary gland, skin, fat, adrenals, lymph nodes and ovary. All organs and tissues contained less than 0.05% of the radiochemical dose except the carcass (0.1%) and skin (0.3%).

After a single oral administration, $^{14}$C- iprodione at 50 or 900 mg/kg bw was rapidly absorbed in rats. The peak concentration of radioactivity in blood was four hours in males and two hours in females after administration of 50 mg/kg bw, and about six hours (males and females) after administration of 900 mg/kg bw. The mean absorption rate constant for 50 mg/kg bw was 1.2 ± 0.7 $h^{-1}$ for males and 0.6 ± 0.5 $h^{-1}$ for females. The mean absorption rate constant at 900 mg/kg bw was 0.35 ± 0.7 $h^{-1}$ for males and 0.81 ± 0.42 $h^{-1}$ for females. The half-life ($t_{½elim.}$).of elimination of radioactivity from blood was 8.9 ± 1.5 hours for males and 6.9 ± 1.7 hours for females given a single oral dose at 50 mg/kg bw. At the higher dose (900 mg/kg), the $t_{½elim}$ from blood was 19.8 ± 3.8 hours for males and 12.5 ± 3.0 hours for females. The area under the blood concentrations–time curve (AUC) for the 50 mg/kg bw dose was 471 ± 110 µg equiv./g/hour for males and 306 ± 70 µg equiv./g/hour for females. The AUC for the 900 mg/kg bw dose was 2870 ± 450 µg equiv./g/hour for males and 1520 ± 480 µg equiv./g/hour for females, indicating saturation of absorption. (Hallifax, 1989)

*Study 3*

In a separate absorption, metabolism and excretion, single dose study, Crl:CD(SD)BR rats (10 per sex) were administered a single oral dose of [*phenyl*-$^{14}$C]iprodione (purity greater than 98%; Batch no. CSL-92-354-81-3) suspended in 0.5% aqueous CMC via gavage at 50 mg/kg bw. Urine and faeces were collected for the periods 0–24, 24–48, 48–72, 72–96, 96–120, 120–144 and 144–168 hours post dose. At each collection of excreta, cage debris was removed and cages rinsed with water. At the end of the collection period, cages were rinsed thoroughly with water and then methanol. At the end, animals were killed by carbon dioxide asphyxiation and carcasses retained for analysis.

Iprodione was rapidly absorbed and excreted from the body following administration of a single oral gavage dose in rats as seen in Table 2.

***Table 2. Elimination of radioactivity and mass balance following oral administration of a single dose of 50 mg/kg bw [phenyl $^{14}$C] iprodione to rats***

| Time period (hours post dose) | Percentage of administered dose in excreta (± SD) | | | |
|---|---|---|---|---|
| | Males | | Females | |
| | Urine | Faeces | Urine | Faeces |
| 0–24 | 33.45 ± 9.206 | 44.42 ± 13.81 | 23.65 ± 11.71 | 44.73 ± 12.05 |
| 24–48 | 2.749 ± 1.356 | 10.07 ± 10.80 | 3.043 ± 1.402 | 4.832 ± 2.354 |
| 48–72 | 0.335 ± 0.225 | 0.930 ± 0.811 | 0.429 ± 0.274 | 0.341 ± 0.218 |
| 72–96 | 0.117 ± 0.143 | 0.571 ± 1.304 | 0.159 ± 0.113 | 0.299 ± 0.326 |
| 96–120 | 0.055 ± 0.036 | 0.045 ± 0.050 | 0.115 ± 0.195 | 0.053 ± 0.036 |
| 120–144 | 0.021 ± 0.010 | 0.019 ± 0.011 | 0.040 ± 0.033 | 0.043 ± 0.023 |
| 144–168 | 0.017 ± 0.005 | 0.009 ± 0.010 | 0.053 ± 0.082 | 0.059 ± 0.119 |
| Total, 0–168 | 36.74 ± 9.123 | 56.06 ± 8.773 | 27.49 ± 11.78 | 50.36 ± 11.94 |
| | Percentage of administered dose other than excreted (± SD) | | | |
| Cage wash | 4.75 ± 2.490 | | 8.373 ± 4.813 | |
| Cage debris | 0.061 ± 0.181 | | 0.187 ± 0.259 | |
| Final wash | 0.188 ± 0.142 | | 1.386 ± 3.548 | |
| Carcass | ND | | ND | |
| Total recovery | 97.80 ± 9.386 | | 87.79 ± 15.43 | |

SD: Standard deviation; ND: Not determined; Source: D'Souza, 1993

Total recovery of radioactivity following oral administration of 50 mg/kg bw [$^{14}$C]iprodione was 97.80% ± 9.39% in males and 87.79% ± 15.43% in females. There were no sex differences in the excretion profile of radioactivity. No detectable radioactivity was found the carcasses. Urinary excretion of radioactivity (including cage washes and final rinse) was about 42% in males and 37% in females. Urinary excretion was greatest in the first 24 hours, amounting to 33.45% in males and 23.65% in females. Subsequently less than 3.1% of AD was recovered at each sampling interval. Faecal excretion accounted for 56.06% ± 8.77% for males and 50.36% ± 11.94% in females. As with urine, radioactivity was recovered primarily within the first 24 hours (44.42% of AD in males; 44.73% females), with a smaller fraction detected between 24 and 48 hours after dosing (10.07% in males, 4.83% females); each of the later collection intervals yielded less than 1% of AD in faeces (D'Souza, 1993).

*Study 4*

In a non-guideline GLP study, male Sprague Dawley rats (Crl:CD(SD)BR) were administered by gavage a single dose of [U-*phenyl*-$^{14}$C]iprodione (purity 98.68%, Batch no. TS 129) suspended in 0.4% aqueous methyl cellulose at a nominal dose level of 70 mg/kg bw. Groups of five rats were sacrificed at 0.5, 1, 2, 4, 6, 10, 24 and 48 hours following dosing, at which points the testes were removed and blood samples taken. Tissues (whole blood, plasma, testis extracts) were analyzed for radioactivity using liquid scintillation counting (LSC). Tissue radioactivity components (plasma, testis extracts) were further investigated using high-performance liquid chromatography (HPLC).

Whole blood radioactivity levels increased rapidly, reaching a maximum level between two and 10 hours after administration and decreasing rapidly thereafter within 24 hours of dosing. A mean ratio of radioactivity in whole blood/plasma of approximately 0.7 ± 0.2 was calculated. The concentration of radioactivity in the testes paralleled those in the whole blood and plasma. The absorption of radioactivity was rapid with detectable levels of radioactivity in the testes only 30 minutes after dose administration. Peak observed levels occurred between 2 and 10 hours after dosing. As with the circulating radioactivity, concentration in the testes fell rapidly thereafter. Comparing the concentrations achieved in the testes and plasma samples at each time point yielded a mean testis/plasma ratio of 0.6 ± 0.2 .

HPLC investigations revealed up to five radioactive peaks in extracts of the testis, the most abundant of which were parent iprodione (up to 8.84 µg equiv./g testis) and metabolite RP36115 (up to 8.89 µg equiv./g testis). Metabolite RP36112 was also identified at lower levels (up to 1.60 µg equiv./g testis) in the testes. These data suggest that concentration in testes of radioactivity from iprodione and its metabolites (representing distribution) was approximately 60% of that observed in the plasma (Fisher, 1998).

**(b) Dermal route**

In a dermal penetration study, four male Crl:CD®BR rats per group and time point were exposed dermally to a single dose of [U-*phenyl*-$^{14}$C]iprodione (purity greater than 98%, Batch no. CSL-92-354-81-3) at dose levels of 0.43, 3.89 and 44.4 mg per rat for 0.5, 1, 2, 4, 10 and 24 hours. In this study Rovral Brand 4 flowable (formulation content was not reported) fungicide containing radiolabelled iprodione was used. The test material was applied to the previously shaved back and shoulder area. At termination the application site was washed with acetone:water mixture (2:1 ratio). Faeces, urine, blood, residual carcass, cage wash and skin wash were analyzed for radioactivity.

The mean total recovery of radioactivity ranged from 96% to 103% of applied dose. At 24 hours, the absorptions of iprodione radioactivity were 7.41%, 3.16%, and 0.19% of the applied dose at 0.4, 4.0, and 40 mg per rat, respectively. Absorption appeared to be saturated at the two highest doses. The amount of iprodione absorbed was small and there was no apparent correlation between dose level and amount absorbed, but in general increased dose correlated with decreased percentage absorption (Cheng, 1994).

## 1.2 Biotransformation

*Study 1*

Samples of urine and faeces collected after administration of 100 mg/kg bw of iprodione to rats were used for metabolite identification (Laurent et al., 1976, described above).

Approximately 10% of the parent compound was found in the faeces and about 5%–6% in the urine. Metabolite RP32490 (N-desisopropyl iprodione) was excreted in the urine (ca 15%–20%) and faeces (2%). Excretion in the urine and faeces of RP36114 [(1-(3,5 dichloro-4-hydroxyphenyl)] biuret (carbamylurea) and its conjugate, amounted to about 11% and 2% of AD respectively (Laurent et al., 1976).

*Study 2*

Iprodione was extensively metabolized by both male and female rats following a single oral dose of 50 or 900 mg/kg bw or repeated administration at 50 mg/kg bw (Halifax, 1989; described above). The urine and faecal samples from the mass balance study were pooled and analyzed by HPLC. Faecal samples were extracted twice with acetonitrile. A further extraction was performed using water followed by hexane. The metabolites identified using HPLC are presented in Tables 3 and 4

***Table 3. Radioactive components separated by HPLC in urine from male and female rats orally administered iprodione;*** **percentages of radioactivity in sample at each retention time ($t_r$) are shown**

| | Dose regime and sampling period | | | | | |
|---|---|---|---|---|---|---|
| | Single dose | | | | Repeat dose [a] | |
| | 50 mg/kg bw | | 900 mg/kg bw | | 50 mg/kg bw | |
| **Metabolites/HPLC peaks** | **0–24 h** | **24–48 h** | **0–24 h** | **24–48 h** | **0–24 h** | **24–48 h** |
| **Males** | | | | | | |
| Iprodione | 1.49 | - | 4.56 | | | |
| RP30228 | - | - | | | | |
| RP36115 and RP36119 | 3.03 | - | 4.04 | 2.76 | 1.57 | 0.65 |
| RP32490 | 25.06 | - | 22.27 | 1.84 | 15.59 | 4.27 |
| RP36112 | - | - | 3.53 | | 2.55 | |
| RP25040 | - | - | | | | |
| RP36116 | 1.69 | - | 1.43 | 1.21 | 1.98 | 3.36 |
| RP36118 | 2.24 | 8.69 | 3.33 | | 4.75 | 2.61 |
| RP36114 | 15.81 | 35.48 | 11.68 | 16.45 | 20.50 | 34.39 |
| Other radioactive peaks | 39.44 | 34.25 | 39.44 | 55.48 | 33.63 | 40.04 |
| Radioactivity not detected as peak | 11.24 | 22.48 | 9.72 | 22.26 | 19.43 | 14.68 |
| **Females** | | | | | | |
| Iprodione | 11.39 | 3.65 | 9.02 | 1.44 | 11.61 | |
| RP30228 | | | | | | |
| RP36115 and RP36119 | 0.93 | | 2.37 | | | |
| RP32490 | 29.43 | 14.35 | 7.77 | 1.75 | 26.86 | 6.45 |
| RP36112 | | | 4.47 | 1.54 | 2.44 | |
| RP25040 | | | | | | |
| RP36116 | 0.50 | | | | 0.95 | |
| RP36118 | 0.91 | | | | 1.0 | |
| RP36114 | 3.72 | | 22.44 | 12.60 | 4.67 | 7.21 |
| Other radioactive peaks | 50.8 | 82.0 | 41.50 | 67.26 | 33.97 | 67.20 |
| Radioactivity not detected as peak | 2.32 | 4.72 | 12.53 | 15.41 | 18.15 | 19.14 |

[a] 14 unlabelled doses followed by one radiolabelled dose; Source: (Hallifax, 1989)

*Table 4. Radioactive components separated by HPLC in acetonitrile extracts of faeces from male and female rats orally administered iprodione; percentages of radioactivity in sample at each retention time ($t_r$) are shown*

| | Dose regime and sampling period | | | | | |
|---|---|---|---|---|---|---|
| | Single dose | | | | Repeat dose [a] | |
| | 50 mg/kg bw | | 900 mg/kg bw | | Repeat dose 50 mg/kg bw | |
| Metabolites/HPLC peaks | 0–24 h | 24–48 h | 0–24 h | 24–48 h | 0–24 h | 24–48 h |
| **Males** | | | | | | |
| Iprodione | 34.03 | 8.51 | 79.35 | 13.79 | 66.88 | 5.08 |
| RP30228 | 1.01 | | 1.24 | 2.07 | 3.48 | |
| RP36115 and RP36119 | 3.62 | 16.98 | 1.91 | 16.34 | 1.28 | 6.28 |
| RP32490 | 9.81 | | 2.01 | 4.72 | 2.49 | |
| RP36112 | 2.20 | | 0.79 | 4.44 | 1.66 | |
| RP25040 | | | | | 0.89 | |
| RP36116 | 5.44 | 13.47 | 1.02 | 6.74 | 0.97 | |
| RP36118 | | | | | 0.91 | |
| RP36114 | 5.90 | 13.17 | 1.85 | 13.44 | 1.56 | |
| Other radioactive peaks | | 3.30 | | 6.16 | | |
| Radioactivity not detected as peak | 37.99 | 42.57 | 11.83 | 32.30 | 19.88 | 88.64 |
| **Females** | | | | | | |
| Iprodione | 31.29 | 4.28 | 85.12 | 17.57 | 31.14 | 5.96 |
| RP30228 | 3.67 | | 1.38 | | 2.96 | |
| RP36115 and RP36119 | 6.81 | 13.13 | | 19.72 | 6.79 | 19.54 |
| RP32490 | 13.63 | 8.88 | | | 8.75 | 5.88 |
| RP36112 | 4.35 | 8.83 | | 12.0 | 2.95 | |
| RP25040 | 2.05 | | | | | |
| RP36116 | 4.76 | | | | 4.35 | 8.29 |
| RP36118 | 10.14 | 7.19 | | | 0.86 | |
| RP36114 | 11.68 | 36.24 | 1.53 | 8.56 | 10.22 | 17.73 |
| Other radioactive peaks | 2.26 | | | 7.18 | | |
| Radioactivity not detected as peak | 9.44 | 21.45 | 11.97 | 34.97 | 29.39 | 42.60 |

[a] 14 unlabelled doses followed by one radiolabelled dose; Source: (Hallifax, 1989)

The major excreted products of iprodione in the urine were found to be compounds RP32490 and RP36114 in males and RP32490 and the parent in females. As a proportion of radioactivity in the sample, RP32490 level decreased from the 0–24 hour to the 24–48-hour period, whilst RP36114 increased. Metabolite RP32490 is the de-alkylated derivative of iprodione; RP36114 is produced by cleavage of the hydantoin ring. Some metabolites were present which were not identified. These were resolved at retention times shorter than any reference compounds, indicating they were more polar than any of the identified metabolites, and as such they were possibly conjugates of metabolites.

The major iprodione-derived components in the acetonitrile extract of faeces was iprodione in males and females from all dose groups. In males, no major metabolites (more than 10% of sample) occurred in the period 0–24 hours. Between 24 and 48 hours, material which corresponded to either RP36115 or RP36119 (not resolved by chromatography) was observed for samples from all dose groups. A similar pattern was observed for the females of all dose groups except that there were higher proportions of RP36114, particularly in the 24–48 hour period. The main differences in the patterns of radiolabelled products in the urine and faeces were that the urine contained relatively little parent compound and a large proportion of unidentified (possibly conjugated) polar metabolites, whereas the

faeces contained a large (but minor, in the low dose group) proportion of parent compound and relatively little material which did not correspond to reference compounds (Hallifax, 1989).

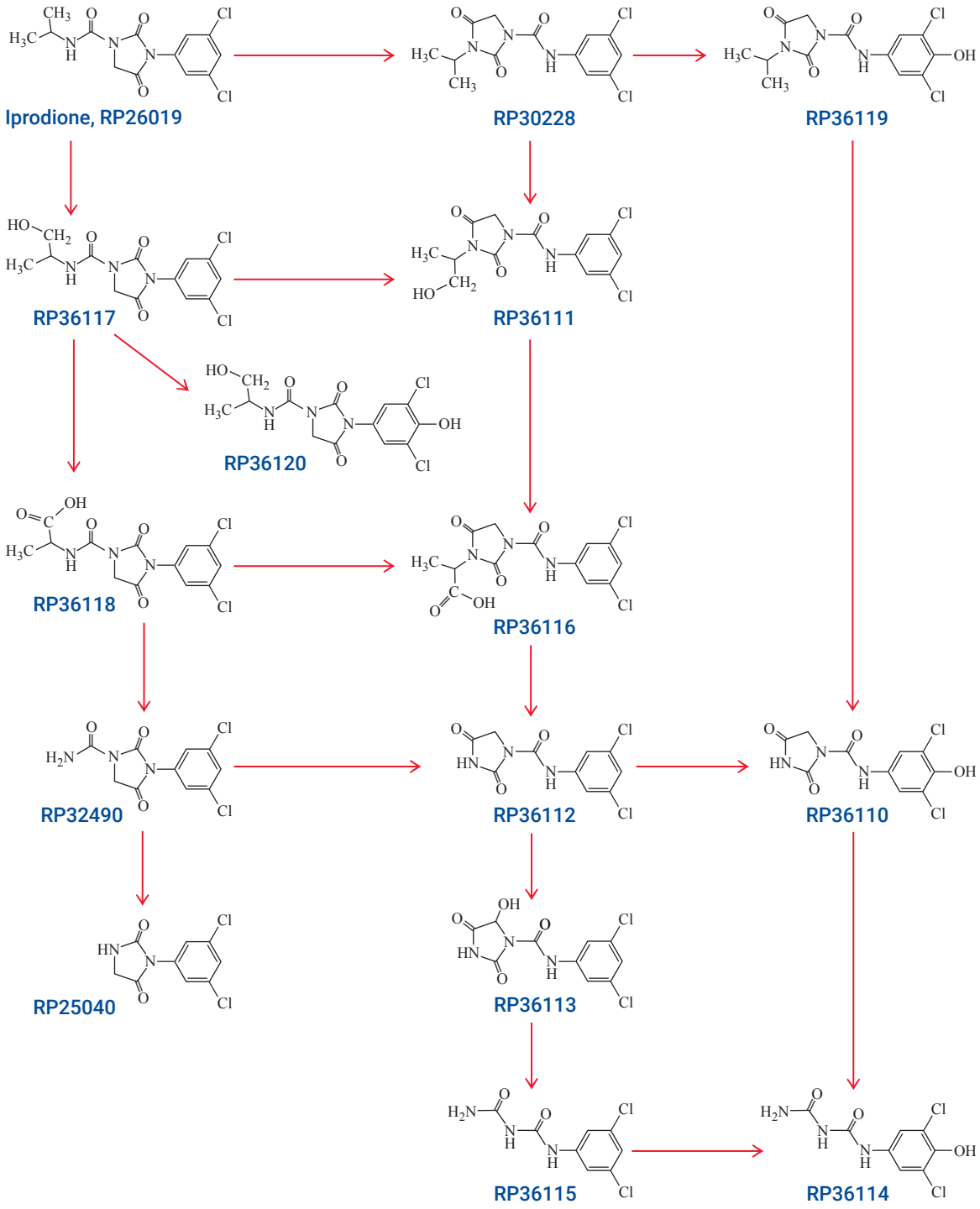
The urine and faeces samples from the study by D'Souza, (1993; study 3 above) were subjected to metabolite identification by thin layer chromatography (TLC) and HPLC. Urine and faecal samples were pooled to give 0–24 hour and 24–48 hour periods. Faecal samples were extracted with acetonitrile three times. Aliquots of urine and reconstituted faecal extracts were added to vials and 0.2 M ammonium acetate buffer (pH 5) and either β-glucuronidase H-l, β-glucuronidase β-l or aryl sulfatase (*Patella vulgata*) extract (1 mL) admixed. The samples were incubated for 24 hours at 37°C. Urine and faecal extracts (24 hour male and female) were investigated by liquid chromatography–mass spectrometry (LC-MS) to provide structural confirmation of the major metabolites present.

Metabolite profiling of untreated urine indicated a very polar region which accounted for approximately 70% of the sample radioactivity. Following deconjugation, which was greater when a non-specific enzyme preparation was used (a mix of β-glucuronidase and aryl sulfatase), the proportion of conjugates was considerably reduced. Two major regions of radioactivity were seen but these were only partially resolved. Analysis by LC-MS confirmed the presence of RP36115, RP32490 and RP36112 and indicated the presence of RP36119 and RP30228 in urine. Faeces contained a large proportion of parent compound and almost all the remaining radioactive material corresponded to the reference articles used. The major faecal metabolites which were confirmed by LC-MS were RP36115, RP36114, RP32490 and RP30228.

In conclusion, TLC and HPLC profiling against reference articles characterized ca 80% of the sample radioactivity (23.57% of the dose) in 24 hour male and female urine. Approximately 90% of the sample radioactivity (48.74% of the dose) in male and female faeces was characterized. Thus in total 72.31% of the dose was identified, which represented almost 90% of the total radioactivity in the samples profiled.

A general metabolic pathway for iprodione in the rat (Fig. 2) indicated that biotransformation results in hydroxylation of the aromatic ring, degradation of the isopropylcarbamoyl chain and re-arrangement, followed by cleavage, of the hydantoin moiety. Structural isomers of iprodione resulting from molecular rearrangement as well as intermediates in the pathway were also detected (D'Souza, 1993).

*Figure 2. Proposed metabolic pathway of iprodione in the rat*




(Redrawn from D'Souza, 1993)

## (c) Effects on enzymes and other biochemical parameters

No data submitted on this topic.

# 2. Toxicological studies

## 2.1 Acute toxicity

### (a) Lethal doses

The results of acute oral, dermal and inhalation toxicity studies are shown in Table 5 and the results of primary skin irritation, primary eye irritation and skin sensitization are shown in Table 6.

***Table 5. Summary of acute toxicity studies with iprodione***

| Species | Strain | Sex | Route | Purity | $LD_{50}/LC_{50}$ | Reference |
|---|---|---|---|---|---|---|
| Rat | CD | M + F | Oral | 97.9% | > 2000 mg/kg bw | Cummins, 1989 |
| Rabbit | New Zealand White | M + F | Dermal | 95.8% | > 2000 mg/kg bw | Plutnick, 1987 |
| Rat | Albino (Sprague Dawley) | M + F | Inhalation | 96.0% | > 3.29 mg/L | Coombs & Clark, 1977 |

M: Male; F: female; $LC_{50}$: Median lethal concentration; $LD_{50}$ Median lethal dose;

***Oral administration***

In an acute oral toxicity study, CD rats (five of each sex per dose; fasted) were administered iprodione (purity 97.9%) via gavage in 0.5% methylcellulose in distilled water at 0, 900, 1342, 2000, 2981 or 4444 mg/kg bw. The gavage dosing volume was 20 mL/kg bw. Treated rats were observed for 14 days. Animals were observed three times during the first hour after dosing and twice during the day for the remaining period. Body weights were recorded before dosing and then on days 1, 8 and 15.

There were no mortalities amongst the male or female rats treated at 900 mg/kg bw. Mortalities in males were 1/5, 1/5, 3/5 and 0/5 at 1342, 2000, 2981 and 4444 mg/kg bw, respectively. In females, no mortalities were observed at other dose levels, but all high-dose females died. All deaths occurred within three days of dosing. At 2000 mg/kg bw and above, clinical signs of toxicity, such as lethargy, decreased motor activity, prone posture, ataxia, unconsciousness, breathing irregularities, piloerection, ungroomed appearance, pigmented orbital secretion, hunched posture, thin body conformation, diarrhoea and reduced body temperature were observed. Clinical signs at 900 and 1342 mg/kg bw were confined to lethargy, decreased motor activity and ataxia. There were no effects on the body weights of surviving animals. Macroscopic findings in the decedents were staining of the body surfaces, altered gastro-intestinal (GI) contents and occasional dark areas on the glandular mucosa of the stomach. The thorax of one animal was found to contain red viscous fluid and pale amorphous material. No macroscopic findings were observed in surviving rats except large lymph nodes in one rat.

Under the study conditions utilized, the acute oral $LD_{50}$ for rats (fasted) was greater than 2000 mg/kg bw (Cummins, 1989).

***Dermal application***

In an acute dermal toxicity study, New Zealand White rabbits (five per sex) were dermally treated with iprodione (purity 95.8%) at 2000 mg/kg bw. The test material was moistened with 0.9% physiological saline and applied to the previously clipped dorsal surface of the shoulder. The treated area was covered with a gauze patch, and the patch secured with tape and plastic sleeve. Upon removal of the occlusive dressings after 24 hours, the test compound was removed from the treated skin areas by distilled water and wiped with paper towels. The rats were kept under observation for up to 14 days.

No mortality occurred. Other clinical signs occurring were soft stool, no stool, staining of the anogenital area, and distended abdomen. All treated rabbits showed increased body weights except one female rabbit which lost weight. Clinical observations included nasal discharge. No abnormalities were observed in eight animals during the gross examination post mortem. One animal displayed staining on the skin around the mouth due to collar in mouth and one animal with clear discharge on fur around nares, distended abdomen, distended small intestines, as well as distended and vascularized caecum and stomach.

Under the study conditions utilized, the acute dermal $LD_{50}$ in rats was greater than 2000 mg/kg bw (Plutnick, 1987).

***Inhalation***

In an acute inhalation study (not GLP), albino rats (seven of each sex per dose) were exposed for four hours to iprodione dust (purity 96%) by whole body exposure at concentrations of 0, 0.65 or 3.29 mg/L. The measured particle size for iprodione was 71% and 63% below 5.5 µm in diameter for the 0.65 and 3.29 mg/L dose groups, respectively. Control rats were immersed in air only. Rats were observed for mortality and signs of toxicity for 14 days post exposure. Individual body weights were recorded prior to exposure and on days 1, 3, 7 and 14. A complete necropsy was performed on each rat and any gross abnormalities observed recorded. Two males and two females from each dose group were killed at the end of exposure to assess any primary lung irritant effects due to iprodione.

No deaths occurred during the study. No treatment-related clinical signs of toxicity were observed. Body weight gains were similar between treated groups and controls. There were no effects of treatment on food or water consumption. Post-mortem examination of the two rats killed immediately after exposure and those rats killed at the end of study showed no gross abnormalities. The calculated lung to body weight ratios were normal for the treated and control group rats.

Under the study conditions utilized, the four-hour acute inhalation $LC_{50}$ was greater than 3.29 mg/L (Coombs & Clark, 1977).

**(b) Dermal irritation**

In a primary skin irritation study, New Zealand White rabbits (four males, two females) were treated once, on intact skin with iprodione (purity 96.2%). One day prior to dosing, fur was clipped from the dorsal area of the trunk. A dose of 0.5 g of iprodione was applied to the previously clipped area over an area of one square centimetre. The test article was moistened with 0.5 mL of distilled water immediately after application and gauze was placed over the treated area and secured to the adjacent skin with tape. After four hours of exposure the patch was removed, and the test sites gently wiped with water to remove any residual test substance. Individual dose sites were scored according to the Draize scoring system at approximately 1, 24, 48 and 72 hours after patch removal. No dermal irritation was observed at the test substance application site.

Under the conditions of this study, iprodione was found to be non-irritating to the skin of rabbits (Bonnette, 1991b).

***Table 6. Summary of acute dermal and eye irritation and skin sensitization studies with iprodione***

| Species | Strain | Sex | End-point | Purity | Result | Reference |
|---|---|---|---|---|---|---|
| Rabbit | NZW | M + F | Eye irritation | 96.2% | Not irritating | Bonnette, 1991a |
| Rabbit | NZW | M + F | Skin irritation | 96.2% | Mild irritant | Bonnette, 1991b |
| Guinea pig | Hartley albino | F | Skin sensitization (Buehler) | 95.8% | Not a dermal sensitizer (Buehler) | Trimmer, 1987 |

M: Male; F: female; NZW: New Zealand White

**(c) Ocular irritation**

In a primary eye irritation study, iprodione (purity 96.2%) was administered once into the conjunctival sac of the right eye of nine New Zealand White rabbits (five males and four females) at a 0.062 g dose (0.1 mL equivalent). The treated eyes of three rabbits were rinsed with 50 mL physiological saline 30 seconds after instillation. The eyes of six rabbits were not rinsed. Ocular irritation was evaluated according to the Draize method at 1, 24, 48 and 72 hours, and up to seven days after dosing.

Iprodione produced conjunctivitis (redness, swelling and discharge) at by one hour after dosing in the eyes of rinsed animals and resolved completely in 72 hours post instillation. In the group with unwashed eyes, corneal opacity was observed in 1/6 animals at 24 hours, and this was resolved completely by 72 hours post dose. Conjunctivitis (redness, swelling and discharge) was noted in 6/6 test eyes at the one-hour scoring time point. Conjunctival irritation diminished over the test period and resolved completely by study day 7.

Under the conditions of this study, iprodione was mildly irritating to the eyes of rabbits (Bonnette, 1991a).

**(d) Dermal sensitization**

In a dermal sensitization study using the Buehler protocol, iprodione (purity 95.8%) diluted to 10% (w/v) in acetone was topically applied to the left side of 10 female Hartley albino Guinea pigs. Exposure was for six hours, and this procedure was repeated three times weekly for three consecutive weeks. Thirty-three days after the initial topical induction, 0.4 mL of 10% (w/v) iprodione in acetone was topically applied to the clipped area on the right flank of all treated animals, and the irritation control group animals. On day 41, the treated animals were rechallenged with 5.0% (w/v) iprodione in acetone.

Following challenge dosing, very slight erythema was noted in 4/5 irritation control group animals. In the treated group, 6/10 animals exhibited very slight erythema, and 1/10 exhibited well defined erythema. Oedema was not noted in either group following challenge dosing. Following rechallenge, very slight erythema was observed in 7/10 treated animals. Slight erythema was observed in 1/5 in the control group. Oedema was not observed in control or treated animals following rechallenge but one treated animal exhibited desquamation. The study author suggested that the slight erythema noted after challenge and rechallenge, and desquamation noted following rechallenge may be attributable to acetone.

Under the study conditions utilized, iprodione was considered non-sensitizing in Guinea pigs (Trimmer, 1987).

## 2.2 Short-term studies of toxicity

**(a) Oral administration**

***Mouse***

Three studies previously reviewed by the JMPR, in which iprodione was administered to mice via the diet for four weeks, were not submitted by the current sponsor. Previous Meetings had concluded the NOAEL in the first study was 1900 ppm (equal to 390 mg/kg bw per day for males and 420 mg/kg bw per day for females) based on liver effects at 6000 ppm (equal to 950 mg/kg bw per day for males and 1000 mg/kg bw per day for females). In the second study the NOAEL was 600 ppm (equal to 115 mg/kg bw per day for males, 137 mg/kg bw per day for females) based on liver changes at 1900 ppm (equal to 366 mg/kg bw per day for males, 439 mg/kg bw per day for females). In the third study, the NOAEL was 1900 ppm (equivalent to 290 mg/kg bw per day) based on crystalline deposits in the urinary bladder, liver enlargement and hepatocellular swelling at 6000 ppm (equivalent to 900 mg/kg bw per day). These studies were not considered critical for the determination of health-based guidance values (HBGVs) for iprodione.

In a 13-week oral toxicity study iprodione (purity 95.7%, Batch no. DA 604) was administered in the diet to Crl:CD-1 (ICR) BR mice (10 of each sex per group) at dose levels of 0, 1500, 3000, 6000 or 12 000 ppm (equal to 0, 260, 509, 1066 and 2130 mg/kg bw per day for males, 0, 327, 659, 1303 and 2608 mg/kg bw per day for females). The objective of the study was to assess toxicity after administration for 13 weeks and thus to select treatment levels for a proposed tumourigenicity study. All animals were observed for mortality and clinical signs during the study and body weights and food consumption were recorded weekly. At termination of treatment animals were subjected to gross necropsy, organ weight analysis and histopathology. Ophthalmology, haematology, blood biochemistry and urine analyses were not performed.

One high-dose female was killed during week 1 and two high-dose males were killed during week 6, all due to poor condition. One control female was killed in week 11, also due to poor condition. The study pathologist concluded that a reaction to crystalline deposits in various tissues (urinary bladder, preputial glands and/or liver) contributed to the poor condition of the two males receiving 12 000 ppm.

A loss of mean body weight and reduced food consumption were observed over the first week of treatment in males and females given 12 000 ppm, but for the remainder of the study body weight gains, food consumption and food utilization for all treated groups were comparable with controls. Dark and/or swollen abdomen, generally occurring from week 1 and persisting throughout the treatment period, was observed for all males and the majority of females given 12 000 ppm, the majority of mice given 6000 ppm and two females receiving 3000 ppm. Lethargy, pilo-erection and/or hunched posture were also observed in several mice given 12 000 ppm.

A dose-dependent increase in liver weight (relative to body weight) was observed in males given 3000, 6000 or 12 000 ppm, and in females at all doses. Absolute adrenal weights were significantly increased compared with controls in males given 6000 or 12 000 ppm, and in females at all dose levels (Table 7). Absolute spleen weights were increased in both sexes at 6000 and 12 000 ppm. In females, absolute uterus weights were significantly lower than controls in the groups treated at 6000 and 12 000 ppm, and relative ovary weights were reduced at 12 000 ppm.

***Table 7. Selected organ weight changes in 13-week toxicity study in mice***

| | Dose level (ppm) | | | | |
|---|---|---|---|---|---|
| **Parameter** | **0** | **1500** | **3000** | **6000** | **12 000** |
| | | | **Males** | | |
| Liver weight; absolute (g) | 2.35 | 2.66 | 3.25 | 5.02 | 5.63 |
| Liver weight; relative to bw (g) | 2.49 | 2.58 | 3.13** | 4.94** | 5.80** |
| Adrenal weight (mg) | 6.1 | 5.8 | 5.7 | 8.7** | 9.0** |
| | | | **Females** | | |
| Liver weight; absolute (g) | 1.80 | 2.14 | 2.43 | 3.78 | 4.65 |
| Liver weight; relative to bw (g) | 1.80 | 2.14** | 2.54** | 3.57** | 4.49** |
| Adrenal weight (mg) | 8.9 | 11.4* | 11.7* | 13.2** | 11.6** |

Statistical difference from control using William's test at: * $p < 0.05$; ** $p < 0.01$; Source: Fryer, 1990a

Crystalline deposits were observed in the liver, heart, kidneys, urinary bladder, preputial glands, brain and skeletal muscle in males and/or females at 12 000 ppm, and in the urinary bladder of males given 6000 ppm. Increased thickness of the urothelium was also observed in the urinary bladder of males at 6000 ppm and above. Hepatocellular hypertrophy was observed in male mice at all dose levels and in females at doses 3000 ppm and above. Scattered, finely vacuolated hepatocytes were observed in male mice given 12 000 ppm and females given 6000 ppm and above. Uterine atrophy and absence of corpora lutea were observed in female mice given 6000 ppm and above. In the adrenal glands, enlargement of cells in the zona glomerulosa and zona fasciculata were observed in males given 12 000 ppm, and enlargement of cells in the zona glomerulosa was observed in females given 6000 ppm and above. Minimal vacuolation of cells of the zona glomerulosa was observed in one mouse of both sexes at 12 000 ppm. Vacuolation of cells of the zona fasciculata was observed in 0/10, 1/10, 3/10, 6/10 and 4/9 females receiving 0, 1500, 3000, 6000 and 12000 ppm, respectively, and in 2/8 males given 12 000 ppm (Table 8). The severity was minimal in all animals except for one female given 6000 ppm, where moderate vacuolation was observed. An increased incidence of extramedullary haemopoiesis was observed in females at 6000 ppm and in both sexes at 12 000 ppm.

***Table 8. Selected histopathological changes at terminal examination in the liver and adrenals in a 13-week toxicity study in mice (number affected/number examined shown)***

| | Dose level (ppm) | | | | |
|---|---|---|---|---|---|
| **Parameter** | **0** | **1500** | **3000** | **6000** | **12 000** |
| | | | **Males** | | |
| Centrilobular hepatocyte enlargement | 0/10 | 2/10 | 10/10 | 10/10 | 8/8 |
| Scattered, finely vacuolated hepatocytes | 0/10 | 0/10 | 0/10 | 0/10 | 6/8 |
| Enlargement of zona glomerulosa cells | 0/10 | 0/10 | 0/10 | 0/10 | 8/8 |
| Enlargement of zona fasciculata cells | 0/10 | 0/10 | 0/10 | 0/10 | 3/8 |
| Vacuolation of cells of zona glomerulosa | 0/10 | 0/10 | 0/10 | 0/10 | 1/8 |
| Vacuolation of cells of zona fasciculata | 0/10 | 0/10 | 0/10 | 0/10 | 2/8 |
| | | | **Females** | | |
| Centrilobular hepatocyte enlargement | 0/9 | 0/10 | 2/10 | 10/10 | 9/9 |
| Scattered, finely vacuolated hepatocytes | 0/9 | 0/10 | 0/10 | 10/10 | 9/9 |

| Parameter | Dose level (ppm) | | | | |
|---|---|---|---|---|---|
| | 0 | 1500 | 3000 | 6000 | 12 000 |
| Enlargement of zona glomerulosa cells | 0/9 | 0/10 | 0/10 | 6/10 | 8/9 |
| Enlargement of zona fasciculata cells | 0/9 | 0/10 | 0/10 | 0/10 | 0/9 |
| Vacuolation of cells of zona glomerulosa | 0/9 | 0/10 | 0/10 | 0/10 | 1/9 |
| Vacuolation of cells of zona fasciculata | 0/9 | 1/10 | 3/10 | 6/10 | 4/9 |

Statistical difference from control using William's test at: * $p < 0.05$; ** $p < 0.01$; Source: Fryer, 1990a

The Meeting considered it was not appropriate to establish a NOAEL from this study in the absence of ophthalmology, haematology, blood biochemistry and urine analyses (Fryer, 1990a).

***Rat***

In a 13-week oral toxicity study iprodione (purity 95.7%, Batch no. DA 604) was administered in the diet to Crl:CD (SD) BR rats (10 of each sex per group) at concentrations of 0, 1000, 2000, 3000 or 5000 ppm (equal to 0, 78, 151, 252 and 351 mg/kg bw per day for males, 0, 89, 184, 266 and 408 mg/kg bw per day for females). The objective of the study was to assess toxicity after administration for 13 weeks and thus select treatment levels for a proposed tumourigenicity study. Animals were observed for mortality and clinical signs, body weights and food consumption were recorded weekly. At termination of treatment animals were subjected to ophthalmology (control and 3000 ppm groups), gross necropsy, organ weight analysis and histopathology of selected tissues (adrenals, liver, uterus and ovaries). Haematology, blood biochemistry and urine analyses were not performed. In a subsequent study amendment (not previously reviewed by JMPR) additional histopathological examinations of the testes, prostate, epididymides and seminal vesicles were reported.

The 5000 ppm group was killed during week 8 due to signs of excessive toxicity including substantial reductions in food intake and body weight, deterioration in clinical condition and the death of one male in week 7. Histopathological changes in these animals included enlarged cells of the zona glomerulosa and vacuolation of the zona fasciculata in the adrenal glands, atrophy of the uterus and reduced numbers of corpora lutea, atrophy of the seminiferous tubules, abnormal or absent spermatozoa, testicular interstitial cell hyperplasia, atrophy in the prostate and reduced secretion in the seminal vesicles.

Clinical signs (hunched posture, pilo-erection, pale and/or cold extremities and emaciated appearance) were observed in males and females receiving 3000 ppm. Body weight gain, food intake and food utilization were reduced at 2000 and 3000 ppm. No treatment-related changes in ophthalmology were observed. Absolute ovary weights were reduced at 2000 and 3000 ppm compared with controls, and absolute uterus weights were reduced at 3000 ppm (Table 9).

***Table 9. Body weight, body weight change and selected organ weights in female rats in a 13-week dietary study***

| Parameter | Dietary dose (ppm) | | | |
|---|---|---|---|---|
| | 0 | 1000 | 2000 | 3000 |
| Terminal body weight (g) | 275 | 266 | 237 | 193 |
| Body weight change (g/rat) | 135 | 126 | 94** | 52** |
| Absolute uterus weight (g) | 0.58 | 0.56 | 0.58 | 0.15** |
| Absolute ovary weight (mg) | 76.4 | 64.6 | 60.3** | 37.2** |

Statistical difference from control using William's test at: * $p < 0.05$; ** $p < 0.01$; Source: Fryer 1990b

Vacuolation of the zona fasciculata of the adrenals was observed in males given 2000 and 3000 ppm and in females receiving 3000 ppm. Enlarged cells of the zona glomerulosa were observed in both sexes at 2000 and 3000 ppm. The uterus showed signs of atrophy and ovaries contained a reduced numbers of corpora lutea at 2000 and 3000 ppm. Increased incidence of testicular interstitial cell hyperplasia, atrophy in the prostate and reduced secretion in the seminal vesicles were observed in rats given 3000 ppm, with one animal given 2000 ppm also showing prostate atrophy.

The Meeting considered it was not appropriate to establish a NOAEL from this study in the absence of haematology, blood biochemistry and urine analyses (Fryer, 1990b; Gopinath, 1993).

***Dog***

In a one-year toxicity study in dogs, iprodione (purity 96.5%) was administered in the diet for 52 weeks to groups of six male and six female beagle dogs at a concentration of 0, 100, 600 or 3600 ppm (equal to 0, 4.1, 24.9 and 145 mg/kg bw per day for males, 0, 4.3, 28.3 and 153 mg/kg bw per day for females). Animals were inspected for clinical signs and mortality regularly throughout the day. Food consumption was measured daily. Body weights were measured weekly. A detailed physical examination was conducted before the commencement of the study and after 3, 8, 11, 16, 24, 27, 31, 35, 39, 43, 47 and 51 weeks of treatment. Ophthalmology, haematology, clinical chemistry, and urinalysis were performed periodically over the course of the study. Bone marrow samples were biopsied several days before necropsy. At necropsy, a macroscopic examination was conducted, and several organs were removed and weighed. Selected tissues were prepared for histopathological examination. Photomicrographs were prepared of the heart, left kidney, liver, lungs, and spleen of one male and one female from the control and high-dose (3600 ppm) groups to illustrate treatment-related micropathology findings.

There were no treatment-related deaths, and no adverse effects were observed on body weight, food consumption, clinical signs, or urinalysis in either sex. No treatment-related abnormalities were observed at physical examination. The composition of bone marrow was unaltered by treatment. Slight retinal hyper-reflection was noted, more frequently in males receiving 3600 ppm and females receiving 600 or 3600 ppm, however, severity did not increase over time or with dose level. The incidence of retinal hyper-reflections in males was 1/6, 0/6, 3/6 and 4/6, and in females 3/6, 4/6, 5/6 and 4/6, both at 0, 100, 600 and 3600 ppm, respectively. One male and one female dog from the 600 ppm dose group had the condition prior to commencement of treatment. Hyper-reflections were not seen consistently in the same animals over time. In two male dogs in the 3600 ppm dose group, fine, scattered, evenly distributed opalescent particles (asteroid bodies) were observed in the vitreous humor from week 13 onwards.

Erythrocytic characteristics (packed cell volume, haemoglobin concentration and erythrocyte counts) were reduced in males receiving 3600 ppm at various observations (excepting week 18) when compared to pretreatment values and contemporary controls. Females receiving 3600 ppm were also similarly affected on several sampling occasions. The erythrocytic characteristics of dogs receiving 100 or 600 ppm were undisturbed by treatment. The platelet count of male and female dogs receiving 3600 ppm were higher (statistically significant) than those of their respective controls at all examination during the treatment period. Activated partial thromboplastin times (APTTs) of females in the 3600 ppm group were increased to a statistically significant extent on all occasions during the study compared to controls. Heinz bodies were observed in moderate numbers of erythrocytes from male and female dogs receiving 3600 ppm. A small number of erythrocytes were also affected up to week 18 in dogs receiving 600 ppm but not subsequently (Table10). The incidence of Heinz bodies in the erythrocytes of dogs in the 100 ppm group was similar to that for controls. Treatment-related changes in clinical chemistry parameters were observed only in high-dose animals. Plasma alkaline phosphatase (ALP) activities of male and female dogs at 3600 ppm were consistently higher than for controls. In females at 3600 ppm there was a transient increase in alanine transaminase (ALT) activity compared to controls. The total bilirubin concentrations for female dogs were marginally higher than those of the controls at all samplings, except for weeks 22 and 52. A transient increase in serum lactate dehydrogenase activity was observed in females receiving 3600 ppm.

***Table 10. The incidence (sum of severity scores per group) of red blood cells containing Heinz bodies in dogs orally administered iprodione for one year[a]***

| Week of treatment | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | 0 | 100 | 600 | 3600 | 0 | 100 | 600 | 3600 |
| 5 | 1 | 0 | 8 | 27 | 0 | 1 | 6 | 28 |
| 9 | 3 | 3 | 11 | 23 | 0 | 2 | 4 | 24 |
| 14 | 0 | 0 | 6 | 25 | 0 | 1 | 5 | 25 |
| 18 | 4 | 7 | 12 | 24 | 1 | 3 | 1 | 20 |

| Week of treatment | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | 0 | 100 | 600 | 3600 | 0 | 100 | 600 | 3600 |
| 22 | 0 | 0 | 0 | 9 | 0 | 0 | 0 | 9 |
| 26 | 0 | 0 | 1 | 25 | 0 | 0 | 0 | 25 |
| 39 | 0 | 0 | 4 | 20 | 0 | 0 | 0 | 24 |
| 52 | 4 | 2 | 8 | 26 | 6 | 2 | 3[b] | 26 |

Source: Broadmeadow, 1984; Broadmeadow & Duffen, 1985; Brown & Broadmeadow, 1986

[a] None was noted before treatment commenced.

[b] One animal dead

There was an increase in absolute and relative (to body weight) liver and adrenal weights in both sexes at 3600 ppm. There was a statistically significant decrease in absolute prostate weight in males at 600 and 3600 ppm, and relative prostate weight (to body weight) at 3600 ppm; the decrease at 600 ppm was by ca 20% and considered not to be adverse. Macroscopic examination indicated enlarged adrenals in two high-dose females (apparently confirmed by their organ weights), and swollen liver in one high-dose male.

***Table 11. Selected organ weights and histopathology findings in dogs orally administered iprodione for one year***

| Parameter | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | 0 | 100 | 600 | 3600 | 0 | 100 | 600 | 3600 |
| **Organ and body weights (mean values ± standard deviation)** | | | | | | | | |
| Terminal body weight (kg) | 12.9 ± 2.3 | 12.7 ± 1.3 | 11.7 ± 1.3 | 12.3 ± 1.9 | 10.8 ± 1.1 | 10.3 ± 1.4 | 10.3 ± 0.6 | 10.1 ± 1.2 |
| Liver weight; absolute (g) | 393 ± 41 | 424 ± 64 | 399 ± 49 | 556 ± 55*** | 392 ± 75 | 342 ± 46 | 378± 62 | 464 ± 75 |
| Liver weight; relative (%) | 3.1 ± 0.3 | 3.3 ± 0.3 | 3.4 ± 0.3 | 4.6 ± 0.6*** | 3.6 ± 0.6 | 3.4 ± 0.6 | 3.6 ± 0.5 | 4.6 ± 0.6* |
| Adrenal weight; absolute (g) | 1.23 ± 0.22 | 1.42 ± 0.32 | 1.36 ± 0.18 | 1.68 ± 0.40* | 1.44 ± 0.10 | 1.28 ± 0.17 | 1.41 ± 0.19 | 2.06 ±0.48** |
| Adrenal weight; relative (% × 1000) | 9.6 ± 1.3 | 11.1 ± 1.6 | 11.7 ± 1.6 | 13.6 ± 2.5*** | 13.5 ± 1.3 | 12.6 ± 1.9 | 13.7 ± 2.1 | 20.4 ± 3.7*** |
| Prostate weight; absolute (g) | 8.8 ± 1.8 | 8.0 ± 1.0 | 6.9 ± 1.5* | 5.2 ± 1.3*** | NA | NA | NA | NA |
| Prostate weight; relative (% × 1000) | 70 ± 20 | 64 ± 10 | 59 ± 10 | 43 ± 1.2** | NA | NA | NA | NA |
| **Histopathological findings (number affected in each group of six)** | | | | | | | | |
| Kidney lipofuscin | 2 | 1 | 2 | 4 | 2 | 0 | 4 | 4 |
| Adrenal, pale zona glomerulosa | 2 | 0 | 3 | 5 | 0 | 0 | 1 | 5 |
| Prostate, chronic inflammation | 2 | 0 | 0 | 2 | NA | NA | NA | NA |

NA: Not applicable Source: Broadmeadow, 1984; Broadmeadow & Duffen, 1985; Brown & Broadmeadow, 1986

Statistical difference from control at: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Histopathological examination indicated treatment-related changes in the adrenals, kidneys, gall bladder, liver and urinary bladder. Changes in the adrenals, kidneys and gall bladder were considered to be exacerbations of the physiological changes which were seen in high-dose dogs.

Histopathological changes in the adrenals and kidneys were also seen in dogs dosed at 600 ppm but changes were less extensive compared to those in high-dose dogs and were considered not to be adverse. In high-dose dogs, liver changes comprised increased size and frequency of agglomerates of pigmented macrophages and hepatic atrophy, whilst in the urinary bladder there appeared small granulomata, containing crystals, in the immediate submucosa. In addition, polymorphonuclear leucocyte infiltration within the transitional epithelium was observed in two high-dose dogs.

The NOAEL was 600 ppm (equal to 24.9 mg/kg bw per day) based on increased incidence of Heinz bodies and histopathological changes in adrenals and kidneys seen at the LOAEL of 3600 ppm, (equal to 145 mg/kg bw per day) (Broadmeadow, 1984; Broadmeadow & Duffen, 1985; Brown & Broadmeadow, 1986).

In a second one-year dog study, iprodione (purity 96.2%, Lot no. 89602 01) was administered in the diet for 52 weeks to groups of six male and six female beagle dogs at concentrations of 0, 200, 300, 400 or 600 ppm (equal to 0, 7.8, 12.4, 17.5 and 24.6 mg/kg bw per day for males, 0, 9.1, 13.1, 18.4 and 26.4 mg/kg bw per day for females). All animals were examined for clinical signs, mortalities, and behaviour twice daily. Body weights were recorded weekly during the pretreatment and treatment periods. Food consumption was measured weekly. Ophthalmoscopic examinations were conducted prior to the start of treatment and on all surviving animals during weeks 4, 8, 12, 20, 28, 36 and 52. Haematological parameters were evaluated prior to start of treatment and again during weeks 4, 8, 12, 20, 28, 36 and 52 of the treatment periods on all animals. Macroscopic examination was conducted on all surviving animals. At termination, adrenals, kidneys and prostate (males) were weighed and preserved for histopathological examination. Additional tissue samples to elucidate abnormal macroscopic findings were taken at the discretion of the supervising pathologist.

No treatment-related mortalities or clinical signs of toxicity were observed during the study. Body weight, body weight gain, food consumption and food efficiency were not affected by the treatment. The ophthalmoscopic examination did not reveal any treatment-related changes.

There were statistically significant decreases in red blood cell (RBC) counts, haemoglobin (Hb) and haematocrit (Ht) values in high-dose males and females compared to controls. However, these decreases were not considered adverse due to the small size of changes, within normal range and/or similar to pretreatment values.

No Heinz bodies were observed in any control or treated animals at any of the sampling periods. No treatment-related effects were observed on liver, kidney or prostate weights. No treatment-related gross or histopathological findings were seen in any of the animals examined.

The NOAEL for this study was 600 ppm (equal to 24.6 mg/kg bw per day) the highest dose tested (Kangas,1995).

The overall NOAEL for one-year dog studies was 600 ppm (equal to 24.9 mg/kg bw per day) based on the effects described above at 3600 ppm (equal to 145 mg/kg bw per day).

**(b) Dermal application**

In a 21-day dermal toxicity study, New Zealand White rabbits (five of each sex per group) were exposed to iprodione (purity 96.2%, Lot no. 89062 01) dermally at 0, 100, 500 or 1000 mg/kg bw per day for 21 days. A gauze dressing 101 × 152 mm, moistened with water and test article was applied to the previously clipped area of the back region. The gauze was secured in place using athletic tape. A plastic Elizabethan collar was then fitted on the rabbit. The test material was in contact with the skin for six hours. After daily exposure for six hours, the wraps and collar were removed, and any residual test article wiped from the skin using sterile water. Clinical signs were monitored once daily. The skin at the application site was examined for signs of erythema, oedema, desquamation and other adverse skin reactions. Body weight was recorded weekly and food consumption was measured daily. Blood samples for haematological parameters and clinical chemistry evaluation were taken from each rabbit pretest (–7 days) and at termination on day 22. At termination, a complete gross necropsy was performed on all animals. Selected organs were weighed. The liver and kidneys from controls and high-dose animals, and the treated skin, untreated skin and gross lesions from animals in all dose groups, were examined microscopically.

There was no mortality during the study. No treatment-related clinical signs of toxicity were observed. No signs of dermal irritation were observed in 100 and 500 mg/kg bw per day males or 100 mg/kg bw per day females. A slight desquamation of dorsal and lateral surfaces was observed occasionally in two 500 mg/kg bw per day females, one 1000 mg/kg bw per day female and three 1000 mg/kg bw per day males. The treated sites were normal at termination. Body weight, body weight gain and food consumption were not affected by the treatment. No adverse treatment-related effects were observed on haematological or clinical chemistry parameters. No statistically significant differences in absolute or relative-to-body organ weights were observed amongst the treated rabbits. Subcutaneous oedema was observed in the treated skin area of one 1000 mg/kg bw per day male at necropsy. This change was not confirmed microscopically. No treatment-related histopathological findings were observed in the treated or untreated skin of animals from all groups, nor in the liver or kidneys of high-dose animals.

The NOAEL in this study was 1000 mg/kg bw per day; the highest dose tested (Siglin, 1991).

**(c) Exposure by inhalation**

In an inhalation toxicity study, Sprague Dawley rats (15 males and 10 females per dose) were exposed to iprodione (purity 96.3%, Lot no. PL-14-0056) via nose-only inhalation at 0, 0.043, 0.21 and 1.07 mg/L (giving nominal exposures of 0, 10, 50 and 250 mg/kg bw per day) for six hours per day, five days a week for four weeks. At the end of the exposure period (day 26 necropsy), 10 males and five females were killed and necropsied. Five males and five females were observed for an additional 28 days without any exposure, to assess any potential delayed onset of findings and/or the reversibility of any effects. Ten naïve males were assigned to a separate group to serve as negative controls and for testosterone measurements. Mortality, clinical signs of toxicity, body weights, food consumption, serum and testicular fluid testosterone analysis and clinical and anatomic pathology were evaluated during the study. Respiratory measurements were performed (respiratory rate, tidal volume, and minute volume); the minute volume data was used for the calculation of the measured inhaled dosage.

The aerosol particle size distribution analysis indicated that the mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were 2.6–2.8 µm and 1.8, respectively. The achieved iprodione technical aerosol concentrations were 0.0430, 0.217 and 1.130 mg/L per day for the low-, mid-, and high-dose groups, respectively. The calculated inhaled doses for the males were 7.3, 38, and 207 mg/kg bw per day inhaled iprodione technical, for the target 10, 50 and 250 mg/kg bw per day groups, respectively. The calculated inhaled doses for the females were 9.0, 43, and 241 mg/kg bw per day inhaled iprodione technical for the target 10, 50, and 250 mg/kg bw per day groups, respectively.

No treatment-related effects were observed on mortality. There were no clinical signs of toxicity, on urinalysis, clinical chemistry or haematological parameters, nor on serum and interstitial testicular testosterone concentrations or gross necropsy findings. Statistically lower group mean values for body weight and group mean food consumed per day were noted for high-dose group males following the completion of exposures. However these changes were not considered adverse due to the small size of the decreases. Treatment-related microscopic findings were present in the adrenal gland and the larynx of the high-dose male and female rats. At the high exposure concentrations, minimal hypertrophy of the adrenal cortical zona fasciculata cells, which correlated with increased adrenal weight, was observed. At day 54, hypertrophy of the adrenal cortical zona fasciculata cells was noted in one male in each of the 50 and 250 mg/kg bw per day groups. Rats exposed to the test substance also exhibited epithelial alteration (metaplasia and hyperplasia) of the transitional epithelium that lined the base of the epiglottis of the larynx, but this was not evident on day 54.

The no-observed-adverse-effect concentration (NOAEC) in this study was 0.217 mg/L per day (equivalent to 38 mg/kg bw per day) based on increased adrenal weights and microscopic findings in the adrenal gland seen at the highest concentration of 1.13 mg/L per day (equivalent to 207 mg/kg bw per day (Elsass, 2016).

## 2.3 Long-term studies of toxicity and carcinogenicity

The long-term toxicity and carcinogenic potential of iprodione was evaluated in two studies in mice and two studies in rats.

***Mouse***

*Study 1*

In a non-GLP chronic toxicity and carcinogenicity study, iprodione (purity not reported, Lot no. 9 CA 7331900) was administered to groups of 60 male and 60 female Carworth CF-1 albino mice in the diet for 18 months at 0, 200, 500 or 1250 ppm (equal to 0, 27.5, 67.6 or 171.4 mg/kg bw per day for males, 0, 31.2, 78.2 or 192.7 mg/kg bw day for females). An ophthalmic examination, haematology, blood biochemistry and urinalysis were performed at 6, 12 and 18 months. Groups of five males and five females from each group were killed after 6 and 12 months of treatment, and all surviving animals were killed after 18 months. Gross necropsy, organ weights and histopathology were evaluated.

There were no treatment-related effects on body weight, food consumption, organ weights, clinical signs, ophthalmology, haematology, blood biochemistry or urinalysis determinations and no findings at necropsy. Histopathology results did not indicate a treatment relationship with neoplastic or non-neoplastic findings.

The NOAELs for toxicity and carcinogenicity were both 1250 ppm (equal to 171.4 mg/kg bw per day), the highest dose tested (Hastings, 1978a, b).

*Study 2*

In a chronic toxicity and carcinogenicity study, iprodione (purity 95.7%; Batch no. DA 604) was administered in the diet to Crl:CD-1 (ICR) BR mice (50of each sex per group) for 99 weeks at dose levels of 0, 160, 800 or 4000 ppm (equal to 0, 23, 115 and 604 mg/kg bw per day for males, 0, 27, 138 and 793 mg/kg bw per day for females). As the terminal procedures took 17 days to complete, treated animals continued to receive the test compound in their diet until the day they were killed, but the duration of treatment was nevertheless reported as 99 weeks. Satellite groups of 15 mice of each sex per group received the same dietary concentrations for 52 weeks. Animals were observed daily for mortality and general clinical signs during the study and their body weights and food consumption periodically recorded. At termination of treatment all surviving animals were killed, blood samples were collected for differential white blood cell counts (samples collected during weeks 52, 80 and 100) and clinical chemistry analysis (using week 52 samples only), and animals were subjected to necropsy, organ weight analysis and histopathology. Other haematological parameters and urinalysis were not investigated.

Mortality was not affected by treatment and no treatment-related clinical signs were observed. Body weight and food consumption were comparable to controls during the first 18 weeks of treatment. Mean body weight gain was lower for mice treated with 4000 ppm compared to controls during weeks 18 to 45, but subsequently mean body weight gain was similar to controls. Food consumption was similar between treated groups and controls, except for slightly elevated food consumption among females in the 4000 ppm group from week 19 to study termination. No treatment-related changes in differential white blood cell counts were observed. The only treatment-related change in clinical biochemistry was increased ALT and aspartate transaminase (AST) in both sexes at 4000 ppm.

After 52 weeks of treatment significantly increased liver weights (adjusted for body weight) and slightly increased absolute adrenal weights were observed in both sexes given 4000 ppm. There was no treatment-related effect on any tumour type or the incidence of tumours at this time point. Microscopic examination revealed various non-neoplastic findings in the liver, adrenals, ovaries, and testes of animals at the highest dose. In the liver there was an increased incidence of hepatocellular hypertrophy in both sexes, and females also displayed centrilobular hepatocyte vacuolation. Changes in the adrenals consisted of hypertrophy of the cells of the zona fasciculata in females. In testes, generalized vacuolation and hypertrophy of the interstitial cells were observed. In a number of females at the highest dose, luteinization of the interstitial cells of the ovary was noted.

Organ weight analysis after 99 weeks of treatment revealed significant changes in animals given 4000 ppm: increased liver weights in both sexes (relative to body weight in males, absolute in females), increased relative thyroid weights in males, increased relative kidney weights in females and decreased

relative uterus weights in females. The incidence of benign and malignant liver tumours was increased in males and females given 4000 ppm (Table 12). A slight but significant increase in luteoma of the ovaries of female mice was also observed at 4000 ppm (Table 12). None of these changes were observed at lower doses. When all four dose groups were considered, the trends for these neoplastic changes were significant, but when the group at the highest dose was excluded from the analysis the trends were not significant.

***Table 12. Mouse carcinogenicity study: summary of neoplastic lesions in main study animals; premature decedent and scheduled termination animals (affected/examined shown)***

| | Dose level (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Parameter** | **0** | **160** | **800** | **4000** | **0** | **160** | **800** | **4000** |
| Hepatocellular adenomas & carcinomas | 7/50 | 6/50 | 10/50 | 26/50** | 2/50 | 2/50 | 2/50 | 21/50** |
| Luteoma | NA | NA | NA | NA | 0/50 | 2/50 | 1/50 | 5/50* |
| Luteoma[a] | NA | NA | NA | NA | 0/50 | 1/50 | 0/50 | 4/50 |
| Survival (%) | 58 | 50 | 64 | 44 | 50 | 42 | 44 | 40 |

Source: Chambers, 1993, Hildebrandt, 2001

[a] A pathology peer review and pathology working group reviewed the histopathologically proliferative lesions using contemporaneously accepted nomenclature and diagnostic criteria (Hildebrandt 2001);

Statistically significant at: * $p < 0.05$; ** $p < 0.01$; (one-tail pairwise comparison with control using the IARC analyses for tumour incidence)

Non-neoplastic changes observed after 99 weeks of treatment (Table 13) included hepatocellular hypertrophy in females at 800 ppm and in both sexes at 4000 ppm, increased incidence of enlarged eosinophilic and fat-containing hepatocytes in both sexes at 4000 ppm and increased incidence and severity of pigmented macrophages and hepatocellular vacuolation in males at 4000 ppm. The incidence of generalized vacuolation and hypertrophy of the testicular interstitial cells was observed in males at 800 and 4000 ppm. Increased incidence of hyperkeratosis of the non-glandular stomach was observed in males given 800 and 4000 ppm. In females, luteinization of the interstitial cells of the ovary and absence of corpora lutea was observed at 4000 ppm. Haemosiderosis in the spleen and amyloidosis and cortical scarring in the kidneys were reported in female mice at 4000 ppm. No treatment-related changes in the adrenals were observed after 99 weeks of treatment.

***Table 13. Mouse carcinogenicity study: summary of selected non-neoplastic lesions in main study animals; premature decedent and scheduled termination animals (affected/examined shown)***

| | Dose level (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Parameter** | **0** | **160** | **800** | **4000** | **0** | **160** | **800** | **4000** |
| **Liver** | | | | | | | | |
| Areas of enlarged eosinophilic hepatocytes | | | | | | | | |
| | 2/50 | 1/50 | 0/50 | 10/50* | 0/50 | 0/50 | 0/50 | 8/50** |
| Area of enlarged eosinophilic hepatocytes | | | | | | | | |
| | 0/50 | 3/50 | 4/50 | 7/50** | 0/50 | 0/50 | 0/50 | 5/50* |
| Centrilobular hepatocyte enlargement | | | | | | | | |
| | 12/50 | 12/50 | 20/50 | 38/50** | 3/50 | 3/50 | 12/50* | 27/50** |
| Centrilobular hepatocyte vacuolation | | | | | | | | |
| | 11/50 | 7/50 | 12/50 | 20/50* | 10/50 | 12/50 | 2/50 | 8/50 |
| Pigmented macrophages | | | | | | | | |
| Total | 1/50 | 2/50 | 4/50 | 11/50* | 2/50 | 3/50 | 3/50 | 4/50 |
| minimal | 1/50 | 1/50 | 3/50 | 5/50 | 1/50 | 2/50 | 2/50 | 3/50 |
| moderate | 0/50 | 1/50 | 1/50 | 6/50 | 1/50 | 1/50 | 1/50 | 1/50 |

| | Dose level (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Parameter** | **0** | **160** | **800** | **4000** | **0** | **160** | **800** | **4000** |
| **Testes;** generalized vacuolation and hypertrophy of interstitial cells | | | | | | | | |
| | 4/50 | 0/50 | 25/50** | 27/50** | NA | NA | NA | NA |
| **Ovaries;** | | | | | | | | |
| Luteinization of the interstitial cells[a]; | | | | | | | | |
| Original study report | NA | NA | NA | NA | 6/50 | 10/50 | 11/50 | 23/50** |
| Re-evaluation | NA | NA | NA | NA | 7/50 | 7/50 | 13/50 | 30/50[b] |
| Corpora lutea absent | NA | NA | NA | NA | 12/50 | 16/50 | 11/50 | 23/50** |

Source: Chambers, 1993; Hildebrandt, 2001

[a] A pathology working group re-evaluation classified the changes as interstitial cell hyperplasia with luteinization; incidences after 99 weeks of treatment were 7/50, 7/50, 13/50 and 30/50 in mice at 0, 160, 800 and 4000 ppm, respectively;

[b] Statistical significance not reported;

Statistically significant at: * $p < 0.05$; ** $p < 0.01$; (Fisher's test: Group 1 compared with Groups 2, 3 and 4)

A Histopathology Peer Review and Pathology Working Group review of the proliferative ovarian lesions observed in this study was performed to determine the incidence of ovarian tumours and interstitial cell hyperplasia with luteinization using currently accepted nomenclature and diagnostic criteria. The working group concluded that the incidence of luteomas following 99 weeks of treatment was 0/50, 1/50, 0/50 and 4/50 in mice given 0, 160, 800 and 4000 ppm, respectively. The incidence in the high-dose group was within the laboratory historical control range for this mouse strain (reported as 0%–8% in the original study report). The working group also concluded there was an increased incidence and severity of interstitial cell hyperplasia with luteinization in treated mice at 800 and 4000 ppm compared with controls, both in the animals dosed for 52 weeks and those treated for 99 weeks. This change was considered most probably mediated by an overproduction of pituitary gonadotrophic hormones (especially luteinizing hormone).

The NOAEL for toxicity was 160 ppm (equal to 23 mg/kg bw per day for males, 27 mg/kg bw per day for females), based on microscopic changes in the testes and ovaries at 800 ppm (equal to 115 mg/kg bw per day for males, 138 mg/kg bw per day for females).

The NOAEL for carcinogenicity was 800 ppm (equal to 115 mg/kg bw per day) based on an increased incidence of benign and malignant liver tumours in both sexes at 4000 ppm (equal to 604 mg/kg bw per day) (Chambers, 1993, 1995; Hildebrandt, 2001).

***Rat***

*Study 1*

In a two-year rat study previously reviewed by JMPR but unavailable to the current sponsor, no evidence of tumourigenic activity was observed following administration of dietary concentrations of 0, 125, 250 or 1000 ppm iprodione. A slight reduction in body weight gain, accompanied by some reduction in food intake, were observed at 1000 ppm. No other treatment-related neoplastic or non-neoplastic findings were reported.

*Study 2*

In a chronic toxicity and carcinogenicity study, groups of 60 male and 60 female Crl:CD(SD)BR rats were fed diets for 104 weeks containing iprodione (purity 94.5%–95.7%, Batch no. DA 604) at concentrations of 0, 150, 300 or 1600 ppm (equal to 0, 6.1, 12.4 or 69 mg/kg bw per day for males, 0, 8.4, 16.5 or 95 mg/kg bw per day for females). Satellite groups of 10 rats of each sex per group at each dose were treated for 12 months. Animals were observed daily for clinical signs; body weight and food consumption were recorded weekly. Ophthalmoscopy was performed on animals in the control and high-dose groups during weeks 52 and 104. Samples were collected for haematology, blood biochemistry and urinalysis evaluations from satellite group rats in weeks 26 and 52, and from main group rats in weeks 78 and 104. Animals were subjected to necropsy, organ weight analysis and histopathology. As

the terminal procedures took 15 days to complete the treated animals continued to receive the test item in their diet until the day on which they were killed.

No dose-related increase in mortality was observed and treatment did not result in clinical signs. The survival rate of animals at the highest dose was greater than that of the other groups: survival rates at 0, 150, 300 and 1600 ppm were 52%, 58%, 53% and 77% in males and 44%, 48%, 50% and 58% in females, respectively. Body weight gain by animals of each sex at the highest dose was lower than that of controls during various periods of treatment, resulting in a body weight that was lower overall than controls by 5% at the end of the study in females and 10% in males. Food consumption of males was slightly lower than for controls throughout the treatment period, and that of females during some weeks of the study. Ophthalmological, haematological, and biochemical investigations and urinalysis revealed no consistent treatment-related effects.

In animals killed at 52 weeks, organ weight analysis revealed a decrease in mean adrenal weights (adjusted for body weight) in treated females compared with controls; this occurred at all dose levels but was not dose-related. The effect was considered probably due to an unusually high mean control value. Microscopic examination revealed a dose-related increase in the incidence of centrilobular hepatocyte enlargement at 300 and 1600 ppm in animals of each sex. Increased incidences of extramedullary haematopoiesis and haemosiderosis were seen in the spleens of females at the highest dose. All males and some females in the high-dose group showed enlargement of cells of the zona glomerulosa. Vacuolation was observed in the zona fasciculata of some males and females at the high dose, and vacuolation of the zona reticularis was also observed in some high-dose males. A slightly increased incidence of minimal interstitial cell hyperplasia was observed in the testes of rats receiving 1600 ppm compared with controls (3/10 versus 1/10). No neoplastic findings were noted at the interim kill.

After 104 weeks of treatment, increased relative (to body weight) liver weights compared with controls were observed in males receiving 300 or 1600 ppm. Significantly increased testis weights were found in high-dose males. Macroscopic changes at 1600 ppm included testicular masses and small seminal vesicles with minimal contents. Small prostates were apparent in some males at 300 and 1600 ppm.

A significantly increased incidence of interstitial cell (Leydig cell) adenomas of the testes was observed at 1600 ppm compared with controls (Table 14). Increases at the low and mid doses were not statistically significant and were within the laboratory's reported historical control range . No increase in the incidence of any other tumour type was observed in males or females.

***Table 14. Rat carcinogenicity study: incidence of Leydig cell adenomas in main study animals; premature decedent and scheduled termination animals[a] (affected/observed animals shown)***

| | Dietary dose | | | |
|---|---|---|---|---|
| | **0 ppm** | **150 ppm** | **300 ppm** | **1600 ppm** |
| Leydig cell adenomas | 3/60 | 7/60 | 7/60 | 29/60** |

Source: Chambers, 1992

[a] Laboratory historical control range (Gopinath, 1998): Range 0–15%; median 8%.
Based on 10 studies (incidence 0/49, 5/50, 5/50, 1/50, 2/50, 3/50, 5/50, 5/50, 0/60, 8/52);

Statistically significant at: * $p < 0.05$; ** $p < 0.01$; (Fisher's test: Group 1 compared with Groups 2, 3 and 4)

Non-neoplastic changes (Table 15) included an increased incidence of interstitial cell hyperplasia in the testes of rats receiving 300 and 1600 ppm, and an increased incidence of atrophy of seminiferous tubules in terminal rats treated with 1600 ppm, when compared to controls. The authors reported that proliferative changes of the interstitial cells of the testes are age-related alterations which may have been associated with the increased survival of males at the highest dose, although the incidence at this dose (58.3%) was greater than the laboratory historical control range (16%–20%), while at 300 ppm survival was the same as control animals, but the incidence of interstitial cell hyperplasia at this dose (21.6%) was slightly greater than the historical control range. Further changes observed were atrophy of the seminiferous tubules, an increased incidence of reduced or absent spermatozoa, atrophy of the prostate, and reduced secretion and absent/empty secretory colloid in seminal vesicles, some of these changes occurring at 300 ppm and above. Minimal centrilobular hepatocyte enlargement was observed

in 3/14 decedent rats treated with 1600 ppm, but in the absence of similar changes in animals killed at termination this finding was not considered to be of significance. The incidence of mainly minimal spleen haemosiderosis was significantly increased in females receiving 300 or 1600 ppm. Changes in the adrenals similar to those observed at interim sacrifice were seen, including enlargement of the cells of the zona glomerulosa and vacuolation in the zona fasciculata and zona reticularis in male rats at 1600 ppm, and to a lesser degree at 300 ppm. A significant increase in generalized fine vacuolation of the zona reticularis compared to controls was observed in males receiving 150 ppm that survived to scheduled termination. However, the incidence at this dose level was not significant when decedents were included in the analysis, and was within the laboratory historical control range. Therefore the effects in males at 150 ppm were not considered to be adverse. In females at the highest dose, a higher incidence of focal enlargement of cells of the zona glomerulosa was found in some animals. In the kidneys of males, there was a dose-related increase in the incidence of basophilic, dilated cortical tubules containing eosinophilic colloid at 300 and 1600 ppm. This lesion is reported to be present in the early stage of chronic progressive nephropathy (CPN) and is believed to be an age-related finding. In the absence of any change in the incidence of CPN, this was considered of minor toxicological importance.

***Table 15. Rat carcinogenicity study: summary of selected non-neoplastic lesions observed in main study animals (premature decedent and scheduled termination animals)***

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | **0** | **150** | **300** | **1600** | **0** | **150** | **300** | **1600** |
| Survival (%) | 52 | 58 | 53 | 77 | 44 | 48 | 50 | 58 |
| **Testes** | | | | | | | | |
| Interstitial cell hyperplasia[a] | | | | | | | | |
| total | 7/60<br>D: 2/29<br>T: 5/31 | 13/60<br>D: 6/25<br>T: 7/35 | 13/60<br>D: 1/28<br>T: 12/32** | 35/60<br>D: 7/14**<br>T: 28/46** | NA | NA | NA | NA |
| trace | 4/60<br>D: 1/29<br>T: 3/31 | 3/60<br>D: 2/25<br>T: 1/35 | 1/60<br>D: 1/28<br>T: 0/32 | 5/60<br>D: 3/14<br>T: 2/46 | NA | NA | NA | NA |
| minimal | 3/60<br>D: 1/29<br>T: 2/31 | 8/60<br>D: 3/25<br>T: 5/35 | 12/60**<br>D: 0/28<br>T: 12/32** | 28/60**<br>D: 3/14<br>T: 25/46** | NA | NA | NA | NA |
| moderate | 0/60 | 2/60<br>D: 1/25<br>T: 1/35 | 0/60 | 2/60<br>D: 1/14<br>T: 1/46 | NA | NA | NA | NA |
| Atrophy of seminiferous tubules[b] | | | | | | | | |
| total | 10/60<br>D: 5/29<br>T: 5/31 | 14/60<br>D: 5/25<br>T: 9/35 | 14/60<br>D: 4/28<br>T: 10/32 | 24/60<br>D: 2/14<br>T: 22/46** | NA | NA | NA | NA |
| minimal | 4/60<br>D: 2/29<br>T: 2/31 | 9/50<br>D: 4/25<br>T: 5/35 | 7/50<br>D: 3/28<br>T: 4/32 | 5/60<br>D: 1/14<br>T: 4/46 | NA | NA | NA | NA |
| moderate | 2/60<br>D: 2/29<br>T: 0/31 | 2/60<br>D: 1/25<br>T: 1/35 | 3/60<br>D: 1/28<br>T: 2/32 | 6/60<br>D: 0/14<br>T: 6/46* | NA | NA | NA | NA |
| marked | 4/60<br>D: 1/29<br>T: 3/31 | 3/60<br>D: 0/25<br>T: 3/35 | 4/60<br>D: 0/28<br>T: 4/32 | 13/60<br>D: 1/14<br>T: 12/46 | NA | NA | NA | NA |
| **Epididymides** | | | | | | | | |
| Reduced spermatozoa[c] | 2/60<br>D: 2/29<br>T: 0/31 | 3/60<br>D: 0/25<br>T: 3/35 | 7/60<br>D: 2/28<br>T: 5/32* | 6/60<br>D: 0/14<br>T: 6/46* | NA | NA | NA | NA |
| Spermatozoa absent[d] | 6/60<br>D: 3/29<br>T: 3/31 | 6/60<br>D: 2/25<br>T: 4/35 | 4/60<br>D: 1/28<br>T: 3/32 | 10/60<br>D: 1/14<br>T: 9/46 | NA | NA | NA | NA |

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | 0 | 150 | 300 | 1600 | 0 | 150 | 300 | 1600 |
| **Prostate** | | | | | | | | |
| Atrophy | 2/60<br>D: 1/29<br>T: 1/31 | 2/60<br>D: 0/25<br>T: 2/35 | 7/60<br>D: 2/28<br>T: 5/32 | 13/60*<br>D: 0/14<br>T: 13/46** | NA | NA | NA | NA |
| **Seminal vesicles** | | | | | | | | |
| Reduced secretion | 4/60<br>D: 4/29<br>T: 0/31 | 4/60<br>D: 3/25<br>T: 1/35 | 12/60*<br>D: 5/28<br>T: 7/32** | 12/60*<br>D: 2/14<br>T: 10/46** | NA | NA | NA | NA |
| Secretory colloid absent/empty | 1/60<br>D: 1/29<br>T: 0/31 | 1/60<br>D: 1/25<br>T: 0/35 | 4/60<br>D: 3/28<br>T: 1/32 | 6/60<br>D: 0/14<br>T: 6/46 | NA | NA | NA | NA |
| **Spleen** | | | | | | | | |
| Haemosiderosis (affected/observed) | 5/60 | 6/60 | 4/60 | 3/60 | 22/59 | 25/60 | 35/59* | 37/60* |
| total | D: 4/29<br>T: 1/31 | D: 5/25<br>T: 1/35 | D: 4/28<br>T: 0/32 | D: 2/14<br>T: 1/46 | D: 13/33<br>T: 9/26 | D: 14/32<br>T: 11/28 | D: 15/29<br>T: 20/30* | D: 15/25<br>T: 22/35* |
| minimal | 4/60<br>D: 3/29<br>T: 1/31 | 6/60<br>D: 5/25<br>T: 1/35 | 4/60<br>D: 4/28<br>T: 0/32 | 2/60<br>D: 1/14<br>T: 1/46 | 14/59<br>D: 7/33<br>T: 7/26 | 16/60<br>D: 9/32<br>T: 7/28 | 22/59<br>D: 9/29<br>T: 13/30 | 25/60<br>D: 7/25<br>T: 18/35* |
| moderate | 0/60 | 0/60 | 0/60 | 1/60<br>D: 1/14<br>T: 0/46 | 8/59<br>D: 6/33<br>T: 2/26 | 9/60<br>D: 5/32<br>T: 4/28 | 12/59<br>D: 5/29<br>T: 7/30 | 12/60<br>D: 8/25<br>T: 4/35 |
| marked | 1/60<br>D: 1/29<br>T: 0/31 | 0/60 | 0/60 | 0/60 | 0/59 | 0/60 | 1/59<br>D: 1/29<br>T: 0/30 | 0/60 |
| **Adrenals[e] (individuals affected/observed)** | | | | | | | | |
| Focal enlargement of cells of zona glomerulosa | 1/60<br>D: 0/29<br>T: 1/31 | 6/60<br>D: 4/25<br>T: 2/35 | 9/60*<br>D: 3/28<br>T: 6/32 | 17/60*<br>D: 3/14<br>T: 14/46** | 0/59 | 1/60<br>D: 1/32<br>T: 0/28 | 5/60*<br>D: 2/30<br>T: 3/30 | 6/60*<br>D: 4/25*<br>T: 2/35 |
| Generalized enlargement of cells of zona glomerulosa | 0/60 | 0/60 | 2/60<br>D: 1/28<br>T: 1/32 | 13/60<br>D: 7/14**<br>T: 6/46* | 0/59 | 0/60 | 0/60 | 3/60<br>D: 2/25<br>T: 1/35 |
| Generalized rarefaction and fine vacuolation of zona fasciculata | 1/60<br>D: 1/29<br>T: 0/31 | 5/60<br>D: 4/25<br>T: 1/35 | 8/60<br>D: 5/28<br>T: 3/32 | 28/60*<br>D: 5/14*<br>T: 23/46** | 1/59<br>D: 1/33<br>T: 0/26 | 0/60 | 1/60<br>D: 1/30<br>T: 0/30 | 5/60<br>D: 3/25<br>T: 2/35 |
| Generalized fine vacuolation of zona reticularis | 7/60<br>D: 6/29<br>T: 1/31 | 14/60<br>D: 6/25<br>T: 8/35* | 24/60*<br>D: 9/28<br>T: 15/32** | 20/60*<br>D: 8/14*<br>T: 12/46** | 0/59 | 1/60<br>D: 0/32<br>T: 1/28 | 1/60<br>D: 1/30<br>T: 0/30 | 3/60<br>D: 1/25<br>T: 2/35 |

D: Died on test; T: Terminal sacrifice; NA: Not applicable; Source: Chambers, 1992

[a] Laboratory historical control range for interstitial cell hyperplasia: 8/50–10/50 (16%–20%);

[b] Laboratory historical control range for atrophy of seminiferous tubules: 5/50–24/50 (10%–48%);

[c] Laboratory historical control range for reduced spermatozoa: 0/50–16/50 (0%–32%);

[d] Laboratory historical control range for spermatozoa absent: 3/50–8/50 (6%–16%);

[e] Laboratory historical control range for adrenals: focal enlargement of cells of zona glomerulosa: 0/50–4/50 (0%–8%);
generalized enlargement of cells of zona glomerulosa: 0/50–1/50 (0%–2%);
generalized rarefaction and fine vacuolation of zona fasciculata: 3/50–9/50 (6%–18%);
generalized fine vacuolation of zona reticularis: 7/50–14/50 (14%–28%)

Statistically significant at: * $p < 0.05$; ** $p < 0.01$; (Fisher's test: Group 1 compared with Groups 2, 3 and 4)

The NOAEL for toxicity was 150 ppm (equal to 6.1 mg/kg bw per day in males, and 8.4 mg/kg bw per day in females) based on histopathological changes in the adrenal cortex, testicular interstitial cell hyperplasia and reduced secretion and absent/empty secretory colloid of the seminal vesicles in males, and increased haemosiderosis in the spleen in females at 300 ppm (equal to 12.4 mg/kg bw per day in males and 16.5 mg/kg bw per day in females).

The NOAEL for carcinogenicity was 300 ppm (equal to 12.4 mg/kg bw per day) based on an increased incidence of Leydig cell adenomas in males at 1600 ppm (equal to 69 mg/kg bw per day) (Ames, 1996; Ames & Gopinath 1993; Chambers, 1992; Gopinath, 1998; Scarfe & Chambers, 1999).

## 2.4 Genotoxicity

### (a) In vitro and in vivo studies

The results of studies on the genotoxicity of iprodione are summarized in Table 16. Iprodione was negative in a bacterial reverse mutation assay and a forward gene mutation (hypoxanthine–guanine phosphoribosyl transferase [HPRT] locus) assay in mammalian cells, did not cause clastogenicity or sister chromatid exchanges in mammalian cells, and was not clastogenic or aneugenic in an in vivo mouse micronucleus assay. A positive result was found in a DNA damage assay with *Bacillus subtilis*, currently considered obsolete. However a clear dose response was not observed: iprodione was positive at the lowest and highest concentrations in the absence of rat liver supernatant fraction obtained by centrifuging at 9000 g (S9 mix), precipitation of the test material occurred, and positive controls did not produce a response in the majority of test strains. This result was therefore considered unreliable. For the in vivo micronucleus test, clinical signs of toxicity were observed following administration of iprodione at all three doses tested, with mortality reported for 1/20 males and 8/20 females in the high-dose group. In addition, a dose-dependent decrease in the ratio of polychromatic to normochromatic erythrocytes (PCE:NCE ratio) compared to the vehicle control group was observed 48 hours after dosing, indicating systemic exposure to the test item.

It should be noted that all the available studies were conducted 20 or more years ago, in compliance with the relevant OECD Guidelines valid at that time with the result of a general lack of sensitivity/specificity compared to the currently regulated studies.

Overall, the outcome of these studies fulfils the basic requirements, can be considered to cover the three genetic end-points (gene mutations, structural and numerical chromosome aberrations) and it can be concluded that iprodione is unlikely to be genotoxic.

***Table 16. Summary of in vitro and in vivo genotoxicity studies performed with iprodione***

| End-point | Test object | Experimental conditions | Batch/ purity | Results | Reference |
|---|---|---|---|---|---|
| | | **In vitro** | | | |
| Bacterial gene mutation | *Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 | Range-finding study:<br>0, 6.67, 10.0 33.3, 66.7, 100, 333, 667, 1000, 3330, 5000 µg/plate (± S9)<br>Main assays:<br>0, 10, 50, 100, 500, 1000, 5000 µg/plate (+ S9);<br>0, 5, 10, 50, 100 and 250 µg/plate (− S9) | 89062 01 96.2% | Negative [ab] | Lawlor & Valentine, 1990 |
| DNA-repair spot assay | *Bacillus subtilis* | 0, 20.6, 61.9, 185.6, 556.7, 1670 µg/mL (± S9) | 83024-01 96.8% | Positive [cd] | Felkner, 1985 |
| Mammalian gene mutation | Chinese hamster ovary cells (CHO-K1-BF4) | Range-finding study:<br>0, 0.03, 0.1, 0.3, 1.0, 3.3, 10, 33.3, 100, 333, 1000 µg/mL (± S9)<br>Main study:<br>0, 5, 10, 50, 75, 100 µg/mL (− S9);<br>0, 100, 250, 500, 1000, 1500 µg/mL (+ S9) | Not reported | Negative [e] | Godek, 1985 |

| End-point | Test object | Experimental conditions | Batch/ purity | Results | Reference |
|---|---|---|---|---|---|
| Chromosome aberration | Chinese hamster ovary cells (CHO-K1-BF4) | Range-finding study: 0, 0.03, 0.1, 0.3, 1.0, 3.3, 10, 33.3, 100, 333, 1000 µg/mL (± S9)<br>Main study: 0, 15, 75, 150 µg/mL (− S9); 0, 40, 150, 400 µg/mL (+ S9) | Not reported | Negative [f] | San Sebastian, 1985a |
| Sister chromatid exchange | Chinese hamster ovary cells (CHO-K1-BF4) | Range-finding study: 0, 0.03, 0.1, 0.3, 1.0, 3.3, 10, 33.3, 100, 333, 1000 µg/mL (± S9)<br>Main study: 0, 5, 10, 25, 50, 75, 100 µg/mL (− S9); 0, 5, 50, 100, 200, 400 µg/mL (+ S9) | Not reported | Negative [g] | San Sebastian, 1985b |
| | | **In vivo** | | | |
| Micronucleus induction | Bone marrow erythrocytes | Range-finding study, phase 1: 500, 1000, 2000 and 4000 mg/kg bw<br>Range-finding study, phase 2: 1536, 1920, 2400 and 3000 mg/kg bw<br>Main study: 0, 750, 1500 and 3000 mg/kg bw<br>Single doses administered by oral gavage to 15 male and 15 female CD-1 mice (5 additional animals per sex included at the high dose to replace any that may have died); animals killed 24 h, 48 h and 72 h after dosing | 9109801 96.1% | Negative [h] | Proudlock & Elmore, 1994 |

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*;

[a] Incomplete set of bacterial strains (*S. typhimurium* TA102 or *E. coli* tester strain missing);

[b] Decreased mean numbers of revertants and background lawn observed at 250 µg/plate (− S9); precipitate at doses ≥ 500 µg/plate (+ S9);

[c] Test currently considered obsolete and not suitable for risk assessment;

[d] Positive in some strains at 20.6 and 1670 µg/mL without S9, and at 1607 µg/mL with S9; Precipitation visible at concentrations ≥ 556.7 µg/mL and could not be excluded at lower concentrations; Positive controls did not produce a response in the majority of test strains;

[e] Precipitation observed at concentrations ≥ 100 µg/mL

[f] Deviation from current and older versions of the OECD TG 473 for treatment schedule, selection of dose-levels for scoring and determination of cytotoxicity;

[g] This test, although useful for research purposes, is currently not included in the test batteries for genotoxicity risk assessment; the relevant OECD TG 479 has been withdrawn;

[h] Study compliant with the OECD TG 474 (adopted 1983); Clinical signs of toxicity observed in rats administered iprodione at all dose levels in main study; 1/20 males and 8/20 females died at the high dose; Dose-related decrease in the ratio of polychromatic to normochromatic erythrocytes compared to the concurrent vehicle group was observed 48 hours after dosing, indicating bone marrow toxicity.

## 2.5 Reproductive and developmental toxicity

### (a) Multigeneration studies

***Rat***

*Study 1*

In a two-generation reproductive toxicity study, iprodione (purity 96.2%, Lot no. 89062-01) was administered to groups of 28 male and 28 female Crl:CD BR/VAF/Plus rats via the diet at concentrations of 0, 300, 1000 or 3000 ppm for at least 10 weeks before mating and throughout gestation, lactation and weaning. The first parental (F0) animals were mated twice to produce F1a and F1b litters. F1a animals were mated twice to produce F2a and F2b litters. The high dose for F1 animals was reduced to 2000 ppm at the time of first mating of F1a rats because of excessive toxicity in the F1 generation. The mean administered doses, calculated during the premating phase of each generation, were equal to 0, 17, 55 and 159 mg/kg bw per day for F0 males; 0, 21, 71 and 214 mg/kg bw per day for F0 females; 0, 21, 69 and 153 mg/kg bw per day for F1 males; and 0, 25, 82 and 191 mg/kg bw per day for F1 females.

There were no test item-related clinical observations in any of the adult animals, neither in the F0 nor F1a generation. Body weight and body weight gain were reduced compared with controls throughout the treatment period in F0 and F1 males receiving 3000 or 2000/3000 ppm and F0 and F1 females receiving 1000, 3000 or 2000/3000 ppm. Mean food consumption was also significantly lower in F0 and F1 males and females receiving 1000, 3000 or 2000/3000 ppm. In F0 animals, one female from the control group and 11 from the 3000 ppm group were killed between lactation days (LDs) 1 and 25 of the F1b phase following the death of the entire litter.

Reproductive performance was unaffected by iprodione exposure. In both generations, offspring viability, number of pups delivered, number of live pups per litter at days 0 and 4 precull, and pup weights were significantly lower at the high dose compared with controls. Pups from the 3000 ppm (F1a and F1b) and 2000/3000 ppm (F2a and F2b) groups showed signs of toxicity, including reduced overall size, slow movement, unkempt appearance, hunching or tremors during lactation. At necropsy, black mucoid material was observed in the stomachs and/or intestines of F1a culled pups and pups that died. This finding was not seen at lower doses in the F1a cohort and was not observed at any dose in the subsequent cohorts. Necropsy observations during the F1b phase revealed an increased incidence of anophthalmia or microphthalmia in pups from the 3000 ppm group compared with controls (5 and 1 pups with anophthalmia, respectively, with none in the low- and mid-dose groups). However the number of litters with these malformations was one for controls and two for 3000 ppm animals, and the study authors considered this finding was not treatment-related.

The NOAEL for parental toxicity was 300 ppm (equal to 17 mg/kg bw per day) based on reduced body weight, body weight gain and food consumption at 1000 ppm (equal to 55 mg/kg bw per day). The NOAEL for reproductive toxicity was 3000 ppm (equal to 153 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 1000 ppm (equal to 55 mg/kg bw per day), based on clinical signs, reduced offspring viability and reduced pup weight at 3000 ppm (equal to 153 mg/kg bw per day) (Henwood, 1991).

*Study 2*

In a two-generation reproductive toxicity study, iprodione (purity 97.8%, Batch no. 04/0070-4) was administered to groups of 25 male and 25 female Wistar Crl:WI(Han) rats at concentrations of 0, 300, 750 or 1500 ppm from at least 75 days prior to treatment until weaning of their offspring. As lactating females consumed twice as much food as non-lactating females, the concentration of iprodione in the diet of F0 and F1 females was reduced by 50% during the lactation period to maintain the dose levels. Calculated intakes of iprodione for each group are summarized in Table 17.

Due to findings in the F1 offspring, the F2 offspring were observed for additional end-points pertaining to nipple retention in males. A subset of these F2 offspring, comprising five high-dose males with nipple/areolae retention, five high-dose males without nipple/areolae retention and 10 control males, were selected to be raised until test week 10. These were then mated with 20 new control females to test their fertility and produce a F3 litter.

*Table 17. Test substance intake (mg/kg bw per day) in a two-generation reproductive toxicity study*

| Sex | Period of treatment | Generation | Dietary dose | | |
|---|---|---|---|---|---|
| | | | 300 ppm | 750 ppm | 1500 ppm |
| Males | N/A | F0 | 26.9 | 67.8 | 135.6 |
| | | F1 | 27.3 | 67.4 | 136.5 |
| | | F2 | 43.4 (weeks 0–4) | 109.7 (weeks 0–4) | 159.3 (weeks 0–10) |
| Females | Premating period | F0 | 28.4 | 71.8 | 142.5 |
| | | F1 | 28.3 | 70.3 | 138.7 |
| | | F2 | 49.5 (weeks 0–2) | 124.7 (weeks 0–2) | 243.8 (weeks 0–2) |
| | Gestation period | F0 | 24.2 | 61.0 | 120.5 |
| | | F1 | 24.0 | 58.9 | 116.0 |
| | Lactation period | F0 | 26.2 | 66.6 | 135.8 |
| | | F1 | 25.9 | 63.1 | 131.6 |

Source: Schneider, 2014

No treatment-related clinical signs or mortality were observed during the study.

Reduced food consumption, body weight and body weight gain were observed in F0 females in the high-dose group, most distinctly during gestation. There were no treatment-related changes in haematology or blood biochemical parameters in the F0 animals. Absolute and relative adrenal gland weights were significantly increased in high-dose males. Histopathology revealed increased incidence and severity of cytoplasmic vacuolation in the adrenal cortex, especially in the zonae fasciculata and glomerulosa, in both sexes at the high dose (Table 18). In affected males the cytoplasmic vacuolation was characterized either by multiple round, clearly demarcated to coalescing vacuoles of varying size, or by more microvesicular vacuoles. In all affected females the vacuolation patter was only of the microvesicular type, giving the cytoplasm of adrenal cortex cells a foamy appearance.

In F1 parental rats, reduced food consumption, body weight and body weight gain were also observed in high-dose females. Males in the high-dose group had a slightly increased frequency (8%) of abnormal sperm in the cauda epididymis compared with controls (6.7%; significant at $p < 0.05$). This finding was considered incidental, based on comparison with historical control data. and because fertility was unaffected and there were no histopathological changes in the contralateral testes or epididymides. No treatment-related changes in other sperm parameters (motility, head count in testis and cauda epididymis) were observed. Adrenal weights (absolute and relative to body weight) were increased in high-dose animals of both sexes. A dose-related increase in incidence and severity of cytoplasmic vacuolation was seen in the adrenal cortex in of both sexes in the mid- and high-dose groups (Table 18).

*Table 18. Adrenal effects in the two-generation reproductive toxicity study in rats (25 organs/group[a])*

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| Parameter | 0 | 300[a] | 750 | 1500 | 0 | 300 | 750 | 1500 |
| **F0 parental animals** | | | | | | | | |
| Adrenal weight; absolute (mg ± SD) | 60.68 ± 7.273 | 58.208 ± 6.366 | 62.52 ± 8.302 | 67.88 ± 10.072* | 76.12 | 70.36 | 73.0 | 79.08 |
| Relative adrenal gland weight (%) | 0.015 | 0.014 | 0.016 | 0.017** | 0.033 | 0.03* | 0.032 | 0.035 |
| Vacuolation increased | 2 | 1 | 4 | 17 | 1 | 2 | 2 | 13 |
| Grade 1 | 2 | 1 | 2 | 10 | 1 | 2 | 2 | 9 |
| Grade 2 | - | - | 2 | 4 | - | - | - | 4 |
| Grade 3 | - | - | - | 3 | - | - | - | - |

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| Parameter | 0 | 300 [a] | 750 | 1500 | 0 | 300 | 750 | 1500 |
| **F1 parental animals** | | | | | | | | |
| Absolute adrenal gland weight (mg) | 63.52 | 62.16 | 65.8 | 70.0* | 73.4 | 69.56 | 73.56 | 83.8 |
| Relative adrenal gland weight (%) | 0.016 | 0.015 | 0.016 | 0.018* | 0.031 | 0.03 | 0.031 | 0.037** |
| Vacuolation increased | 2 | 3 | 6 | 16 | 1 | 1 | 10 | 23 |
| Grade 1 | 2 | 3 | 5 | 4 | | 1 | 10 | 6 |
| Grade 2 | - | - | 1 | 8 | 1 | - | - | 13 |
| Grade 3 | - | - | - | 4 | - | - | - | 4 |

SD: Standard deviation; Source: Schneider, 2014

[a] Only 24 adrenal glands weighed for F0 males administered 300 ppm, but 25 adrenal glands assessed histopathologically;

Statistically significant using Kruskal–Wallis and Wilcoxon tests at: * $p < 0.05$; ** $p < 0.01$;

There were no indications from clinical examinations, gross observations and histopathology that iprodione adversely affected the fertility or reproductive performance of F0 or F1 animals. Estrous cycle data, mating behaviour, conception, gestation, parturition, lactation and weaning as well as sexual organ weights and gross and histopathological findings in these organs were unaffected by treatment.

F1 pups of both sexes in the high-dose group had decreased body weights when compared to controls. An increased number of male pups with areolae/nipples at weaning (PND 20) were observed in the high-dose group (4 compared to 0 in the control group), although all four affected pups were from the same litter. One F1 male offspring with persisting nipples/areolae was also noted in the mid-dose group, but as this was not seen in the F2 generation it was regarded as a spurious finding. A significant increase in age at preputial separation was observed in males in the mid- and high-dose groups, indicating a delay in onset of male puberty.

Supressed body weights were also observed for F2 pups of both sexes in the high-dose group. Persistent nipple/areolae retention was evident in F2 high-dose, male offspring (5 compared to 0 in the control group). However, F2 males with persistent nipples/areolae produced healthy F3 offspring with no evidence of nipple/areola retention when paired with untreated females. As in the F1 generation, delayed preputial separation was observed in mid- and high-dose F2 male offspring (Table 19). For both F1 and F2 generations, the rise in age of preputial separation corresponded with an increase in body weight, mostly commensurate with the age of the animals, indicating the change was unlikely to be due to slower overall body development.

***Table 19. Days to reach preputial separation and body weights at achievement in F1 and F2 offspring in a two-generation reproductive and developmental study in rats***

| | Dietary dose (ppm) | | | |
|---|---|---|---|---|
| Parameter | 0 | 300 | 750 | 1500 |
| **F1 offspring** | | | | |
| Days to reach criterion (mean ± SD) [a] | 40.6 ± 1.50 | 41.4 ± 1.50 | 44.8 ± 2.32** | 48.2 ± 1.88** |
| Body weight at criterion (g; mean ± SD) [b] | 169.5 ± 12.78 | 170.2 ± 12.86 | 185.7 ± 15.32** | 198.4 ± 16.52** |
| **F2 offspring** | | | | |
| Days to reach criterion (mean ± SD) | 40.1 ± 1.79 | 41.8 ± 1.68** | 45.1 ± 1.86** | 47.9 ± 1.93** |
| Body weight at criterion (g; mean ± SD) | 168.4 ± 12.60 | 168.7 ± 12.71 | 191.1 ± 16.68** | 196.8 ± 14.67** |

SD: Standard deviation; Source: Schneider, 2014

[a] Historical control range: mean age 41.7 days at criterion; range of study means: 39.7–42.5 (based on 331 litters);

[b] Historical control range: mean body weight 170.1 g at criterion; range of study means: 156.5–180.5 (based on 329 litters);

No adverse effects were observed in F2 parental animals or F3 pups;

Statistically significant using Dunnett two-sided test at: * $p < 0.05$; ** $p < 0.01$

The NOAEL for parental toxicity was 750 ppm (equal to 59 mg/kg bw per day) based on decreased body weight and food consumption, increased absolute and relative adrenal weights and an increased incidence of adrenal vacuolation at 1500 ppm (equal to 116 mg/kg bw per day). The NOAEL for reproductive toxicity was 1500 ppm (equal to 116 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 300 ppm (equal to 24 mg/kg bw per day) based on the delayed onset of male puberty at 750 ppm (equal to 59 mg/kg bw per day) (Schneider, 2014).

**(b) Developmental toxicity**

One developmental toxicity study in rats and one in rabbits previously reviewed by the JMPR were not provided by the current sponsor. In the unsubmitted rat study, females given 400 mg/kg bw per day iprodione (but not 100 or 200 mg/kg bw per day) showed maternal toxicity. There was no indication of an embryotoxic or teratogenic effect due to iprodione at maternal doses up to 400 mg/kg bw per day. In the unsubmitted rabbit study, maternal body weight gain compared to controls was slightly reduced at 100 mg/kg bw per day, and a dose-related weight loss occurred at 200 and 400 mg/kg bw per day. Food intake was reduced at 200 mg/kg bw per day. At 400 mg/kg bw per day, the highest dose tested, nine of 17 females died and only one of the four remaining pregnant animals carried to term. Fetal loss was increased at 200 mg/kg bw per day, and fetal weight was reduced at 200 mg/kg bw per day and above. Multiple malformations occurred in one of 68 living fetuses at 200 mg/kg bw per day. Minor malformations were noted in all groups.

***Rat***

*Study 1*

In a range-finding developmental toxicity study, iprodione (purity 84.2%; Lot no. DA237) in 0.5% (w/v) methylcellulose was administered by gavage to pregnant CD rats at doses of 0, 40, 120, 240, 400 or 800 mg/kg bw per day during gestation days (GDs) 6 to 15. Groups of six females were included, with additional groups of eight given 40 or 800 mg/kg bw per day iprodione with the intention of providing blood samples on GD 15 to assess absorption. Surviving females were killed on GD 21 for examination of uterine contents.

Treatment with 400 and 800 mg/kg bw per day resulted in mortality, weight loss, and pronounced clinical signs including piloerection, pallor, ataxia, involuntary mastication and animals occasionally found prone or prostrate. Blood loss from the vagina was observed in approximately half of these animals. One animal receiving 400 mg/kg bw per day and seven of 14 administered 800 mg/kg bw per day were killed in extremis and one animal given 800 mg/kg per day was found dead. All animals given 240 mg/kg bw per day showed flaccid muscles, with similar signs observed occasionally at 120 mg/kg bw per day. Maternal body weight and body weight gain were reduced at 240 mg/kg bw per day and above. Maternal rats had enlarged adrenal glands and gastrointestinal tract effects at 400 mg/kg bw per day and above. Resorptions were observed at 240 mg/kg bw per day and above. Mean plasma iprodione concentrations measured 0.5, 1, 2 and 24 hours after administration of the final dose on GD 15 were 2.987, 2.515, 2.730 and 0.031 µg/mL respectively at 40 mg/kg bw per day and 8.966, 18.633, 18.965 and 5.384 µg/mL respectively at 800 mg/kg bw per day. It was concluded that dose levels in a definitive developmental toxicity study should not exceed 240 mg/kg bw per day (Tesh, 1986a).

*Study 2*

In the main developmental toxicity study following the range-finding study, iprodione (purity 94.2%, Lot no. DA237) was administered in 0.5% (w/v) aqueous methylcellulose by gavage at doses of 0, 40, 90 or 200 mg/kg bw per day to pregnant CD rats (20 per dose group) from GDs 5–15. Animals were killed on GD 21 and uterine contents were examined.

Iprodione produced no adverse effects on maternal health as assessed by mortality, clinical signs, body weight, food consumption or macroscopic observations. There were no treatment-related effects on numbers of implantations, live young, resorptions, pre- or post-implantation loss or mean placental weight. In the offspring, the incidence of space between body wall and organs and of small fetuses (below 2.7g) was increased at the high dose, while group mean fetal body weights were slightly reduced in treated groups. However none of the differences were statistically significant and values were within historical control ranges (Table 20). No treatment-related malformations or variations were observed.

*Table 20. Summary of key offspring findings from the developmental toxicity study in rats*

| Parameter | Dose (mg/kg bw per day) | | | |
|---|---|---|---|---|
| | 0 | 40 | 90 | 200 |
| Small fetus; <2.70 g (% incidence and [no. of litters])[a] | 2.9 [5/20] | 5.1 [4/20] | 4.9 [9/20] | 8.0 [8/19] |
| Fetal weight; mean ± SD (g)[b] | 3.27 ± 0.06 | 3.18 ± 0.06 | 3.16 ± 0.09 | 3.15 ± 0.07 |
| Space between body wall and organs (% incidence and [no. of litters])[c] | 4.3 [3/20] | 5.3 [4/20] | 5.8 [4/20] | 11.2 [6/19] |

SD: Standard deviation; Source: Tesh, 1986b

[a] Historical control mean: 3.57%; range: 0.4%–18.4%

[b] Historical control mean: 3.33 g; range: 3.06%–3.55 g

[c] Historical control mean: 15.91%; range: 1.0%–47.4%

The NOAEL for maternal toxicity was 200 mg/kg bw per day, the highest dose tested. The NOAEL for embryo/fetal toxicity was 200 mg/kg bw per day, the highest dose tested (Tesh, 1986b, 1987).

*Study 3*

In a study aiming to determine effects on pregnancy and sexual differentiation, iprodione (purity 97.1%, Batch no. 9426801) in 0.4% aqueous methylcellulose was administered by gavage to groups of 25 sperm-positive CD rats at doses of 0, 20, 120 or 250 mg/kg bw per day from GDs 6–19. A positive control group was administered 50 mg/kg bw per day flutamide. Animals were killed on GD 20 and the uterine contents were examined.

Clinical signs of toxicity were observed at 250 mg/kg bw per day including episodes of prostration, reduced motor activity and facial and urogenital staining. Six animals in this dose group were killed from GDs 15–18 due to the severity of clinical signs, and an additional three animals were found dead from GDs 18–20. At 120 mg/kg bw per day, three animals had dirty fur on the facial and urogenital area from GDs 19–20. Decreased body weight gain, food consumption and food efficiency were observed at 120 and 250 mg/kg bw per day. At necropsy, enlarged adrenal glands were observed in seven and 20 animals given 120 and 250 mg/kg bw per day, respectively.

No effects on corpora lutea, implantations, litter size, live fetuses or resorptions were observed following treatment with iprodione. A significant reduction in fetal body weight was observed in both sexes at the high dose. There were no effects on the incidence of external abnormalities or sex ratio. Anogenital distance in male fetuses was reduced (statistically significant) at 120 and 250 mg/kg bw per day, but there were no differences compared with controls in the anogenital index of these animals (Table 21). The study authors considered that the statistically significant reductions in anogenital distance for males at 120 and 250 mg/kg bw per day were not biologically relevant, as the differences from controls were slight to minimal, and these dose levels induced maternal toxicity at a critical period of development (GDs 16–20). The study authors concluded that administration of iprodione to pregnant females during organogenesis had no effect on sex differentiation and no specific effect on the anogenital distance of male fetuses. In animals treated with flutamide, all male fetuses had a marked reduction in anogenital distance and anogenital index, and had morphologically abnormal external genitalia (all were recorded as female based on external examination).

***Table 21. Fetal weights, anogenital distance and anogenital index in male rat fetuses in a study to determine the effect of iprodione on pregnancy and sexual differentiation***

| | Dose (mg/kg bw per day) | | | |
|---|---|---|---|---|
| | 0 | 20 | 120 | 250 |
| Fetal weight; mean ± SD (g) | 3.64 ± 0.31 | 3.64 ± 0.33 | 3.61 ± 0.34 | 3.11 ± 0.47** |
| Anogenital distance; mean ± SD (mm) | 2.43 ± 0.09 | 2.44 ± 0.14 | 2.32 ± 0.12* | 2.10 ± 0.19** |
| Anogenital index mean ± SD | 0.67 ± 0.04 | 0.67 ± 0.05 | 0.65 ± 0.06 | 0.68 ± 0.07 |

SD: Standard deviation; Source: Repetto-Larsay, 1997

Statistically significant using Dunnett's or Mann-Whitney's test at: * $p < 0.05$; ** $p < 0.01$

The NOAEL for maternal toxicity was 20 mg/kg bw per day based on decreased body weight gain, food intake, food efficiency and enlarged adrenals at 120 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 120 mg/kg bw per day based on reduced fetal weight in both sexes at 250 mg/kg bw per day (Repetto-Larsay, 1997).

***Rabbit***

In a developmental toxicity study, iprodione (purity 95.0%; Lot no. CA8425101) in 0.5% aqueous methylcellulose was administered by gavage to artificially inseminated New Zealand White rabbits (18 per group) at doses of 0, 20, 60 or 200 mg/kg bw per day from GD 6 to GD 18. Animals were killed on GD 29 and uterine contents were examined.

Rabbits given 200 mg/kg bw per day experienced body weight loss and reduced food consumption throughout the treatment period. Body weight loss and reduced food consumption were observed during the first six days of treatment which, in the absence of measurements for earlier time points following the start of dosing, may be indicative of an acute adverse effect (Table 22). Clinical signs at this dose included hair loss, decreased urination and/or defaecation and loose faeces or soft stools. Seven of 18 females receiving the high dose aborted between GD 18 and GD 23. The incidence of abortions in the 0, 20 and 60 mg/kg bw per day groups was 1/18, 0/18 and 1/18, respectively. Animals in the 60 mg/kg bw per day group showed slight body weight loss during the first six days of treatment and reduced body weight gain compared with controls over the course of the study. Increased post-implantation loss was observed in the high-dose group, attributed to two females with total litter resorptions: the mean number of viable fetuses was slightly decreased at this dose level. Increased incidences of some skeletal variations (thirteenth full rib, malaligned sternebrae and 27 presacral vertebrae) were observed at the high dose, but the incidences were within the laboratory historical control range. Umbilical hernia was observed in one fetus in the 20 mg/kg bw per day group (8.3% of litters) and two in the 60 mg/kg bw per day group (15.4% of litters), compared with none in the control group. These percentages were outside the historical control range (0.0%–6.7%), but no umbilical hernias were observed at 200 mg/kg bw per day. The high dose was considered excessive for a teratology study.

***Table 22. Mean food consumption and body weight changes in maternal rabbits during the first six days of treatment (GDs 6–12) with iprodione***

| | Dose (mg/kg bw per day) | | | |
|---|---|---|---|---|
| **Parameter** | **0** | **20** | **60** | **200** |
| Food consumption (g per day) | 184 | 177 | 171 | 111** |
| Body weight change (g) | 53 | 65 | −6 | −151** |

Statistically significant using Dunnett's test at: * $p < 0.05$; ** $p < 0.01$ Source: Rodwell, 1985

The NOAEL for maternal toxicity was 20 mg/kg bw per day based on decreased body weight gain at 60 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 60 mg/kg bw per day based on increased abortions and post-implantation losses at 200 mg/kg bw per day. The NOAEL for acute maternal toxicity was 60 mg/kg bw, based on body weight loss and reduced food consumption between GD 6 and GD 12 at 200 mg/kg bw per day (Rodwell, 1985).

## 2.6 Special studies

### (a) Neurotoxicity

No neurotoxicity studies were submitted. No evidence of neurotoxicity was reported in routine studies.

### (b) Immunotoxicity

In an immunotoxicity study conducted in accordance with US EPA OPPTS 870.7800, iprodione (purity 97.8%, Batch no. SIN000047) was administered in the diet to female Sprague Dawley (CrlCD(SD)) rats (10 per dose group) at concentrations of 0, 500, 1200 or 3000 ppm (equal to 0, 39, 95 and 225 mg/kg bw per day) for at least 28 days. A positive control group of 10 females was administered cyclophosphamide at 3.5 mg/kg bw per day by gavage. On study day 26 (four days prior to necropsy), all animals received a single intravenous dose of $2.5 \times 10^9$ sheep red blood cells (sRBCs) in phosphate buffered saline. Animals were monitored daily for mortality and clinical signs. Body weight and food consumption were recorded weekly. Blood samples were collected on study day 30, then animals were necropsied, gross pathology observations were performed and spleen and thymus weights were recorded.

There were no mortalities and no treatment-related clinical signs. At the end of the study mean body weights were significantly lower (approximately 10%) than controls at 3000 ppm, but not at 500 or 1200 ppm. Mean cumulative body weight gains were 14%, 21% and 38% lower than controls in animals receiving 500, 1200 and 3000 ppm, respectively (Table 23). Mean food consumption was reduced by approximately 20% at 3000 ppm compared with controls on study day 8. Reduced food consumption compared with controls was also observed at 3000 ppm on study days 15, 22 and 29 (by 13%, 14% and 12% respectively). Food consumption was unaffected by treatment in rats given 500 or 1200 ppm. The study authors concluded that the reduced body weight gains at 500 and 1200 ppm were not adverse because the terminal body weights were not significantly reduced compared with controls at these doses.

There were no differences in anti-sRBC IgM levels in animals treated with iprodione compared to controls. Absolute and relative spleen and thymus weights were not affected by treatment with iprodione, and no macroscopic changes were observed. Mean anti-sRBC IgM levels were significantly lower compared with controls in animals treated with the immunosuppressant cyclophosphamide. Animals treated with cyclophosphamide displayed reduced absolute and relative (to body weight) weights of spleen ($p \leq 0.01$) and thymus (not statistically significant) compared to controls. Treatment-related atrophic/small spleen (5/10 affected) and/or thymus (2/10 affected) were also observed in the groups treated with cyclophosphamide.

***Table 23. Rat immunotoxicity study: body weight and cumulative body weight gain on study day 29 (mean ± standard deviation shown)***

| | Dietary dose (ppm) | | | |
|---|---|---|---|---|
| | **0** | **500** | **1200** | **3000** |
| Body weight (g) | 242 ± 20 | 235 ± 17 | 231 ± 17 | 219 ± 19 |
| Cumulative body weight gain (g) | 58 ± 9 | 50 ± 9 | 46 ± 15 | 36 ± 13** |

Statistically significant using Bartlett, ANOVA and Dunnett's test at: ** $p < 0.01$; Source: Odin-Feurtet, 2010

The NOAEL for systemic toxicity was 1200 ppm (equal to 95 mg/kg bw per day), based on reduced body weight and body weight gain at 3000 ppm (equal to 225 mg/kg bw per day). The NOAEL for functional immunotoxicity was 3000 ppm (equal to 225 mg/kg bw per day), the highest dose tested (Odin-Feurtet, 2010).

## (c) Studies on metabolites

### *Metabolite RP30228*

There were several studies conducted on plant and rat metabolites that were made available to other regulatory bodies but not submitted to JMPR.

In an acute oral toxicity study, fasted Sprague Dawley rats (five of each sex per dose) were administered a single dose of metabolite RP30228 (purity 99.3%, Lot no. EA2025RF4) via gavage in distilled water at a dose level of 2000 mg/kg bw. Treated rats were observed for 14 days.

No clinical signs of toxicity or mortalities were observed. Body weight gains were comparable to controls. A macroscopic examination revealed no abnormalities.

The $LD_{50}$ of metabolite RP30228 was greater than 2000 mg/kg bw (Clouzeau, 1992).

### *3,5-dichloroaniline:*

In bacterial reverse mutation assays 3,5-dichloroaniline was negative for mutagenicity in the presence and absence of metabolic activation (Table 24).

***Table 24. Mutagenicity of 3,5- dichloroaniline, a metabolite of iprodione***

| End-point | Test object | Experimental conditions | Batch/purity | Results |
|---|---|---|---|---|
| Bacterial gene mutation | *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 | Range finding study: 0, 5, 15, 50, 0.15, 150, 1500, 2500 and 5000 µg/plate in DMSO (50 µL) ± S9 | SZBB045XV 99.70% | Negative |
| | *Escherichia coli* WP2 *uvr*A | Main study: 0, 15, 50, 150, 500, and 2500 µg/plate in DMSO (50 µL) ± S9 [a] | | |

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g* ; Source: Dakoulas, 2014

[a] No precipitate but toxicity beginning at 1500 µg/plate;

[b] No precipitate but toxicity beginning at 5000 µg/plate;

[c] Studies compliant with OECD Guideline 471, corrected June 2020

### *Chemoinformatics assessment of iprodione metabolites: quantitative structure–activity relationship (QSAR) predictions*

In silico genotoxicity assessment of iprodione and its many metabolites (RP30228, RP32490, RP36114, RP36115, RP44247, RP36112, DCHPU, RP36221, RP44160, RP37677, RP36119, RP37176, RP35606, RP25040, RP32596, and M610F007) was conducted using a combination of in silico models and read-across methods. The metabolites were grouped into five groups and one single metabolite, based on structural similarity as defined by Benigni et al. (2019). The structures of iprodione and its metabolites were assessed for the presence of reactive functional groups using a profiler for facile chemical reactivity, together with a function group assessment using the OECD QSAR Toolbox (v. 4.5 SPI). The Toolbox was used to codify structural alerts for genotoxicity (DNA binding by OASIS, DNA binding by OECD, protein binding by OASIS and protein binding by OECD). The OECD Toolbox was also used to profile specific end-points such as DNA alerts for AMES, chromosomal aberration (CA) and micronuclei (MNT) by OASIS, protein binding alerts for chromosomal aberration by OASIS, in vitro mutagenicity (Ames test) alerts by ISS and in vivo mutagenicity (micronucleus) alerts by ISS. Structures, chemical names and code numbers for these compounds can be found in Appendix 1.

DEREK Nexus (v. 6.2.1), the knowledge-based tool, was used to evaluate in vitro mutagenicity, in vivo mutagenicity, in vitro chromosome damage, and in vivo chromosome damage. The statistics-based tool SARAH Nexus was used to predict in vitro mutagenicity. The default settings were used for the prediction. The available data on iprodione and its metabolites and publicly available information was used in a weight-of-evidence approach.

The structural similarities and grouping of metabolites based on structural similarity as generated by OECD QSAR Toolbox is shown in the Table 25.

*Table 25. Structural features and properties of iprodione metabolites*

| Compound | Group | % Similarity to iprodione | Molecular weight | Log $K_{ow}$ | Alkane, branched with secondary carbon | Aryl | Aliphatic amine, primary | α-Aminocarboxylic acid | Amine, primary | Aniline | Aryl halide | Carboxylic acid | Hydantoin | Organic amide and thioamide | Imidazolidine | Imide | Isopropyl | Urea derivatives | Phenol |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Iprodione** | A | 100 | 330 | 2.5 | X | X | | | | | X | | X | | X | X | X | X | |
| **RP32490** | A | 82.1 | 288 | 1.68 | | X | | | | | X | | X | | X | X | | X | |
| **RP37677** | A | 83.7 | 346 | 2.31 | X | X | | | | | X | | X | | X | X | X | X | X |
| **RP25040** | A | 72.2 | 245 | 1.5 | | X | | | | | X | | X | | X | X | | X | |
| **RP30228** | B | 81 | 330 | 2.63 | X | X | | | | | X | | X | | X | X | X | X | |
| **RP36112** | B | 66.7 | 288 | 2.03 | | X | | | | | X | | X | | X | X | | X | |
| **RP36119** | B | 65.1 | 346 | 2.34 | X | X | | | | | X | | X | | X | X | X | X | X |
| **RP36114** | C | 32.4 | 264 | 1.78 | | X | | | | | X | | | | | X | | X | X |
| **RP36115** | C | 50 | 248 | 2.07 | | X | | | | | X | | | | | X | | X | |
| **RP44247** | C | 48.5 | 205 | 2 | | X | | | | | X | | | | | X | | X | |
| **DCHPU** | C | 29.4 | 221 | 1.7 | | X | | | | | X | | | | | | | X | X |
| **RP36221** | C | 66.7 | 290 | 2.81 | X | X | | | | | X | | | | | X | X | X | |
| **RP37176** | D | 65 | 304 | 1.86 | X | X | | | | | X | | | X | | | | X | |
| **M610F007** | D | 46.2 | 291 | −0.4 | | X | X | X | X | | X | X | | X | | X | X | X | |
| **RP44160** | E | 65 | 306 | 1.22 | | X | | | | | X | X | | | | X | | X | |
| **RP35606** | E | 79.1 | 348 | 1.96 | X | X | | | | | X | X | | | | X | X | X | |
| **RP32596** | N | 46.7 | 162 | 2.51 | | | X | | X | X | X | | | | | | | | |

N: Not grouped; treated alone; Gray shading indicates functional groups present in iprodione.

Seven distinct structural alerts were flagged by the profiler (Table 26). The knowledge-based DEREK Nexus model predicted neither the parent compound nor the metabolites were mutagenic (inactive for in vitro mutagenicity and no alert for in vivo mutagenicity). However, for some metabolites (RP36112, RP36114, RP36115, RP44247, DCHPU, RP36221, RP44160, RP37176, M610F007, and RP32596) one distinct alert was identified for in vivo chromosomal damage (aromatic amine alert).

SARAH Nexus (v. 3.2.1) did not predict the parent (iprodione), RP30228, RP32490, RP36114, RP36115, RP44247, RP36112, DCHPU, RP36221, RP44160, RP37677, RP36119, RP37176, RP35606, RP25040, RP32596 to be mutagenic. An equivocal prediction for M610F007 was generated for mutagenicity. This result can be overruled by data provided in the software example set, where a close structural analogue (similarity 64%) had negative mutagenicity results.

*Table 26. Summary of structural profiling, DEREK and SARAH nexus results for genotoxicity*

| Grouping | A | A | A | A | B | B | B | C | C | C | C | C | D | D | E | E | Ind |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Profiler (mechanistic/ end-point-specific** | **Iprodion** | **RP32490** | **RP37677** | **RP25040** | **RP30228** | **RP36112** | **RP36119** | **RP36114** | **RP36115** | **RP44247** | **DCHPU** | **RP36221** | **RP37176** | **M610F00** | **RP44160** | **RP35606** | **RP32596** |
| DNA binding by OECD | 1 | 1 | 1 | | 1,2 | 1,2 | 1,2 | 2 | 2 | 2 | 2 | 2 | | | 1,2 | 1,2 | 3 |
| DNA binding by OASIS | | | | | | | | | | | | | | | | | |
| Protein binding by OASIS | | | | | | | | | | | | | | | | | |
| DNA binding by OECD | 4 | 4 | 4 | 4 | 4 | 4 | 4 | | | | | | 4 | 4 | | | |
| DNA alerts for AMES, CA and MNT | | | | | | | | | | | | | | | | | |
| Protein binding alerts for CA | | | 5 | | | | 5 | 5 | | | 5 | | | | | | |
| In vitro mutagenicity | | | | | | | | | | | | | | | | | 6 |
| In vivo mutagenicity | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | | | 7 | 7 | 7 | 7 | 7 | 6 |
| DEREK in vitro mutagenicity | IN | IN | IN | IN | IN | IN | IN | IN | IN | IN | IN | IN | IN | IN | IN | IN | IN |
| DEREK in vivo mutagenicity | | | | | | | | | | | | | | | | | |
| DEREK Chr. damage in vitro | | | | | | | | | | | | | | | | | |
| DEREK Chr. damage in vivo | | | | | | E 8 | | E 8 | E 8 | E 8 | E 8 | E 8 | E 8 | E 8 | E 8 | | E 8 |
| Mutagenicity prediction | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg | E | Neg | Neg | Neg |

Empty cell: No alert; Neg: Negative prediction;
CA: Chromosome aberration; MNT: Micronucleus test; Chr.: Chromosome;

**Alert codes**

1: SN1 – Iminium ion formation; aliphatic tertiary amines
2: SN1 – Nitrenium ion formation – Aromatic phenyl ureas
3: SN1 – Nitrenium ion formation – Primary aromatic amine
4: Acylation – Direct acylation involving a leaving group – acetates
5: AN2 – Michael addition to quinoid structures
6: Primary aromatic amine, hydroxyl amine and its derived esters
7: H-acceptor-path3-H-acceptor
8: Derek alert 952: Aromatic amine
IN Inactive; no classified or unclassified features
E Equivocal

In silico assessment of iprodione metabolites predicted neither iprodione nor any of the 16 metabolites to be mutagenic in vitro, however for some of the metabolites (RP36114, RP36115, RP44247, RP36112, DCHPU, RP36221, RP32596, RP37176, RP44160, RP32596 and M610F007) an equivocal aromatic amine alert for in vivo chromosomal damage was flagged by one of the computational tools that could not be ruled out by QSAR alone. Based on findings from a weight of evidence analysis of structural profiling, experimental data, grouping and read-across, none of the assessed metabolites were predicted to be genotoxic (Piparo, Jackson & Johnson, 2023).

There are no data on the toxicity of these metabolites. They can be considered using a TTC approach in Cramer class III, based on no QSAR indications of mutagenicity.

### (d) Mode of action studies

***Leydig cell tumour mode of action***

A series of in vitro and in vivo studies were undertaken to investigate the mode of action (MOA) for induction of Leydig cell tumours in the rat (Table 27). These studies support the hypothesis that iprodione interferes with testosterone biosynthesis, resulting in decreased testosterone levels and increased luteinizing hormone (LH) secretion, a known MOA for induction of Leydig cell tumours in rats (Cook et al., 1999). However, histopathological changes were also observed in mouse Leydig cells, so it was not possible to reach a conclusion on the human relevance of effects of iprodione on Leydig cells.

***Table 27. Results of mode of action studies for Leydig cell tumours in the rat***

| Study type | Dose | Results | Reference |
|---|---|---|---|
| | | **In vitro** | |
| Receptor-binding study using androgen receptors isolated from untreated rats | NA | Iprodione and its metabolites (RP36119, RP32490, RP36114, RP36115, RP36118, RP25040, RP36112) have low androgen receptor-binding affinity. | Fail, 1994 |
| Testosterone secretion in porcine Leydig cells | 0.03–10 µg/mL | Iprodione and metabolites RP36112 and RP36115 inhibited testosterone secretion following human chorionic gonadotropin (hCG) stimulation.<br>RP25040, RP32490 and RP36118 had no effect.<br>Inhibition by iprodione was reversible following removal from culture medium. | Benahmed, 1995 |
| Testosterone secretion in porcine Leydig cells | 10 µg/mL | Iprodione and its metabolites RP36112 and RP36115 may modulate Leydig cell steroidogenesis by interfering with cholesterol transport and/or activity of certain steroidogenic enzymes. | Benahmed, 1996 |
| | | **In vivo** | |
| Effects on male sexual organs and reproductive hormones in rats | Preliminary study:<br>0, 120, 300 or 600 mg/kg bw per day for 15 days | 15-day study:<br>Decreased relative prostate, seminal vesicle and total accessory sex organ weights at 600 mg/kg bw per day.<br>Increased LH at 600 mg/kg bw per day;<br>Increased FSH at ≥ 300 mg/kg bw per day. | Fail, 1994; Eldridge, 1996 |
| | Main study:<br>0 or 600 mg/kg bw per day for 30 days. | 30-day study:<br>Increased adrenal weights and vacuolization in zona fasciculata;<br>Decreased relative seminal vesicle and total accessory sex organ weights;<br>Increased estradiol; subtle changes in pattern of testosterone and LH secretion; Increased Leydig cell proliferation. | |
| Testosterone secretion in male rats | 0 or 3000 ppm (equal to 175 mg/kg bw per day) for 14 days; testicular sections from treated animals incubated with 0, 1, 10 or 100 µg/mL | Increased relative adrenal and testes weights;<br>decreased relative total accessory sex organ weights;<br>no change in plasma testosterone;<br>increased LH.<br>Dose-related decrease in testosterone secretion from testicular sections treated with iprodione; prior dietary exposure to iprodione had no effect on testosterone secretion with or without hCG. | Fail, 1996a |

| Study type | Dose | Results | Reference |
|---|---|---|---|
| Effects on plasma and intratesticular fluid testosterone levels | 0 or 3000 ppm for 2, 7 or 14 days (equal to 153, 172 and 195 mg/kg bw per day);<br>Animals challenged with hCG 1 h prior to termination. | Increased relative adrenal (2- or 14-day exposure) and testes (7- or 14-day exposure) weights;<br>decreased relative total accessory sex organ weights;<br>no effect on plasma or testicular testosterone concentrations. | Fail, 1996b |
| Effects on plasma testosterone and LH in male rats | 0, 70 or 300 mg/kg bw (single dose); plasma collected 2, 4, 6, 10 and 24 hours post dosing | Decreased testosterone and increased LH at 2 and 4 hours post dosing at 70 mg/kg bw and above | Chuzel, 1998 |
| Effects on plasma testosterone and LH in male rats | 0, 70 or 300 mg/kg bw (single dose); plasma collected 0.5, 1, 2, 4 and 24 hours post dosing | Decreased testosterone from 30 minutes to 4 hours post dosing at ≥ 70mg/kg bw;<br>increased LH from 2–4 hour post dosing, statistically significant at 300 mg/kg bw only | Chuzel, 2001 |
| Effects on plasma testosterone and LH in male rats | 0, 6, 70 or 300 mg/kg bw per day for 14 days; plasma collected 1, 2, 4 and 24 hours after final dose | Decreased testosterone at 300 mg/kg bw per day at 1, 2 and 4 hours post dosing (36%, 37% and 48% compared with controls), and at 70 mg/kg bw per day 2 hours post dosing (44% lower than controls);<br>increased LH at 4 hours post dosing (by 24% and 44% at 70 and 300 mg/kg bw per day, respectively);<br>No effects at 6 mg/kg bw per day | Kennel, 2002a |
| Effects on Leydig cell proliferation in male rats | 0, 6, 70 or 300 mg/kg bw per day for 14 days | Dose-related increases in BrdU labelling index at 70 mg/kg bw per day and above (increased by 36% and 74% at 70 and 300 mg/kg bw per day, respectively);<br>No effect at 6 mg/kg bw per day | Kennel, 2002b |
| Effects on male rat pubertal development | 0, 50, 100 or 200 mg/kg bw per day from PND 23 to PND 51/52 | Increased adrenal weights and decreased seminal vesicle and epididymis weights at 200 mg/kg bw per day;<br>preputial separation delayed at 100 and 200 mg/kg bw per day;<br>Decreased serum testosterone at 50 mg/kg bw per day and above (reduced by 73%, 83% and 89% at 50, 100 and 200 mg/kg bw per day, respectively);<br>No effects on LH;<br>Iprodione exposure in vivo reduced ex vivo testis production of testosterone and progesterone;<br>No evidence of androgen receptor antagonism in vitro at concentrations up to 10 µM iprodione | Blystone et al, 2007 |

| Study type | Dose | Results | Reference |
|---|---|---|---|
| Assessment of anti-androgenic effects in castrated immature male rats | 0, 50, 100 or 200 mg/kg bw per day from PND 49 for 10–11 days; testosterone administered subcutaneously daily; 0, 10, 30, 60 or 100 mg/kg bw per day vinclozolin with or without iprodione (50 mg/kg bw per day) from PND 23 to 55/57 | Adrenal weights increased at 200 mg/kg bw per day; decreased ventral prostate and levator ani/bulbocaverosus muscle (LABC) weights at all doses; Iprodione co-administration increased the delay in preputial separation caused by vinclozolin; greater reduction in glans penis, seminal vesicle, LABC and epididymides weights compared with vinclozolin alone; iprodione antagonized vinclozolin-induced increase in serum testosterone; In vitro studies showed iprodione binds to the human androgen receptor ($IC_{50}$: 86.0 µM), reducing the effects of testosterone on androgen receptor-mediated transcriptional activation | Blystone et al, 2009 |
| Mode of action study on male reproductive system in rats | 0, 2, 6, 24, 140 or 250 mg/kg bw per day for 90 days (dietary administration) | At ≥ 140 mg/kg bw per day and above, increased adrenal weights and cytoplasmic microvacuoles of zona glomerulosa and zona fasciculata, decreased absolute seminal vesicle and prostate weights, and decreased epididymal sperm counts; Decreased sperm percent progressive motility at 250 mg/kg bw per day; increased FSH on day 90 at ≥ 140 mg/kg bw per day; increased number of Leydig cells per testis and Leydig cell volume at ≥ 140 mg/kg bw per day no effect on intratesticular testosterone concentrations; NOAEL: 24 mg/kg bw per day | Robaire, 2017 |

LH: Luteinizing hormone; FSH: Follicle-stimulating hormone; hCG: Human chorionic gonadotropin; BrdU: Bromodeoxyuridine; $IC_{50}$: Half-maximal inhibitory concentration;

***Hepatocellular adenoma/carcinoma mode of action – in vivo studies***

In a non-guideline but GLP-compliant mechanistic study, iprodione (purity 97.1%; Batch no. 9426801) was administered in the diet for 14 days to groups of 15 male CD-1 mice at concentrations of 0, 4000 or 12 000 ppm (equal to 0, 696 or 2138 mg/kg bw per day, respectively). At the end of the treatment period livers were weighed, and clinical biochemistry, hepatic cellular proliferation, liver cytochrome P450 (CYP450) content, isoenzyme profile and histopathology were examined. Additional groups of animals were administered ketoconazole, phenobarbital or cyproterone acetate. Satellite subgroups of 15 mice were included for each treatment and killed after three days of treatment to assess hepatic cell proliferation and liver histopathology.

One mortality was observed on day 7 in the iprodione, high-dose group. Clinical signs observed in this animal between days 4 and 7 included coldness to touch, tremors, prostration, palpebral ptosis and general pallor. No other animals showed treatment-related clinical signs. Increases in total protein, albumin, total cholesterol, AST and ALT were observed in high-dose animals. Dose-related increases in absolute and relative (to body weight) liver weights were observed after 3 and 14 days of iprodione administration, accompanied by liver enlargement and an accentuated lobular pattern after 14 days. Centrilobular or diffuse hypertrophy, increased number of hepatocyte mitoses and centrilobular/midzonal fine vacuolation were observed at 12 000 ppm on days 4 and 15. No microscopic liver changes were observed at 4000 ppm after three days of administration, while an increased incidence of centrilobular hypertrophy and centrilobular/midzonal fine vacuolation were observed after 14 days of treatment. Iprodione treatment for 14 days induced a dose-related increase in hepatocellular microsome total CYP450, CYP2B and CYP3A content, but CYP1A1 or CYP2E1 were not affected. Dose-related increases in pentoxyresorufin-*O*-deethylase (PROD) and benzoxyresorufin-*O*-dealkylase (BROD) activities were observed, with smaller increases in ethoxyresorufin-*O*-deethylase (EROD) activity

and lauric acid hydroxylation. A dose-related increase in hepatocellular proliferation as measured by proliferating cell nuclear antigen (PCNA) immunohistochemistry was observed after 3 or 14 days of iprodione treatment. The profile of liver changes observed after phenobarbital treatment were similar to those observed following treatment with iprodione (Bigot, 1996).

The data suggest that iprodione produces liver tumours by a phenobarbital-like MOA, but this was not reliably established. No alternative MOA has been investigated and no investigations in human cells were reported. There are no data to demonstrate that the liver tumours produced by iprodione are not relevant to humans.

## 3. Microbiological aspects

There was no information available in the public domain and no experimental data were submitted, that addressed the possible impact of iprodione residues on the human intestinal microbiome.

## 4. Observations in humans

No data on observations in humans were available for the previous JMPR evaluations or the present assessment. The sponsor stated that with respect to uses registered by their company, there were no reports of adverse effects in humans during manufacturing or application of iprodione to crops.

# Comments

## Biochemical aspects

After oral administration to CD rats of a single radiolabelled dose of iprodione by gavage at 50 or 900 mg/kg bw and repeated administration of 50 mg/kg bw of unlabelled iprodione for 14 days followed by one radiolabelled dose, [$^{14}$C]iprodione was rapidly and nearly completely absorbed. More than 70% of the label was excreted within 48 hours. Urinary excretion of iprodione was 53%, 43% and 46% of the administered dose (AD) at 50 mg/kg bw, 900 mg/kg bw and after repeated exposure, respectively. Faecal excretion of iprodione was 39%, 56% and 52% of AD at 50 mg/kg bw, 900 mg/kg bw and after repeated exposure, respectively. At 168 hours after oral administration of [$^{14}$C]iprodione, tissue concentrations (including carcass) of radioactivity were about 0.2% of AD. Concentrations greater than 0.1 µg equiv./g were found in liver, stomach, small and large intestine, pancreas, kidney, uterus, salivary gland, adrenals, lymph nodes, ovary, fat, and blood. The peak concentration of radioactivity in blood was observed four hours (males) and two hours (females) after administration of 50 mg/kg bw, and about six hours (males and females) after administration of 900 mg/kg bw. Blood levels did not increase proportionately with dose. The half-life of elimination of radiolabelled iprodione from blood was 8.9 ± 1.5 hours for males and 6.9 ± 1.7 hours for females, both given a single oral dose at 50 mg/kg bw. At the higher dose (900 mg/kg bw), the half-life of elimination from blood was 19.8 ± 3.8 hours for males and 12.5 ± 3.0 hours for females (Hallifax, 1989).

About 1.5% to 4.5% unmetabolized iprodione was excreted in the urine. The excretion of intact iprodione was dose-dependent, ranging from 34% to 80% of the radioactivity found in faeces in 24 hours in males, and 31% to 85% in females during the same period. The major metabolites in the urine were found to be compounds RP32490 and RP36114 in males, and RP32490 and parent compound in females (Hallifax, 1989). The main routes of metabolism were hydroxylation of the aromatic ring, degradation of the isopropylcarbamoyl chain and rearrangement followed by cleavage of the hydantoin moiety (D'Souza, 1993). No significant difference in absorption or metabolism were seen according to sex.

## Toxicological data

The acute oral median lethal dose ($LD_{50}$) of iprodione was greater than 2000 mg/kg bw and the dermal $LD_{50}$ was greater than 2000 mg/kg bw. The inhalation median lethal concentration ($LC_{50}$) was greater than 3.29 mg/L (Coombs & Clarke, 1977; Cummins, 1989; Plutnick, 1987). Iprodione was not irritating

to skin but mildly irritating to the eyes in rabbits, and was not skin sensitizing in the Guinea pig Buehler protocol (Bonnette, 1991a, b; Trimmer, 1987).

In repeat-dose toxicity studies on mice, rats and dogs, the main effects were on body weight, adrenal glands, erythrocytes, spleen, ovaries, uterus and testes.

In a one-year dietary toxicity study in dogs, iprodione was administered at concentrations of 0, 100, 600 or 3600 ppm (equal to 0, 4.1, 24.9 and 145 mg/kg bw per day for males, 0, 4.3, 28.3 and 153 mg/kg bw per day for females). The on-observed-adverse-effect level (NOAEL) was 600 ppm (equal to 24.9 mg/kg bw per day) based on increased incidence of Heinz bodies, and histopathological changes in adrenals and kidneys at 3600 ppm (equal to 145 mg/kg bw per day) (Broadmeadow, 1984; Broadmeadow & Duffen, 1985; Brown & Broadmeadow, 1986).

In a second one-year dog study, iprodione was administered in the diet for 52 weeks to dogs at concentrations of 0, 200, 300, 400 or 600 ppm (equal to 0, 7.8, 12.4, 17.5 and 24.6 mg/kg bw per day for males, 0, 9.1, 13.1, 18.4 and 26.4 mg/kg bw per day for females). The NOAEL was 600 ppm, (equal to 24.6 mg/kg bw per day), the highest dose tested (Kangas, 1995).

The overall NOAEL for one-year dog studies was 600 ppm (equal to 24.9 mg/kg bw per day) based on effects at 3600 ppm (equal to 145 mg/kg bw per day).

In an 18-month combined chronic toxicity and carcinogenicity study in mice, iprodione was administered in the diet at concentrations of 0, 200, 500 or 1250 ppm (equal to 0, 27.5, 67.6 and 171 mg/kg bw per day for males, 0, 31.2, 78.2 and 193 mg/kg bw day for females). The NOAEL for toxicity and carcinogenicity was 1250 ppm (equal to 171 mg/kg bw per day), the highest dose tested (Hastings, 1987a, b).

In a 99-week combined chronic toxicity and carcinogenicity study in mice, iprodione was administered in the diet at concentrations of 0, 160, 800 or 4000 ppm (equal to 0, 23, 115 and 604 mg/kg bw per day for males, 0, 27, 138 and 793 mg/kg bw per day for females). The NOAEL for toxicity was 160 ppm (equal to 23 mg/kg bw per day), based on microscopic changes in the testes (generalized vacuolation and hypertrophy of the testicular interstitial cells, Leydig cells) and ovaries (interstitial cell hyperplasia with luteinization) at 800 ppm (equal to 115 mg/kg bw per day). The NOAEL for carcinogenicity was 800 ppm (equal to 115 mg/kg bw per day) based on an increased incidence of hepatocellular carcinomas and adenomas in both sexes at 4000 ppm (equal to 604 mg/kg bw per day) (Chambers, 1993, 1995; Hilderbrandt, 2001).

In a combined chronic toxicity and carcinogenicity study in rats, iprodione was administered for 104 weeks in the diet at concentrations of 0, 150, 300 or 1600 ppm (equal to 0, 6.1, 12.4 and 69 mg/kg bw per day for males, 0, 8.4, 16.5 and 95 mg/kg bw per day for females). The NOAEL for toxicity was 150 ppm (equal to 6.1 mg/kg bw per day), based upon histopathological changes in the adrenal cortex, testicular interstitial cell hyperplasia and reduced secretion and absent/empty secretory colloid of the seminal vesicles in males, and increased haemosiderosis in the spleen in females, all at 300 ppm (equal to 12.4 mg/kg bw per day). The NOAEL for carcinogenicity was 300 ppm (equal to 12.4 mg/kg bw per day) based upon an increased incidence of Leydig cell adenomas in males at 1600 ppm (equal to 69.0 mg/kg bw per day) (Ames, 1996; Ames & Gopinath 1993; Chambers, 1992; Gopinath, 1998; Scarfe & Chambers, 1999).

The Meeting concluded that iprodione is carcinogenic in mice and male rats, but not in female rats.

A series of in vitro and in vivo studies was undertaken to investigate the mode of action (MOA) for induction of Leydig cell tumours in the rat. These studies supported the hypothesis that iprodione interferes with testosterone biosynthesis, resulting in decreased testosterone levels and increased luteinizing hormone (LH) secretion, a known MOA for induction of Leydig cell tumours in rats. However, histopathological changes were also observed in mouse Leydig cells, so it was not possible to reach a conclusion on the human relevance of the effects on Leydig cells.

In a study investigating the MOA for induction of hepatocellular adenomas and carcinomas in mice, induction of CYP2B and CYP3A enzymes and hepatocellular proliferation were observed. The profile of liver changes observed after iprodione treatment was similar to that observed following treatment with phenobarbital, suggesting possible activation of the CAR/PXR receptor. The data suggest that iprodione may induce liver tumours by a phenobarbital-like MOA, but this was not reliably established.

Iprodione was tested for genotoxicity in an adequate range of in vitro and in vivo assays and gave negative results in all relevant studies (Felkner, 1985; Godek, 1985; Lawlor & Valentine, 1990; Proudlock & Elmore, 1994; San Sebastien, 1985a, b).

The Meeting concluded that iprodione is unlikely to be genotoxic.

As iprodione is unlikely to be genotoxic and there is a clear threshold for liver tumours in mice and Leydig cell tumours in male rats, the Meeting concluded that iprodione is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation reproductive toxicity study in rats, iprodione was administered at dietary concentrations of 0, 300, 1000 or 3000 ppm. The high dose for F1 animals was reduced to 2000 ppm at the time of first mating of F1a rats because of excessive toxicity in the F1 generation. The mean administered doses, calculated during premating phases, were equal to 0, 17, 55 and 159 mg/kg bw per day for F0 males, and for F0 females 0, 21, 71 or 214 mg/kg bw per day. The NOAEL for parental toxicity was 300 ppm (equal to 17 mg/kg bw per day) based on reduced body weight and body weight gain, and reduced food consumption at 1000 ppm (equal to 55 mg/kg bw per day). The NOAEL for reproductive toxicity was 3000 ppm (equal to 159 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 1000 ppm (equal to 55 mg/kg bw per day) based on clinical signs, reduced offspring viability and reduced pup weight at 3000 ppm (equal to 159 mg/kg bw per day) (Henwood, 1991).

In a second two-generation reproductive toxicity study in rats, iprodione was administered in the diet at concentrations of 0, 300, 750 or 1500 ppm (equal to 0, 27, 67 and 136 mg/kg bw per day for males, 0, 24, 59 and 116 mg/kg bw per day for females). The NOAEL for parental toxicity was 750 ppm (equal to 59 mg/kg bw per day) based on decreased body weight and food consumption, increased absolute and relative adrenal weights and an increased incidence of adrenal vacuolation at 1500 ppm (equal to 116 mg/kg bw per day). The NOAEL for reproductive toxicity was 1500 ppm (equal to 116 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 300 ppm (equal to 24 mg/kg bw per day), based on the delayed onset of male puberty at 750 ppm (equal to 59 mg/kg bw per day) (Schneider, 2014).

In a developmental toxicity study in rats, iprodione was administered by gavage from gestation day (GD) 5 to GD 15 at doses of 0, 40, 90 or 200 mg/kg bw per day. The NOAELs for maternal toxicity and embryo/fetal toxicity were 200 mg/kg bw per day, the highest dose tested (Tesh, 1986b, 1987).

In a study aiming to determine effects of iprodione on pregnancy and sex differentiation, it was administered to rats from GD 6 to GD 19 by gavage at doses of 0, 20, 120 or 250 mg/kg bw per day. The NOAEL for maternal toxicity was 20 mg/kg bw per day based on decreased body weight gain, food intake, food efficiency and enlarged adrenals, at 120 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 120 mg/kg bw per day, based on reduced fetal body weight at 250 mg/kg bw per day.

In a developmental toxicity study in rabbits, iprodione was administered by gavage from GD 6 to GD 18 at doses of 0, 20, 60 or 200 mg/kg bw per day. The NOAEL for maternal toxicity was 20 mg/kg bw per day based on decreased body weight gain at 60 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 60 mg/kg bw per day based on increased abortions and post-implantation losses at 200 mg/kg bw per day. Animals in the high-dose group experienced body weight loss and reduced food consumption during the first six days of treatment and this persisted throughout the study, which may be indicative of an acute adverse effect. A NOAEL for acute maternal toxicity of 60 mg/kg bw was identified, based on body weight loss between GD 6 and GD 12 at 200 mg/kg bw per day (Rodwell, 1985).

The Meeting concluded that iprodione is not teratogenic.

No specific data were submitted regarding neurotoxicity, but no evidence of neurotoxicity was reported in routine toxicological studies with iprodione.

The Meeting concluded that iprodione is unlikely to be neurotoxic.

In an immunotoxicity study in female rats, iprodione was administered at dietary concentrations of 0, 500, 1200 or 3000 ppm (equal to 0, 39, 95 and 225 mg/kg bw per day). The NOAEL for systemic toxicity was 1200 ppm (equal to 95 mg/kg bw per day), based on reduced body weight and body weight gain

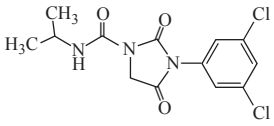
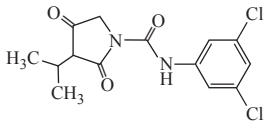
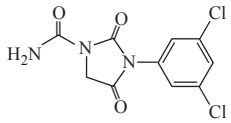
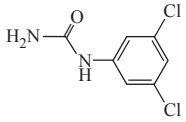
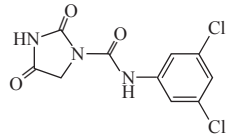
at 3000 ppm (equal to 225 mg/kg bw per day). The NOAEL for functional immunotoxicity (T-helper cell-mediated antibody production after immunization with sheep red blood cells) was 3000 ppm (equal to 225 mg/kg bw per day), the highest dose tested (Odin-Feurtet, 1010).

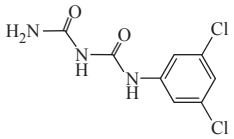
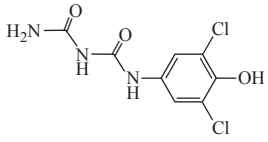
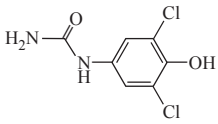
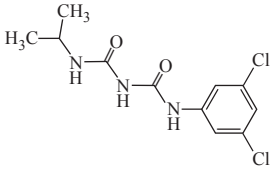
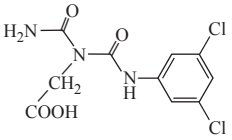
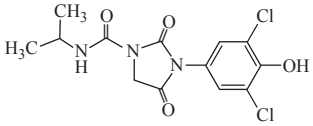
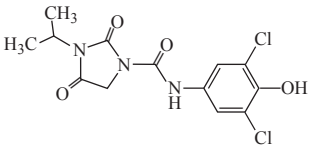
The Meeting concluded that iprodione is not immunotoxic.

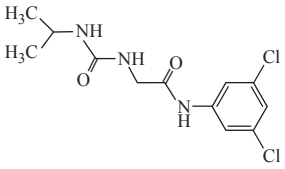
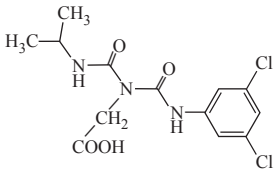
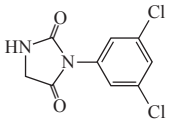
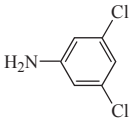
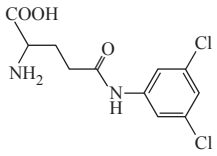
## Toxicological data on metabolites and/or degradates

A toxicological evaluation was performed on metabolites and Table 28 below shows an overall summary of the toxicological characterization of those metabolites requested for consideration in the residue definition.

***Table 28. Summary of toxicological characterization of plant/livestock metabolites***

| **Compound, codes and structure** | **Major rat metabolite** (> 10% AD) | **Genotoxicity assessment** (data, QSAR, read-across) | **General toxicity** | **Toxicological reference values** |
|---|---|---|---|---|
| **Iprodione**<br>RP26019<br> | Parent | Not genotoxic (data) | Full data set | ADI:<br>0–0.06 mg/kg bw<br>ARfD:<br>0.6 mg/kg bw |
| **RP30228**<br>*N*-(3,5-Dichlorophenyl)-3-isopropyl-2,4-dioxoimidazolidine-1-carboxamide<br> | No | Not genotoxic (QSAR; RA) | $LD_{50}$:<br>greater than 2000 mg/kg bw | |
| **RP32490**<br>3-(3,5-Dichlorophenyl)-2,4-dioxoimidazolidine-1-carboxamide<br> | Yes<br>(15%<br>in urine) | Not genotoxic as covered by parent | Covered by parent | Parent ADI and ARfD |
| **RP44247**<br>Amino-*N*-(3,5-dichlorophenyl)amide<br> | No | Not genotoxic (QSAR; RA) | No information | TTC Cramer class III value:<br>1.5 µg/kg bw per day |
| **RP36112**<br>*N*-(3,5-dichlorophenyl)-2,4-dioxoimidazolidine-1-carboxamide<br> | No | Not genotoxic (QSAR; RA) | No information | TTC Cramer class III value:<br>1.5 µg/kg bw per day |

| Compound, codes and structure | Major rat metabolite (> 10% AD) | Genotoxicity assessment (data, QSAR, read-across) | General toxicity | Toxicological reference values |
|---|---|---|---|---|
| **RP36115**<br>Amino-*N*-[*N*-(3,5-dichlorophenyl) carbamoyl]amide<br> | Yes (>15% in urine) | Not genotoxic as covered by parent | Covered by parent | Parent ADI and ARfD |
| **RP336114**<br>*N*-(3,5-Dichloro-4-hydroxyphenyl)-2-carbamoylacetamide<br> | Yes\| (> 15% in urine) | Not genotoxic as covered by parent | Covered by parent | Parent ADI and ARfD |
| **DCHPU**<br>Amino-*N*-(3,5-dichloro-4-hydroxyphenyl)amide<br> | No | Not genotoxic (QSAR; RA) | No information | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **RP36221**<br>1-(3,5-Dichlorophenyl)-5-isopropyl biuret<br> | No | Not genotoxic (QSAR; RA) | No information | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **RP44160**<br> | No | Not genotoxic (QSAR; RA) | No information | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **RP37677**<br> | No | Not genotoxic (QSAR; RA) | No information | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **RP36119**<br> | No | Not genotoxic (QSAR; RA) | No information | TTC Cramer class III value: 1.5 µg/kg bw per day |

| Compound, codes and structure | Major rat metabolite (> 10% AD) | Genotoxicity assessment (data, QSAR, read-across) | General toxicity | Toxicological reference values |
|---|---|---|---|---|
| **RP37176**<br>*N*-(3,5-Dichlorophenyl)-2-[(isopropylcarbamoyl)amino] acetamide<br> | No | Not genotoxic (QSAR; RA) | No information | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **RP35606**<br>*N*-(3,5-Dichlorophenylcarbamoyl) -*N*-isopropylcarbamoyl-glycine<br> | No | Not genotoxic (QSAR; RA) | No information | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **RP25040**<br>3-(3,5-Dichlorophenyl)-imidazolidine-2,4-dione<br> | No | Not genotoxic (QSAR; RA) | No information | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **RP32596**<br>3,5-Dichloroaniline<br> | No | Not genotoxic (Ames test; QSAR; RA) | No information | TTC Cramer class III value: 1.5 µg/kg bw per day |
| ***N*-(3,5-Dichlorophenyl)-*L*-glutamine**<br> | No | Not genotoxic (QSAR; RA) | No information | TTC Cramer class III value: 1.5 µg/kg bw per day |

RA: Read-across; TTC: Threshold of toxicological concern; QSAR: Quantitative structure–activity relationship analysis; ADI: Acceptable daily intake; ARfD: Acute reference dose

## Microbiological aspects

There was no information available in the public domain and no experimental data were submitted that addressed the possible impact of iprodione residues on the human intestinal microbiome.

## Human data

No information was provided on the health of workers involved in the manufacture or use of iprodione. No information on accidental or incidental poisoning in humans was available.

The Meeting concluded that the existing database on iprodione was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

# Toxicological evaluation

The Meeting re-affirmed the acceptable daily intake (ADI) for iprodione of 0–0.06 mg/kg bw on the basis of a NOAEL of 150 ppm (equal to 6.1 mg/kg bw per day) in the two-year chronic toxicity and carcinogenicity study in rats, and using a safety factor of 100. This gives a margin of 10 000 to the lowest dose causing tumours in mice and 1150 for the corresponding margin in rats.

This ADI also applies to metabolites RP32490, RP36114 and RP36115.

The Meeting established an acute reference dose (ARfD) for the general population of 0.6 mg/kg bw for iprodione, on the basis of a NOAEL of 60 mg/kg bw, based upon body weight loss and reduced food consumption between gestation day 6 and gestation day 12, at 200 mg/kg bw per day in the developmental toxicity study in rabbits, and using a safety factor of 100.

This ARfD also applies to metabolites RP32490, RP36114 and RP36115.

***Levels relevant to risk assessment of iprodione***

| Species | Study | Effect | NOAEL | LOAEL |
|---|---|---|---|---|
| Mouse | 78-week study of toxicity and carcinogenicity [a] | Toxicity | 1250 ppm, equal to 171 mg/kg bw per day [c] | - |
| | | Carcinogenicity | 1250 ppm, equal to 171 mg/kg bw per day [c] | - |
| | 99-week study of toxicity and carcinogenicity [a] | Toxicity | 160 ppm, equal to 23 mg/kg bw per day | 800 ppm, equal to 115 mg/kg bw per day |
| | | Carcinogenicity | 800 ppm, equal to 115 mg/kg bw per day | 4000 ppm, equal to 604 mg/kg bw per day |
| Rat | Two-year studies of toxicity and carcinogenicity [a, d] | Toxicity | 150 ppm, equal to 6.1 mg/kg bw per day | 300 ppm, equal to 12.4 mg/kg bw per day |
| | | Carcinogenicity | 300 ppm, equal to 12.4 mg/kg bw per day | 1600 ppm, equal to 69.0 mg/kg bw per day |
| | Two-generation study of reproductive toxicity [a] | Reproductive toxicity | 3000 ppm, equal to 159 mg/kg bw per day [c] | - |
| | | Parental toxicity | 300 ppm, equal to 17 mg/kg bw per day | 1000 ppm, equal to 55 mg/kg bw per day |
| | | Offspring toxicity | 1000 ppm, equal to 55 mg/kg bw per day | 3000 ppm, equal to 159 mg/kg bw per day |
| | Two-generation study of reproductive toxicity [a] | Reproductive toxicity | 1500 ppm, equal to 116 mg/kg bw per day [c] | - |
| | | Parental toxicity | 750 ppm, equal to 59 mg/kg bw per day | 1500 ppm, equal to 116 mg/kg bw per day |
| | | Offspring toxicity | 300 ppm, equal to 24 mg/kg bw per day | 750 ppm, equal to 59 mg/kg bw per day |
| | Developmental toxicity study [b] | Maternal toxicity | 20 mg/kg bw per day | 120 mg/kg bw per day |
| | | Embryo/fetal toxicity | 120 mg/kg bw per day | 250 mg/kg bw per day |

| Species | Study | Effect | NOAEL | LOAEL |
|---|---|---|---|---|
| Rabbit | Developmental toxicity study [b] | Maternal toxicity | 20 mg/kg bw per day | 60 mg/kg bw per day |
| | Developmental toxicity study [b] | Acute maternal toxicity | 60 mg/kg bw per day | 200 mg/kg bw per day |
| | One-year studies of toxicity [a, d] | Embryo/fetal toxicity | 60 mg/kg bw per day | 200 mg/kg bw per day |
| | | Toxicity | 600 ppm, equal to 24.9 mg/kg bw per day | 3600 ppm, equal to 145 mg/kg bw per day |
| Dog | One-year studies of toxicity [a, d] | Toxicity | 600 ppm, equal to 24.9 mg/kg bw per day | 3600 ppm, equal to 145 mg/kg bw per day |

[a] Dietary administration; [b] Gavage administration; [c] Highest dose tested [d] Two or more studies combined; [e] Lowest dose tested

*Acceptable daily intake (ADI) applies to iprodione, RP32490, RP36114 and RP36115, expressed as iprodione.*

0–0.06 mg/kg bw

*Acute reference dose (ARfD) applies to iprodione, RP32490, RP36114 and RP36115, expressed as iprodione.*

0.6 mg/kg bw

## Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure.

***Critical end-points for setting guidance values for exposure to iprodione***

| | |
|---|---|
| ***Absorption, distribution, excretion and metabolism in mammals*** | |
| Rate and extent of oral absorption | Rapidly and almost completely absorbed |
| Dermal absorption | No data |
| Distribution | Distributed rapidly and eliminated quickly from the tissues Highest levels were found in liver, stomach, small and large intestine, pancreas and kidney |
| Potential for accumulation | No evidence of accumulation |
| Rate and extent of excretion | Rapid and nearly complete; 82% in urine and faeces within 48 hours |
| Metabolism in animals | Extensive metabolism in rats, primary route of metabolism was hydroxylation of the aromatic ring, degradation of the isopropylcarbamoyl chain and rearrangement followed by cleavage of the hydantoin moiety |
| Toxicologically significant compounds in animals and plants | Iprodione (parent), RP32490, RP36114 and RP36115 |
| ***Acute toxicity*** | |
| Rat, $LD_{50}$, oral | > 2000 mg/kg bw |
| Rat, $LD_{50}$, dermal | > 2000 mg/kg bw |
| Rat, $LC_{50}$, inhalation | > 3.29 mg/L |
| Rabbit, dermal irritation | Not irritating |
| Rabbit, ocular irritation | Mildly irritating |
| Mouse, dermal sensitization | No data |
| Guinea pig, dermal sensitization | Not sensitizing (Buehler) |

| | |
|---|---|
| ***Short-term studies of toxicity*** | |
| Target/critical effect | Increased incidence of Heinz bodies; histopathological changes in adrenals and kidneys (dog) |
| Lowest relevant oral NOAEL | 24.9 mg/kg bw per day (dog) |
| Lowest relevant dermal NOAEL | 1000 mg/kg bw per day, highest dose tested (rabbit) |
| Lowest relevant inhalation NOAEC | 0.217 mg/L (rat) |
| ***Long-term studies of toxicity and carcinogenicity*** | |
| Target/critical effect | Histopathological changes in adrenal cortex, testes, seminal vesicles and spleen (rat) |
| Lowest relevant NOAEL | 6.1 mg/kg bw per day (rat) |
| Carcinogenicity | Carcinogenic in mice and male rats [a] |
| ***Genotoxicity*** | Unlikely to be genotoxic |
| ***Reproductive toxicity*** | |
| Target/critical effect | Reduced body weight, body weight gain and food consumption (parental); delayed onset of male puberty |
| Lowest relevant parental NOAEL | 17 mg/kg bw per day (rat) |
| Lowest relevant offspring NOAEL | 24 mg/kg bw per day (rat) |
| Lowest relevant reproductive NOAEL | 116 mg/kg bw per day, highest dose tested (rat) |
| ***Developmental toxicity*** | |
| Target/critical effect | Decreased body weight gain (rat and rabbit maternal); Enlarged adrenals (rat maternal) Decreased fetal weight (rat offspring) |
| Lowest relevant maternal NOAEL | 20 mg/kg bw per day (rat, rabbit) |
| Lowest relevant embryo/fetal NOAEL | 60 mg/kg bw per day (rabbit) |
| ***Neurotoxicity*** | |
| Acute neurotoxicity NOAEL | No evidence from routine studies |
| Subchronic neurotoxicity NOAEL | No evidence from routine studies |
| Developmental neurotoxicity NOAEL | No data |
| ***Other toxicological studies*** | |
| Immunotoxicity NOAEL | 225 mg/kg bw per day, highest dose tested (rat) |
| ***Studies on toxicologically relevant metabolites*** | No data submitted |
| ***Microbiological data*** | No data submitted |
| ***Human data*** | No data submitted |

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

## Summary

| | Value | Study | Safety factor |
|---|---|---|---|
| ADI | 0–0.06 mg/kg bw [a] | Two-year study of toxicity and carcinogenicity (rat) | 100 |
| ARfD | 0.6 mg/kg bw [a] | Developmental toxicity study (rabbit) | 100 |

[a] Applies to iprodione, RP32490, RP36114 and RP36115, expressed as iprodione.

## References

All unpublished references below were supplied to WHO by FMC Corporation, Philadelphia, PA 19104, United States of America.


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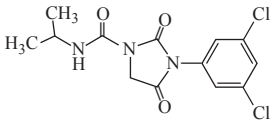
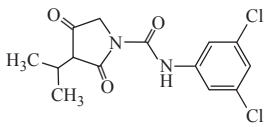
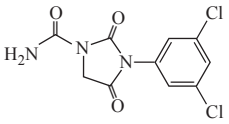
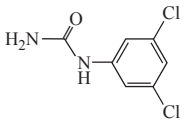
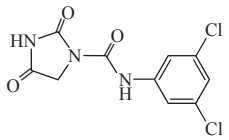
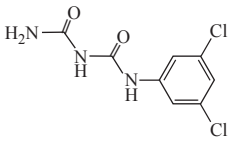
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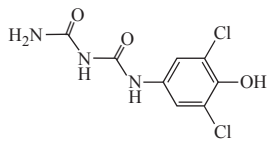
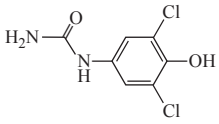
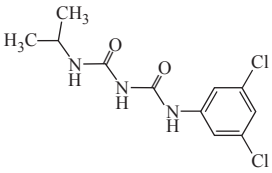
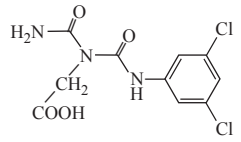
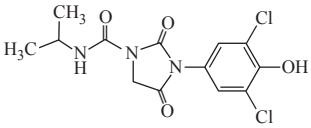
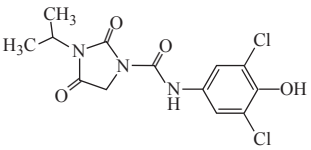
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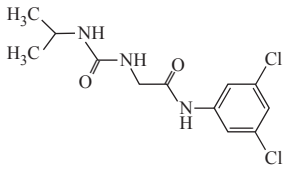
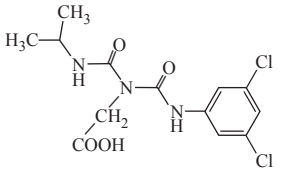
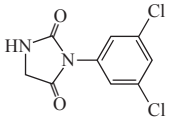
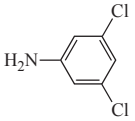
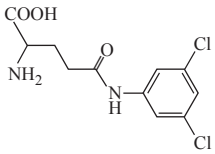
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# Annex 1
# Detailed data on metabolites of iprodione

*Table A1. Data for metabolites of iprodione relevant to this evaluation*

| Name/ abbreviation or code | Chemical structure and SMILES code | Chemical name, molecular formula and molecular weight | Found in/comments |
|---|---|---|---|
| | **Parent compound** | | |
| **Iprodione**<br>RP26019<br>Code: 101169 | <br>SMILES code:<br>O=C2N(c1cc(Cl)cc(Cl)c1)C(=O)CN2C(=O)NC(C)C | 3-(3,5-Dichlorophenyl)-*N*-isopropyl-2,4-dioxoimidazolidine-1-carboxamide<br>Molecular formula: $C_{13}H_{13}Cl_2N_3O_3$<br>Molecular weight: 330.17 g/mol | Primary and rotational crops<br>Poultry, and ruminant tissues |
| | **Metabolites** | | |
| **RP30228**<br>M610F001<br>Code: 5079647<br>Iprodione isomer | <br>SMILES code:<br>O=C2CN(C(=O)Nc1cc(Cl)cc(Cl)c1)C(=O)N2C(C)C | *N*-(3,5-Dichlorophenyl)-3-isopropyl-2,4-dioxoimidazolidine-1-carboxamide<br>Molecular formula: $C_{13}H_{13}Cl_2N_3O_3$<br>Molecular weight: 330.17 g/mol | Primary and rotational crops<br>Minor in several poultry and ruminant tissues |
| **RP32490**<br>Code: 5079628 | <br>SMILES code:<br>O=C2CN(C(=O)N)C(=O)N2c1cc(Cl)cc(Cl)c1 | 3-(3,5-Dichlorophenyl)-2,4-dioxoimidazolidine-1-carboxamide<br>Molecular formula: $C_{10}H_7Cl_2N_3O_3$<br>Molecular weight:288.09 g/mol | Primary crops<br>Major in all poultry and ruminant tissues. |
| **RP44247**<br>Unknown Y<br>Code: 89517 | <br>SMILES code:<br>NC(=O)Nc1cc(Cl)cc(Cl)c1 | amino-*N*-(3,5-dichlorophenyl) amide<br>Molecular formula: $C_7H_6Cl_2N_2O$<br>Molecular weight: 205.04 g/mol | Rotational crops<br>Hen liver, kidney and eggs |
| **RP36112**<br>Code: 5079623 | <br>SMILES code:<br>O=C2NC(=O)CN2C(=O)Nc1cc(Cl)cc(Cl)c1 | *N*-(3,5-Dichlorophenyl)-2,4-dioxoimidazolidine-1-carboxamide<br>Molecular formula: $C_{10}H_7Cl_2N_3O_3$<br>Molecular weight: 288.09 g/mol | Found in hen eggs, goat and hen liver and kidney |
| **RP36115**<br>Code: 5079624 | <br>SMILES code:<br>NC(=O)NC(=O)Nc1cc(Cl)cc(Cl)c1 | Amino-*N*-[*N*-(3,5-dichlorophenyl)carbamoyl]amide<br>Molecular formula: $C_8H_7Cl_2N_3O_2$<br>Molecular weight: 248.07 g/mol | Found in hen eggs, goat and hen liver and kidney |

| Name/ abbreviation or code | Chemical structure and SMILES code | Chemical name, molecular formula and molecular weight | Found in/comments |
|---|---|---|---|
| **RP36114**<br>Code: 5079627 | <br>SMILES code: NC(=O)NC(=O)Nc1cc(Cl)c(O)c(Cl)c1 | *N*-(3,5-Dichloro-4-hydroxyphenyl)-2-carbamoylacetamide<br>Molecular formula: $C_8H_7Cl_2N_3O_3$<br>Molecular weight: 264.07 g/mol | Found in cow milk and in goat and hen kidney and liver |
| **DCHPU**<br>Unknown X<br>Code: 5932706 | <br>SMILES code: NC(=O)Nc1cc(Cl)c(O)c(Cl)c1 | Amino-*N*-(3,5-dichloro-4-hydroxyphenyl)amide<br>Molecular formula: $C_7H_6Cl_2N_2O_2$<br>Molecular weight: 221.04 g/mol | Found in goat kidney |
| **RP36221**<br>M610F002<br>Code: 5079618 | <br>SMILES code: Clc1cc(NC(=O)NC(=O)NC(C)C)cc(Cl)c1 | 1-(3,5-Dichlorophenyl)-5-isopropyl biuret<br>Molecular formula: $C_{11}H_{13}Cl_2N_3O_2$<br>Molecular weight: 290.15 g/mol | Found in goat and hen liver and kidney |
| **RP44160**<br>Code: – | <br>SMILES code: NC(=O)N(CC(=O)O)C(=O)Nc1cc(Cl)cc(Cl)c1 | 2-[Carbamoyl-[(3,5-dichlorophenyl) carbamoyl]-amino]acetic acid<br>Molecular formula: $C_{10}H_9Cl_2N_3O_4$<br>Molecular weight: 306.1 g/mol | Found in goat liver and kidney |
| **RP37677**<br>Code: – | <br>SMILES code: CC(C)NC(=O)N1CC(=O)N(C1=O)c2cc(Cl)c(O)c(Cl)c2 | 3-(3,5-Dichloro-4-hydroxy-phenyl)-*N*-isopropyl-2,4-dioxo-imidazolidine-1-carboxamide<br>Molecular formula: $C_{13}H_{13}Cl_2N_3O_4$<br>Molecular weight: 346.17 g/mol | Found in goat and hen liver and kidney |
| **RP36119**<br>Code: 5079629 | <br>SMILES code: CC(C)N1C(=O)CN(C(=O)Nc2cc(Cl)c(O)c(Cl)c2)C1=O | *N*-(3,5-Dichloro-4-hydroxy-phenyl)-3-isopropyl-2,4-dioxo-imidazolidine-1-carboxamide<br>Molecular formula: $C_{13}H_{13}Cl_2N_3O_4$<br>Molecular weight: 346.17 g/mol | Found in goat liver and kidney |

| Name/ abbreviation or code | Chemical structure and SMILES code | Chemical name, molecular formula and molecular weight | Found in/comments |
|---|---|---|---|
| **RP37176**<br>Code: 5079612 | <br>SMILES code: CC(C)NC(=O)NCC(=O)Nc1cc(Cl)cc(Cl)c1 | *N*-(3,5-dichlorophenyl)-2-[(isopropylcarbamoyl)amino] acetamide<br>Molecular formula: $C_{12}H_{15}Cl_2N_3O_2$<br>Molecular weight: 304.18 g/mol | Found in processed commodities (hydrolysis) and in soil. |
| **RP35606**<br>Code: 5079626 | <br>SMILES code: – | *N*-(3,5-dichlorophenylcarbamoyl)-*N*-isopropylcarbamoyl-glycine<br>Molecular formula: $C_{13}H_{15}Cl_2N_3O_4$<br>Molecular weight: 348.2 g/mol | Found after hydrolysis as a function of pH. Found in soil.<br>Intermediate product (ring opening) in the formation of RP30228. |
| **RP25040**<br>M610F004<br>Code: 207099 | <br>SMILES code: Clc1cc(Cl)cc(c1)N2C(=O)CNC2=O | 3-(3,5-Dichlorophenyl)-imidazolidine-2,4-dione<br>Molecular formula: $C_9H_6Cl_2N_2O_2$<br>Molecular weight: 245.1 g/mol | Rotational crops and photolytic (soil) degradation |
| **RP32596**<br>3,5-DCA<br>M610F012<br>Code: 85831 | <br>SMILES code: Nc1cc(Cl)cc(Cl)c1 | 3,5-dichloroaniline<br>Molecular formula: $C_6H_5Cl_2N$<br>Molecular weight: 162.02 g/mol | Not observed in unbound form in plants.<br>Found after processing (hydrolysis) and in photolysis studies.<br>Found in goat liver and kidney. |
| M610F007<br>Code: 5916256 (L-Form) | <br>SMILES code: – | *N*-(3,5-dichlorophenyl)-*L*-glutamine<br>Molecular formula: $C_{11}H_{12}Cl_2N_2O_3$<br>Molecular weight: 291.13 g/mol | L-glutamine complex with 3,5-DCA; Found in carrot metabolism study, but this study was not submitted. |

# Isocycloseram

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## Explanation

Isocycloseram is the International Organization for Standardization (ISO)-approved common name for 4-[5-(3,5-dichloro-4-fluorophenyl)-5-(trifluoromethyl)-4,5-dihydro-1,2-oxazol-3-yl]-*N*-(2-ethyl-3-oxo-1,2-oxazolidin-4-yl)-2-methylbenzamide (IUPAC), with the Chemical Abstracts Service number 2061933-85-3. The substance is a new insecticide and miticide belonging to the chemical group of isoxazolines. It binds the GABA receptor, blocking inhibitory neurotransmission results in hyperexcitation and finally death of target insects. It is intended for control of a variety of pests in agricultural and ornamental crops but may also play a role in vector control, for example to prevent transmission of malaria. However, suitability of this compound for the latter purpose is beyond the scope of this evaluation.

Isocycloseram has not previously been evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed by the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR). All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with current test guidelines, unless otherwise specified. Public scientific literature that might complement the toxicological studies is scarce and did not contribute to the toxicological evaluation but provided some insight into the insecticidal mode of action.

## Evaluation for acceptable daily intake

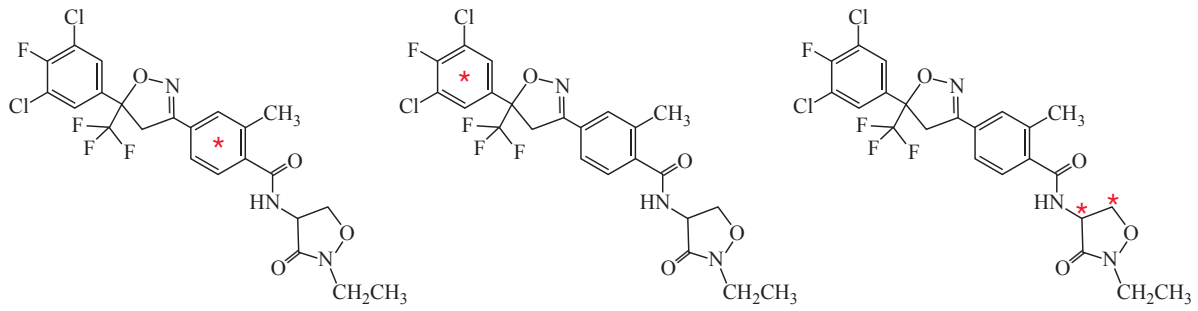
### 1 Biochemical aspects

Isocycloseram (SYN547407) is a mixture of four diastereomers. The main isomer is SYN548088, the (5*S*, 4*R*)-isomer, accounting for 89.2% to 99.2% across all batches used in toxicological testing and absorption, distribution, metabolism and excretion (ADME) studies. The other isomers are SYN548090, SYN548089, and SYN548091.

It is worth noting that toxicological effects of SYN548089 and SYN548090 were investigated, in parallel to the racemic mixture, in a comparative 28-day feeding study in rats (Laidlaw & Robertson, 2023; see below) revealing no major differences in toxicity, and in particular no evidence of any higher toxicity due to these isomers when compared with technical isocycloseram.

Figure 1 shows the chemical structure of isocycloseram together with the positions of the different radiolabels used in the ADME studies reported below.

***Figure 1. Chemical structure of isocycloseram with indications of the respective positions of radiolabelling used in the ADME studies***




[*Methylphenyl*-$^{14}$C]isocycloseram [*Halophenyl*-$^{14}$C]isocycloseram [*Oxoisoxazolidinyl*-$^{14}$C]isocycloseram

* indicates position of $^{14}$C label

## 1.1. Absorption, distribution and excretion

### (a) Oral route

*Study 1*

In a preliminary study (not GLP), the toxicokinetics, absorption, metabolism and excretion of methylphenyl ring-labelled [$^{14}$C]isocycloseram were investigated in small groups of male and female Han Wistar rats following either single oral doses of 3 or 300 mg/kg body weight (bw), or a single intravenous (i.v.) dose of 1 mg/kg bw. For oral application, the vehicle was 0.5% aqueous carboxymethyl cellulose (CMC) whereas the test item was dissolved for i.v. injection in a mixture of dimethysulfoxide (DMSO), polyethylene glycol (PEG 400), and 20% aqueous hydroxypropyl-β-cyclodextrin (HPβCD) in a ratio of 10:20:70 by volume.

One male and one female per dose level received either an oral or i.v. dose for the measurement of radioactivity in excreta, the samples being obtained over a seven-day period. Following oral doses only, radioactivity was also measured in expired air collected over the first two days. Thereafter, the rats were killed and residual radioactivity determined in the gastrointestinal (GI) tract and remaining carcass. Excreta were also examined for metabolites.

Groups of six males and females received the low or high oral dose and had blood samples taken at various intervals up to three days after treatment. All rats were sampled twice. From each animal an interim sample of 1.5 mL was taken at a pre-defined time point, whereas a larger sample of 5–10 mL was collected at termination at 0.5, 4, 8, 24, 30 or 72 hours post dosing. In addition, one male and one female received an i.v. dose of 1 mg/kg bw and a small amount of blood (0.1 mL) was withdrawn on 11 occasions during a two-day period. Blood samples were retained for measurement of radioactivity and also for detection and identification of metabolites in plasma.

Following single oral administration of [$^{14}$C]isocycloseram, recovery of radioactivity was essentially complete in both sexes and at all dose levels. The major route of elimination was always via the faeces, accounting for 77%–94% of the administered radioactivity following the oral doses and 84%–90% after i.v. injection. Urinary excretion contributed much less to overall elimination (3.9%–7.4% in the various groups) and exhalation via expired air was negligible. The majority of the administered radioactivity (70%–94%) was eliminated by 72 hours post dose. However, excretion was incomplete at 168 hours in the low dose oral group and in the i.v. group, 7% or 9% of total applied radioactivity, respectively, remaining in the carcass or GI tract. In contrast, elimination was nearly complete in the animals receiving the high oral dose of 300 mg/kg bw suggesting lower oral absorption and a larger amount directly eliminated from the intestines. There were no clear-cut sex differences in the elimination pattern.

Toxicokinetic analysis revealed maximum blood and plasma concentrations at 4–8 hours after administration in the rats receiving the low oral dose. After administration of the high dose of 300 mg/kg bw, absorption was delayed, with the time to reach maximum concentration ($T_{max}$) observed at 24–48 hours post dose. Using dose-normalized area under the concentration–time curve ($AUC_{0-t}$) estimates, total systemic exposure increased with dose but in a sub-proportional manner in both males and females. The absolute oral bioavailability of radiolabelled isocycloseram was estimated to be 88%–97% after the low dose but only 22%–26% after dosing with 300 mg/kg bw. Saturation of oral absorption seems obvious from this data but it must be acknowledged that there was a very large spacing between the doses. There were no consistent sex-related differences in toxicokinetics. Following the single intravenous dose of 1 mg/kg bw to male and female rats, the concentration of total radioactivity in blood plateaued at 30 hours post dose, with a slight decrease being observed at the final measurement 48 hours after dosing.

In the oral dose groups, blood to plasma ratios of below one suggested that total radioactivity remained predominately in plasma rather than entering blood cells. The contrary was seen in the main study (see Table 3) but the time points of measurement were different and this might explain any difference.

Information on metabolites and metabolic pathways is summarized in the following sub-section on biotransformation to better compare it with data coming from other studies. (Tomlinson, Hutton & O'Neill, 2015)

In a preliminary (and only partly GLP) quantitative, whole-body, autoradiography study, isocycloseram was $^{14}$C-radiolabelled in the methylphenyl moiety and administered as a single oral dose of either 3 or 50 mg/kg bw to groups of six male and six female Han:Wistar rats. The originally-intended high dose of 300 mg/kg bw was not tolerated by the animals which refused to eat or drink and displayed prone position and heavy chromodacryorrhea. The test substance (radiochemical purity 99.2%) was suspended in 0.5% CMC and given by oral gavage at a volume of 7.5 mL/kg bw (low dose) or 10 mL/kg bw (high dose). One animal per sex and dose was subject to autoradiography at six sampling times in total, ranging from 6–168 hours for the low-dose group and from 30–168 hours for the high-dose group.

It was demonstrated that radioactivity was widely distributed throughout the body and was, in nearly all organs and tissues, above the blood levels. Peak concentrations ($C_{max}$) in most tissues were largely proportional to the administered dose (AD) but were achieved after different time intervals. In the case of the low-dose group $C_{max}$ was observed between 6 and 12 hours, but between 30 and 48 hours after administration of the high dose. Apparently absorption of the high dose was delayed. Elimination of both the low and high dose was incomplete since radioactivity was still observed in most tissues at termination. Terminal half-lives in blood ranged from 44 to 59.2 hours, and elimination profiles from tissues were similar, regardless of sex or dose. Irrespective of dose, highest concentrations of radioactivity were found in bile ducts, liver, kidney cortex and adrenals in both males and females (Lewsley & Akroyd, 2016).

*Study 2*

The absorption, elimination and tissue distribution of isocycloseram after single oral gavage application at two different dose levels was investigated in a comprehensive mass balance study in male and female Han:Wistar rats. In total, 40 animals were used of which eight per sex were surgically prepared, under anaesthesia, with in-dwelling bile duct and duodenal cannulas to allow measurements of biliary excretion, whilst the other rats were left intact. Most experiments were run with [*methylphenyl-U-*$^{14}$C]isocycloseram but a few animals also received either [*halophenyl-*$^{14}$C]isocycloseram or [*oxoisoxazolidinyl-*$^{14}$C]isocycloseram in order to investigate any possible impact of the position of radiolabel. The test compound was dissolved at 0.5% (w/v) in aqueous CMC (oxoisoxazolidinyl label) or at 0.5% (w/v) in aqueous CMC containing 0.5% Tween 80 for the other two labelled test items. Table 1 shows an overview of the study design and allocation of animals to the various study groups.

***Table 1. Summary of the study design in the ADME study with single oral application of isocycloseram***

| Test group | Number and sex | Dose (mg/kg bw) | Radiolabel position | Samples taken for radio-analysis |
|---|---|---|---|---|
| Group 1 (intact rats) | 4 males, 4 females | 1 | Methylphenyl | Excreta collection over 168 hours, terminal blood sample, selected; Tissues taken for analysis in addition to residual carcass and GIT with contents |
| Group 2 (intact rats) | 4 males, 4 females | 10 | Methylphenyl | |
| Group 3 (intact rats) | 1 male, 1 female | 1 | Halophenyl | Excreta collection over 168 hours; Terminal blood sample, residual carcass and GIT with contents |
| Group 4 (intact rats) | 1 male, 1 female | 10 | Halophenyl | |
| Group 5 (intact rats) | 1 male, 1 female | 1 | Oxoisoxazolidinyl | |
| Group 6 (intact rats) | 1 male, 1 female | 10 | Oxoisoxazolidinyl | |
| Group 7 (bile duct cannulation) | 4 males, 4 females | 1 | Methylphenyl | Excreta and bile collection over 72 hours; Terminal blood sample, residual carcass and GIT with contents |
| Group 8 (bile duct cannulation) | 4 males, 4 females | 10 | Methylphenyl | |

The following tissues were removed from the intact animals of groups 1 and 2: adrenals, brain, heart, kidneys, liver, lungs, ovaries (females), pancreas, spleen, testes, thymus, thyroid and uterus

together with representative samples of bone mineral (tibia, fibula), fat (renal) and muscle. All samples were analyzed for radioactivity by liquid scintillation counting (LSC). These measurements were performed either directly or following sample oxidation.

The nature and identity of metabolites present in the bile and excreta were also investigated but are reported separately in the section 1.2 Biotransformation, below.

With all three radiolabels, total recovery of radioactivity was around 100% for all groups. As a general observation, the majority of the administered radioactivity was excreted within 72 hours post dose. However, elimination was not complete at termination after 168 hours in intact rats, since residual radioactivity in carcass and GI tract combined represented up to 7.2% in low-dose animals and up to 6.2% in the high-dose group, suggesting some potential for accumulation that, of course, cannot be definitely assessed on the basis of a single-dose study. The major route of elimination was consistently via the faeces, with urinary elimination being much less important. This elimination pattern was not altered much by sex, dose or position of radiolabel.

Table 2 summarizes the mass balance, including overall tissue and carcass residues at study termination, for the groups of four intact males and females receiving methylphenyl-labelled isocycloseram. The findings with the other two radiolabels were similar, with the only possible exception being higher urinary elimination after administration of the oxoisoxazolidinyl-labelled test article (8.1% and 8.4% at the low dose, 5.9% and 5.7% after the high dose treatment in the single male or female rat, respectively). Because of the small number of animals on test, the reproducibility and significance of this observation was not clear.

***Table 2. Mass balance of radioactivity in excreta and tissues after a single oral dose of [methylphenyl-$^{14}$C]isocycloseram to rats (expressed as group mean % of total applied radioactivity)***

| Matrix / Time intervals (hours) | | Low-dose group 1 (1 mg/kg bw) | | High-dose group (10 mg/kg bw) | |
|---|---|---|---|---|---|
| | | Males (*n* = 4) | Females (*n* = 4) | Males (*n* = 4) | Females (*n* = 4) |
| Urine | 0–8 | 0.2 | 0.3 | 0.5 | 0.5 |
| | 8–24 | 1.9 | 1.0 | 2.2 | 1.4 |
| | 24–48 | 0.8 | 0.8 | 1.1 | 0.9 |
| | 48–72 | 0.4 | 0.4 | 0.4 | 0.5 |
| | 72–96 | 0.2 | 0.2 | 0.2 | 0.3 |
| | 96–120 | 0.1 | 0.1 | 0.1 | 0.2 |
| | 120–144 | 0.1 | 0.1 | 0.1 | 0.1 |
| | 144–168 | 0.1 | 0.1 | 0.1 | 0.1 |
| | Subtotal | 3.8 | 3.0 | 4.7 | 3.9 |
| Faeces | 0–24 | 39 | 39 | 40 | 32 |
| | 24–48 | 27 | 26 | 27 | 28 |
| | 48–72 | 11 | 11 | 13 | 15 |
| | 72–96 | 4.8 | 4.7 | 5.5 | 8.7 |
| | 96–120 | 2.6 | 2.6 | 2.7 | 3.7 |
| | 120–144 | 1.6 | 1.8 | 1.4 | 3.0 |
| | 144–168 | 1.2 | 1.4 | 1.4 | 1.4 |
| | Subtotal | 87 | 86 | 91 | 91 |
| Cage wash (total) | | 1.3 | 1.4 | 1.3 | 0.8 |
| Tissues (selected range) | | 1.6 | 2.1 | 1.4 | 1.8 |
| Gastrointestinal tract | | 0.3 | 0.4 | 0.2 | 0.3 |
| Gastrointestinal tract contents | | 0.5 | 0.5 | 0.5 | 0.5 |
| Residual carcass | | 4.8 | 5.3 | 4.1 | 5.0 |
| Total recovery | | 99 | 98 | 103 | 104 |

Source: Hutton, 2017a

An overview of tissue distribution at termination of radioactivity (same groups as in Table 2) is given in Table 3. Seven days after administration of both the low and high dose radioactive residues were detected in all tissues examined and their levels were consistently above the concentrations in circulating blood or plasma. By far the highest mean concentrations were observed in the kidneys. The absolute residue levels reflect the ten-fold increase in dose, and the overall tissue distribution pattern did not differ with regard to dose level or sex.

***Table 3. Group mean residues (µg equiv./g) in tissues/organs of intact rats at 168 hours after administration of a single oral dose of [methylphenyl-U-$^{14}$C]isocycloseram***

| Organ/tissue | Low dose (1 mg/kg bw) | | High dose (10 mg/kg bw) | |
|---|---|---|---|---|
| | Males (*n*=4) | Females (*n*=4) | Males (*n*=4) | Females (*n*=4) |
| Adrenals | 0.277 | 0.304 | 1.88 | 2.26 |
| Bone | 0.051 | 0.066 | 0.43 | 0.51 |
| Brain | 0.061 | 0.080 | 0.48 | 0.68 |
| Fat (renal) | 0.068 | 0.097 | 0.63 | 0.86 |
| Heart | 0.076 | 0.092 | 0.62 | 0.83 |
| Kidneys | 0.589 | 0.527 | 4.41 | 4.55 |
| Liver | 0.190 | 0.308 | 1.53 | 2.52 |
| Lungs | 0.190 | 0.169 | 1.09 | 1.49 |
| Muscle | 0.050 | 0.057 | 0.37 | 0.50 |
| Ovaries | - | 0.302 | - | 2.09 |
| Pancreas | 0.162 | 0.198 | 1.36 | 1.79 |
| Spleen | 0.336 | 0.336 | 2.45 | 2.62 |
| Testes | 0.065 | - | 0.49 | - |
| Thymus | 0.219 | 0.210 | 1.59 | 1.78 |
| Thyroid | 0.145 | 0.156 | 1.51 | 1.92 |
| Uterus | - | 0.142 | - | 1.14 |
| Whole blood | 0.037 | 0.035 | 0.29 | 0.31 |
| Plasma | 0.015 | 0.016 | 0.13 | 0.13 |

Source: Hutton, 2017a

***Table 4. Elimination and fate of radioactivity after administration of [methylphenyl-$^{14}$C]isocycloseram to bile duct-cannulated rats (expressed as percentage of total applied radioactivity)***

| Compartment / oral absorption estimate | 1 mg/kg bw | | 10 mg/kg bw | |
|---|---|---|---|---|
| | Males (n = 4) | Females (n = 4) | Males (n = 4) | Females (n = 4) |
| Urine (0–72 hours) | 1.7 | 2.9 | 2.3 | 3.9 |
| Faeces (0–72 hours) | 30 | 32 | 47 | 46 |
| Bile (0–72 hours) | 50 | 46 | 41 | 33 |
| Cage wash (total) | 0.5 | 0.6 | 0.5 | 0.5 |
| Total excretion (0–72 hours) | 82 | 82 | 91 | 84 |
| Carcass at termination (72 hours) | 15 | 16 | 9.7 | 15 |
| Oral absorption | 67 | 65 | 54 | 52 |

Source: Hutton, 2017a

The experiments in bile-duct cannulated rats revealed that the major route of elimination was via the bile and, accordingly, oral absorption was certainly higher than suggested by the very low urinary excretion in intact rats. Biliary excretion peaked in the time intervals between 12 and 48 hours after administration. Following a single high oral dose of 10 mg/kg bw to males, excretion of radioactivity

was approximately evenly distributed to faeces and bile while more was excreted via faeces in females. In the low dose group receiving 1 mg/kg bw, however, bile was the predominant route of excretion in both sexes suggesting some saturation of absorption when a ten-fold higher dose was administered. Urinary excretion was confirmed to be low at both dose levels. A summary of mass balance data for bile-fistulated rats and the resulting estimates of oral absorption rate are provided in Table 4, above.

As in intact rats, the total recovery of administered radioactivity was nearly complete in both sexes by 72 hours post dose, but 11%–17% of radioactivity still remained in the carcass or GI tract at termination suggesting rather slow elimination.

Oral absorption rate may be calculated as the sum of biliary excretion, urinary excretion and remaining radioactivity in carcass at 72 hours post dosing, excluding the GI tract and its contents. On this basis, oral absorption at the low dose approached 70% whereas little more than 50% of the applied radioactivity was absorbed in the group receiving the high dose (Hutton, 2017a).

*Study 3*

The toxicokinetics of isocycloseram was studied in male and female Han:Wistar rats. Groups of 20 animals per sex received a single oral dose of either 1 or 10 mg/kg bw. In addition, four rats per sex were given a single intravenous dose of 1 mg/kg bw of isocycloseram. The larger number of animals in the oral dose groups reflected the variety of blood sampling times allowing for allocation of rats to a total of five test groups per dose level. The test item was [*methylphenyl-U-*$^{14}$C]isocycloseram. The respective vehicles were 0.5% (w/v) CMC containing 0.5% Tween 80 for oral administration, and a mixture of DMSO, polyethylene glycol (PEG 400) and 20% aqueous HPβCD in a ratio of 10:20:70 by volume for intravenous injection.

Serial and terminal blood samples were taken over a period of four days (oral treatment) or three days (i.v. administration) to measure total radioactivity in blood and, following oral dosing only, also the radioactivity in plasma. Subsequent to oral administration, all rats were sampled twice, whereas five samples (but smaller amounts) were drawn from animals after i.v. injection. All samples were analyzed for radioactivity by LSC, in the case of blood after previous oxidation. Oral bioavailability was determined by comparing the dose-normalized exposure after oral and i.v. applications. In addition, the nature and identity of plasma metabolites were investigated in the orally-treated groups with the results reported in section 1.2 Biotransformation, below.

Following a single intravenous dose of 1 mg/kg bw, the concentration of radioactivity in blood in both sexes plateaued until 24 hours post dose and then steadily declined. The systemic exposure ($AUC_{0-t}$) was similar at 15.2–15.4 µg equiv. h/g, irrespective of sex. In males however an apparent terminal phase could not be reliably estimated.

Subsequent to a single oral administration of 1 mg/kg bw, peak blood and plasma concentrations ($C_{max}$) were observed at 6–8 hours post dose. At the higher dose of 10 mg/kg bw, the $C_{max}$ was observed after 12 or 4 hours post dose in males and females, respectively. Systemic exposure to total radioactivity (based on $C_{max}$ and $AUC_{0-t}$ estimates) increased in a subproportional manner between the 1 mg/kg bw and 10 mg/kg bw dose levels in whole blood and plasma, for both males and females. The absolute bioavailability was estimated to be 96%–100% at the low dose, suggesting that absorption was complete. At the high dose bioavailability was estimated to be 88%.

The outcome just described may appear, to some extent contradictory to the results of the mass balance study (Hutton, 2017a) in which a lower oral absorption had been calculated for the same oral dose, but on the basis of biliary and urinary excretion and carcass residues. However, the estimate of nearly complete oral absorption for the low dose and an oral absorption of at least greater than 80% for the high dose was supported by further evidence. On one hand, elimination of radioactivity via the faeces was similar after oral and i.v. administration in the preliminary study by Tomlinson, Hutton & O'Neill (2015, see above). On the other hand, extensive biotransformation of isocycloseram has been demonstrated for material excreted in faeces following oral administration of doses up to 10 mg/kg bw, with chemically unchanged parent accounting for at most 3.5% (Tomlinson, Hutton & O'Neill, 2015; Real Sesma, 2018). On balance, the Meeting considered the higher estimate coming from this kinetic study to be more reliable. An explanation for the obvious discrepancy compared with the mass balance study by Hutton (2017a) could be non-biliary excretion of radioactivity bound to isocycloseram

metabolites into the intestinal lumen, in addition to and besides the predominant biliary route.

In the oral dose groups, blood to plasma ratios suggested first that total radioactivity remained predominantly in plasma rather than the cellular component of whole blood. However, the blood to plasma ratio appeared to increase at later time points, tending to an even distribution or even greater amounts in the cellular fraction than plasma. This data is indicative of a shift with time, and confirmed the respective (and at first glance contradictory) evidence from the two studies reported above (Hutton, 2017b).

*Study 4*

In a further study, tissue distribution and depletion of radioactivity were investigated in 80 Han Wistar rats (20 of each sex per dose) receiving a single oral dose of 1 or 10 mg/kg bw of [$^{14}$C]labelled isocycloseram. They were divided into subgroups of four animals per sex and dose and were humanely killed at predetermined time points. For low-dose animals, time points 8, 24, 72, 120 and 168 hours post dose were chosen, whereas for the high-dose group, rats were killed after 4, 24, 72, 144 and 192 hours. These time points had been selected on the basis of toxicokinetic data obtained in a prior study (Hutton, 2017b) with the first one reflecting the $C_{max}$ observed in blood.

An additional group of 12 male rats were given an oral dose of 10 mg/kg bw of radiolabelled test item once a day for up to 14 consecutive days. Subgroups of four were killed at 24 hours, after the seventh dose, 24 hours after the fourteenth dose (on day 15), or 10 days after the final administration.

For all these experiments, isocycloseram was once again labelled in the methylphenyl ring and the vehicle was 0.5% (w/v) aqueous CMC containing 0.5% Tween 80. For all animals, residual radioactivity was measured in selected tissues/organs and the remaining carcasses. In the animals receiving multiple treatments, excretion of radioactivity in urine and faeces was monitored in subgroups of four male rats for a period of 24 hours following the first and seventh doses, and then for ten days at 24-hour intervals following the final (fourteenth) dose. The nature and identity of metabolites in selected plasma and excreta samples from all experimental groups were also investigated, but this data is reported in section 1.2 Biotransformation, below.

Following single oral administration of labelled isocycloseram, radioactivity was widely distributed to the tissues in both sexes with peak mean tissue concentrations observed at the first sampling time (eight hours post dose) in liver and kidneys, followed by adrenals. Thereafter, all tissue concentrations declined steadily but the total mean residues in tissues and carcass at 168 hours post dose still accounted for 11% and 12% of the dose in males and females, respectively, suggesting slow elimination. At this last termination time point, radioactivity was most abundant in kidneys, followed by spleen, adrenals and liver. With very few exceptions, organ and tissue concentrations were consistently above those in circulating blood. As an example, group mean tissue residues over the course of time for male rats are shown in Table 5; the pattern was very similar for females.

***Table 5. Distribution of radioactivity in organs and tissues (µg equiv./g or µg equiv./mL) at various time points after single oral administration of 1 mg/kg bw of [$^{14}$C]isocycloseram to male rats***

| | Time after dosing (hours) | | | | |
|---|---|---|---|---|---|
| **Organ/tissue** | **8** | **24** | **72** | **120** | **168** |
| Adrenals | 2.212 | 1.154 | 0.471 | 0.272 | 0.242 |
| Bone (mineral) | 0.334 | 0.291 | 0.120 | 0.057 | 0.050 |
| Brain | 0.191 | 0.165 | 0.122 | 0.073 | 0.061 |
| Fat (renal) | 1.323 | 1.026 | 0.273 | 0.111 | 0.084 |
| Heart | 1.067 | 0.572 | 0.213 | 0.104 | 0.142 |
| Kidneys | 2.919 | 2.365 | 1.284 | 0.649 | 0.536 |
| Liver | 3.753 | 1.994 | 0.663 | 0.278 | 0.224 |
| Lungs | 1.027 | 0.658 | 0.309 | 0.157 | 0.152 |
| Muscle | 0.511 | 0.293 | 0.113 | 0.057 | 0.050 |
| Pancreas | 1.455 | 0.915 | 0.433 | 0.204 | 0.179 |

| | Time after dosing (hours) | | | | |
|---|---|---|---|---|---|
| **Organ/tissue** | **8** | **24** | **72** | **120** | **168** |
| Plasma | 0.534 | 0.322 | 0.091 | 0.024 | 0.019 |
| Spleen | 0.765 | 0.635 | 0.431 | 0.279 | 0.297 |
| Testes | 0.326 | 0.254 | 0.138 | 0.075 | 0.061 |
| Thymus | 0.714 | 0.549 | 0.326 | 0.196 | 0.194 |
| Thyroid | 1.507 | 0.728 | 0.299 | 0.161 | 0.158 |
| Whole blood | 0.351 | 0.210 | 0.084 | 0.037 | 0.031 |

Source: Hutton, 2017c

Following single oral administration of a high dose of 10 mg/kg, radioactivity was also widely distributed to the tissues with a pattern similar to the one observed after low-dose application and, again, without major sex differences. Table 6 provides the data for females. Peak mean tissue concentrations were measured at the first sampling time of four hours post dose and, in both sexes these were highest in liver and adrenals. Thereafter, all tissue concentrations declined steadily up to 192 hours post dose when total mean residues in tissues and carcass still accounted for 7.1% and 8.8% of the applied dose in males and females respectively. At termination radioactivity was most abundant in kidneys. Virtually all tissue concentrations were above those in circulating blood.

***Table 6. Distribution of radioactivity in organs and tissues (µg equiv./g or µg equiv./mL) at various time points after single oral administration of 10 mg/kg bw of [$^{14}$C]isocycloseram to female rats***

| | Time after dosing (hours) | | | | |
|---|---|---|---|---|---|
| **Organ/tissue** | **4** | **24** | **72** | **144** | **192** |
| Adrenals | 25.01 | 13.88 | 5.39 | 3.37 | 2.47 |
| Bone (mineral) | 2.86 | 2.17 | 0.99 | 0.61 | 0.41 |
| Brain | 3.44 | 1.85 | 1.19 | 0.92 | 0.60 |
| Fat (renal) | 20.82 | 22.73 | 4.27 | 1.40 | 0.80 |
| Heart | 10.01 | 6.18 | 2.29 | 1.21 | 0.78 |
| Kidneys | 14.45 | 15.57 | 9.42 | 5.52 | 3.92 |
| Liver | 22.09 | 15.58 | 5.71 | 3.30 | 2.15 |
| Lungs | 9.07 | 6.37 | 3.10 | 1.91 | 1.34 |
| Muscle | 5.40 | 3.01 | 1.23 | 0.70 | 0.46 |
| Ovaries | 11.80 | 7.98 | 3.82 | 3.51 | 2.23 |
| Pancreas | 16.27 | 10.00 | 4.16 | 2.61 | 1.64 |
| Plasma | 3.65 | 3.20 | 0.94 | 0.23 | 0.14 |
| Spleen | 6.45 | 5.64 | 3.86 | 3.24 | 2.52 |
| Thymus | 6.99 | 5.05 | 2.82 | 2.23 | 1.72 |
| Thyroid | 12.25 | 7.49 | 3.23 | 2.24 | 1.20 |
| Uterus | 5.79 | 5.79 | 2.34 | 1.84 | 1.28 |
| Whole blood | 2.42 | 1.87 | 0.80 | 0.39 | 0.28 |

Source: Hutton, 2017c

At both dose levels, tissue depletion half-lives appeared similar in male and female animals but reliable estimates were not always available. In fact, the terminal phase half-life estimates in plasma were ca 34–39 hours for both the low- and high-dose groups and were the shortest that could be calculated. Longer half-lives were estimated for whole blood (51–79 hours for the low dose, 68–78 hours for the high dose). The calculated half-lives for tissue depletion were variable with the longest being 91 hours obtained for pancreas.

Since previous experiments did not reveal meaningful sex differences in toxicokinetics, it appears acceptable that the impact of repeated administration of isocycloseram was studied only in male rats. In line with what observed in single dose experiments, the major route of elimination was via the faeces also when isocycloseram was administered for 7 or 14 days, with a relatively minor proportion of the dose excreted in the urine. Unfortunately, the available data does not allow to provide a complete overview on mass balance to be obtained, nor the calculation of total recovery in the repeated dose experiment but some information on faecal and urinary excretion is summarized in Table 7. On balance, the repeated dosing, therefore, did not appear to fundamentally change the excretion profile of isocycloseram.

***Table 7. Elimination and recovery of radioactivity in different subgroups (n = 4) of male rats receiving multiple doses of 10 mg/kg bw [$^{14}$C]isocycloseram (% of total cumulative dose applied)***

| | | Matrix | | |
|---|---|---|---|---|
| **Subgroup; time interval** | | **Urine** | **Faeces** | **Cage wash** |
| First subgroup; 0–24 hours after first dose | | 2.2 | 26 | 0.3 |
| Second subgroup; 0–24 hours after seventh dose | | 0.9 | 14 | 0.2 |
| Third subgroup; 0–240 hours after fourteenth dose | | 1.1 | 25 | 0.6 |
| **Percentages at termination** | | | | |
| Third subgroup; tissues at termination | 0.6 | | | |
| Carcass | 2.1 | | | |
| Gastrointestinal tract | 0.2 | | | |
| Gastrointestinal tract contents | 0.1 | | | |
| Total residues at termination | 2.9 | | | |

Source: Hutton, 2017c

As in the single dose studies, radioactivity was widely distributed to the organs and tissues in male rats receiving the test compound for a longer period (Table 8).

***Table 8. Distribution of radioactivity in organs and tissues (µg equiv./g or µg equiv./mL) at various time points during repeated oral administration of 10 mg/kg bw of [$^{14}$C]isocycloseram to male rats***

| | Time after dosing | | |
|---|---|---|---|
| | **24 h after 7$^{th}$ dose** | **24 h after final of 14 doses** | **10 days after final of 14 doses** |
| Adrenals | 30.50 | 38.49 | 12.16 |
| Bone (mineral) | 6.57 | 8.33 | 3.00 |
| Brain | 6.05 | 7.87 | 3.08 |
| Fat (renal) | 46.01 | 36.96 | 2.53 |
| Heart | 15.46 | 17.17 | 3.64 |
| Kidneys | 61.76 | 88.57 | 31.89 |
| Liver | 39.29 | 46.27 | 7.97 |
| Lungs | 16.09 | 21.38 | 7.31 |
| Muscle | 7.90 | 9.00 | 2.02 |
| Pancreas | 25.90 | 30.12 | 7.66 |
| Plasma | 6.87 | 7.30 | 0.38 |
| Spleen | 20.99 | 32.16 | 19.84 |
| Testes | 7.49 | 8.91 | 2.76 |
| Thymus | 17.24 | 25.28 | 12.34 |
| Thyroid | 19.84 | 25.08 | 7.42 |
| Whole blood | 5.10 | 6.59 | 2.09 |

h: Hours

Source: Hutton, 2017c

Radioactive residues were detectable in all tissues at the first sampling time point, that is 24 hours after the seventh dose. Because of the repeated dosing and the relatively long half-lives, it was no surprise that the concentrations were higher than at 24 hours after a single administration (see Table 7). With the exception of renal fat, higher mean tissue concentrations were still observed at 24 hours after the fourteenth and final dose. Kidneys and liver consistently, contained the highest levels of radioactivity, followed by adrenals, spleen, pancreas and renal fat. All tissue concentrations were above those in circulating blood. At the final sampling point of 10 days after the final dose, the levels of radioactivity in all tissues had declined, with total mean residues in tissues and carcass still accounting for 2.9% of the total dose. Even though the decline in levels with time after cessation of treatment was remarkably variable for the individual organs and tissues, it may be considered an indication of slow elimination rather than any real potential for accumulation. This view is supported, in particular, by the marked reduction in radioactivity observed to be stored in fat tissue (Hutton, 2017c).

**(b) Dermal route**

In a four-week dermal study in rats (reported in greater detail below), mass balance was not observed, but blood samples were taken from four animals of each sex per dose in week 4, either at 2 and 12 hours or at 6 and 24 hours following dosing. At all three dose levels (100, 300 and 1000 mg/kg bw per day) isocycloseram was present in circulating blood and highest concentrations were measured 24 hours post dose. The $C_{max}$ values were virtually identical for low-dose males and females (148 and 152 ng/mL) but were much higher in female rats at the two upper dose levels. In females receiving 300 mg/kg bw per day, $C_{max}$ was 646 ng/mL compared to 355 ng/mL in males. At the top dose level the difference was even more pronounced; 2940 ng/mL in females and 559 ng/mL in males. The mean blood concentration–time curve over 24 hours post dose reflected this pattern, suggesting a sublinear increase in blood levels of isocycloseram with dose in males, in contrast to an approximately proportional increase in females. No explanation for this apparent sex difference was available (Cooper, 2019).

## 1.2 Biotransformation

Analysis of metabolites was performed in samples of urine and faeces obtained in the preliminary toxicokinetic study in which single oral doses of 3 or 300 mg/kg bw or an i.v. dose of 1 mg/kg bw had been administered to small groups of male and female rats. Plasma samples were only examined in the animals receiving oral doses.

In total, in addition to the parent, nine metabolites were identified in this study (SYN549105, SYN549106, and Metabolites 1–7). No attempts were made by the sponsor or during review to compare their structures to those elucidated in the subsequent, more definitive studies. Further metabolites were detected to occur in excreta but could not be identified.

In urine at least 16 metabolites were detected of which only one (Metabolite 5) could be identified. This minor metabolite accounted for 0.8% of the applied dose in male rats after intravenous administration. It must be acknowledged that urine is a minor route of elimination (less than 7% of the dose in this study) and that the amounts of the individual metabolites were very small making identification a challenge.

In faeces, at least 17 radiolabelled components were detected and 49% to 77% of the applied radioactivity in the various groups could be assigned to identified metabolites. Unchanged parent was present as a relatively large proportion of the total dose in high-dose males and females (17% and 30% respectively) representing most likely the unabsorbed portion since it was not found with low-dose animals. Metabolite 3 was the most abundant, in particular in the low-dose groups, followed by Metabolites 1, 2, 4 and 5. It is unclear how the metabolites in this study correspond to the better characterized ones identified in subsequent studies.

In plasma, four radiolabelled components were detected of which SYN549105 was the most abundant in most groups, accounting for 68% and 58% of the AUC in low- and high-dose males respectively, and for 66% and 34% in corresponding female rats. At the request of JMPR the sponsor confirmed SYN549105 to be an isomer of SYN54936, making the latter the most abundant circulating metabolite in rat plasma, with the possible exception of high-dose females.

In the high-dose female group SYN549106 was the main metabolite, accounting for 55% of the AUC, whereas the same compound was a minor metabolite in low-dose females and low-dose males and was not detected at all in high-dose males. It is a presumed downstream metabolite of SYN549105 but may also directly result from biotransformation of the parent. It is worth noting that neither SYN549105 nor SYN549106 were detected in excreta.

Unchanged parent isocycloseram was detected in low- and high-dose males (making up 12% and 26% of the AUC, respectively) but only in females only at the low dose (15% of the AUC). In addition, one more compound (Metabolite 5) was found in small amounts, but only in male rats receiving the low dose. Some sex differences in metabolism of isocycloseram could have been assumed from these observations, but this was not confirmed in the subsequent definitive studies (Tomlinson, Hutton & O'Neill, 2015).

In the definitive biotransformation study, urine, bile, faeces and plasma samples from various studies (see Table 9) were analyzed for metabolites. In these studies, radiolabelled isocycloseram had been given to rats as single or repeated doses of 1 or 10 mg/kg bw, respectively. Urine and bile were prepared for analysis by centrifugation and the supernatants analyzed. Their radioactive contents were determined by LSC. The majority of radioactivity in faeces, as well as plasma samples, were extracted with acetonitrile and acetonitrile/water mixtures. Solvent extracts containing significant radioactivity were pooled, concentrated and analyzed. Metabolites were identified by means of a combination of liquid chromatography and mass spectrometry (LC-MS), comparison with standard reference compounds, accurate mass measurement and fragmentation pattern.

***Table 9. Origin of samples for characterization of metabolites after oral dosing***

| Reference | Samples | Radiolabel | Animal type | Dose (mg/kg bw) | Sex |
|---|---|---|---|---|---|
| Hutton, 2017a | Urine, faeces | MP | Intact | 1 (single dose) | M |
| | | | | | F |
| | | | | 10 (single dose) | M |
| | | | | | F |
| | Urine, faeces | HP | Intact | 1 (single dose) | M |
| | | | | | F |
| | | | | 10 (single dose) | M |
| | | | | | F |
| | Urine, faeces | Oxo | Intact | 1 (single dose) | M |
| | | | | | F |
| | | | | 10 (single dose) | M |
| | | | | | F |
| | Bile, urine, faeces | MP | Bile duct-cannulated | 1 (single dose) | M |
| | | | | | F |
| | | | | 10 (single dose) | M |
| | | | | | F |
| Hutton, 2017b | Plasma | MP | Intact | 1 (single dose) | M |
| | | | | | F |
| | | | | 10 (single dose) | M |
| | | | | | F |
| Hutton, 2017c | Urine, faeces | MP | Intact | 10 (single dose) | |
| | | | | 10 (7 daily doses) | M |
| | | | | 10 (14 daily doses) | |

M: Male; F: Female; MP: Methylphenyl; HP: Halophenyl; Oxo: Oxoisoxazolidinyl

A number of metabolites were identified. Metabolite pattern in excreta of male and female rats was not substantially affected by sex or location of radiolabelling. This is demonstrated in Table 10

for single administration at the low dose of 1 mg/kg bw. Differences at the high dose were of a similar magnitude. Therefore the following, results are reported only for the methylphenyl labelled compound.

***Table 10. Metabolites in faeces and urine (combined) after single oral administration of 1 mg/kg bw of isocycloseram labelled at different positions; (expressed as percentage of administered dose)***

| Metabolite | Males | | | Females | | |
|---|---|---|---|---|---|---|
| | Oxo | MP | HP | Oxo | MP | HP |
| Parent isocycloseram | 0.4 | ND | 0.5 | 0.4 | ND | 1.0 |
| Metabolite B | 0.5 | 1.1 | 1.0 | ND | ND | 0.9 |
| Metabolite D | 2.8 | 1.1 | 3.3 | 1.6 | <0.1 | 3.3 |
| Metabolite A | 1.9 | 2.0 | 3.4 | 2.7 | 1.7 | 2.8 |
| SYN549551 | 2.9 | 3.9 | 3.3 | 3.9 | 2.7 | 3.3 |
| SYN549553 | 9.7 | 11.2 | 8.7 | 25.0 | 11.8 | 14.4 |
| SYN550455 | ND | 1.4 | 1.9 | ND | ND | 2.7 |
| SYN549554 | 4.6 | 4.7 | 12.1 | 6.7 | 5.5 | 9.4 |
| SYN550602/SYN549432 | 3.9 | 10.3 | 1.3 | 5.3 | 2.8 | ND |
| SYN549544/SYN549549 | 2.5 | 4.1 | 7.6 | 5.3 | 4.8 | 7.4 |
| Metabolite C | 0.5 | 0.6 | 2.0 | ND | 0.9 | 2.0 |
| SYN549543 | 0.9 | 1.2 | 6.9 | 1.0 | 1.2 | 3.6 |
| SYN549433 | 0.8 | ND | ND | ND | ND | ND |
| SYN549436 | 0.5 | 0.6 | 1.5 | 1.3 | 0.8 | 1.1 |
| SYN549431 | ND | 0.9 | 1.2 | ND | 1.1 | 1.6 |

MP: Methylphenyl; HP: Halophenyl; Oxo: Oxoisoxazolidinyl; ND: Not detected; Source: Hutton 2017a, b, c

As might be expected due to predominantly biliary and faecal elimination, much higher levels of all metabolites were present in faeces or in bile than in urine. Urine contributed very little to the total abundance of metabolites in excreta. This is demonstrated in Table 11 in samples that were collected over 72 hours following administration of a low dose of isocycloseram to bile duct-cannulated rats.

***Table 11. Metabolite profile in excreta of bile duct-cannulated rats following a single oral dose of [methylphenyl-U-$^{14}$C]isocycloseram at 1 mg/kg bw; (expressed as percentage of administered dose)***

| Metabolites | Males | | | | Females | | | |
|---|---|---|---|---|---|---|---|---|
| | Urine | Bile | Faeces | Total | Urine | Bile | Faeces | Total |
| Parent isocycloseram | ND | ND | 1.3 | 1.3 | ND | 0.9 | 1.1 | 2.0 |
| Metabolite B | <0.1 | 5.4 | ND | 5.4 | 0.1 | 4.5 | ND | 4.6 |
| Metabolite D | <0.1 | ND | ND | <0.1 | 0.1 | ND | ND | 0.1 |
| SYN549551 | ND | 0.6 | 0.7 | 1.3 | 0.1 | 0.6 | 0.5 | 1.2 |
| SYN549553 | <0.1 | 0.6 | 8.6 | 9.2 | 0.1 | ND | 5.7 | 5.8 |
| SYN550455 | ND | ND | ND | ND | ND | 1.9 | ND | 1.9 |
| SYN549554 | ND | ND | 2.3 | 2.3 | <0.1 | ND | 2.0 | 2.0 |
| SYN549432/SYN550602 | ND | ND | ND | ND | <0.1 | ND | ND | <0.1 |
| SYN549544/SYN549549 | ND | 0.9 | 1.6 | 2.5 | <0.1 | 1.0 | 1.2 | 2.2 |
| SYN549543 | 0.1 | 11.0 | 0.3 | 11.4 | 0.1 | 8.8 | 0.3 | 9.2 |
| SYN549433 | <0.1 | 0.5 | 1.5 | 2.0 | <0.1 | ND | 0.9 | 0.9 |
| SYN549436 | <0.1 | 3.3 | 0.4 | 3.7 | 0.1 | 4.3 | 0.2 | 4.6 |
| Metabolite C[a] | ND | 1.1 | ND | 1.1 | ND | 5.9 | ND | 5.9 |
| SYN549431 | <0.1 | 0.9 | 0.3 | 1.2 | <0.1 | 1.5 | 0.3 | 1.8 |

[a] Glucuronide of SYN549436 (treated as sum of both); ND: Not detected; Source: Hutton, 2017a

At the low dose level of 1 mg/kg bw, the metabolite SYN549543 was observed to occur in the bile in a quantity that accounted for 11% of administered dose in males and 8.8% in females, followed by Metabolite B (5.4% and 4.5% in males and females respectively) and SYN549436 (3.3% or 4.3% respectively). In females, Metabolite C (the glucuronide of SYN549436) accounted for 5.9% of administered dose, whereas the bile of males contained much less.

The metabolite pattern in bile after administration of the high dose was similar, but it is worth noting that the parent compound accounted for 3.5% in the bile of male rats and up to 2.8% in females.

In plasma the parent compound accounted for 3.8%–6.4% of total recovered radioactivity (TRR) following the low dose, and 12.5%–13.9% TRR after administration of 10 mg/kg bw. In both sexes, SYN549436 accounted for the largest proportion of the TRR (57%–80%). Minor plasma metabolites comprised unresolved SYN549544/SYN549549 and SYN549431, SYN549543 and unresolved SYN550602/SYN549432.

Repeated administration of isocycloseram to male rats had no major impact on the metabolite pattern as shown in Table 12.

***Table 12. Metabolite profile in excreta of male rats following single or repeated oral dosing with 10 mg/kg bw of [methylphenyl-U-$^{14}$C]isocycloseram; (expressed as percentage of administered dose)***

| | Dosage regime and matrix | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Single dose | | | 7 daily doses | | | 14 daily doses | | |
| Metabolite | Urine | Faeces | Total excreta | Urine | Faeces | Total excreta | Urine | Faeces | Total excreta |
| Parent compound | ND | 0.7 | 0.7 | ND | 0.2 | 0.2 | ND | 0.3 | 0.3 |
| Metabolite B | <0.1 | 0.2 | 0.2 | <0.1 | 0.1 | 0.1 | <0.1 | 0.4 | 0.4 |
| Metabolite D | <0.1 | 0.8 | 0.8 | <0.1 | 0.1 | 0.1 | <0.1 | 0.5 | 0.5 |
| Metabolite A | ND | 0.5 | 0.5 | ND | 0.3 | 0.3 | ND | 0.9 | 0.9 |
| SYN549551 | ND | 0.9 | 0.9 | ND | 0.4 | 0.4 | ND | 1.3 | 1.3 |
| SYN549553 | ND | 1.5 | 1.5 | <0.1 | 1.0 | 1.0 | <0.1 | 3.2 | 3.2 |
| SYN550455 | ND | ND | ND | <0.1 | ND | ND | <0.1 | 0.3 | 0.3 |
| SYN549554 | ND | 0.4 | 0.4 | ND | 0.3 | 0.3 | ND | 1.1 | 1.1 |
| SYN550602/ SYN549432 | ND | 1.8 | 1.8 | ND | 0.6 | 0.6 | ND | 1.7 | 1.7 |
| SYN549544/ SYN549549 | ND | 0.5 | 0.5 | ND | 0.4 | 0.4 | <0.1 | 1.4 | 1.4 |
| Metabolite C | ND | 0.2 | 0.2 | ND | 0.2 | 0.2 | <0.1 | 0.7 | 0.7 |
| SYN549543 | ND | 0.1 | 0.1 | ND | 0.1 | 0.1 | ND | 0.3 | 0.3 |
| SYN549433 | <0.1 | 0.6 | 0.6 | <0.1 | 0.2 | 0.2 | <0.1 | 0.4 | 0.4 |
| SYN549436 | ND | 0.1 | 0.1 | ND | 0.1 | 0.1 | ND | 0.3 | 0.3 |
| SYN549431 | ND | 0.1 | 0.1 | ND | 0.1 | 0.1 | ND | 0.5 | 0.5 |

ND: Not detected; Source: Hutton, 2017c

The metabolic pathways of isocycloseram in the rat comprised ring opening of the isoxazole ring, ring opening and cleavage of the oxazolidinone ring, oxidative defluorination and glucuronic acid conjugation. In detail, the biotransformation of the parent molecule proceeded by the following reactions:

- De-ethylation of the oxazolidinone ring to form SYN549436 with subsequent ring opening to give SYN549544. Hydrolysis of the amide group to the carboxylic acid generated SYN549543.
- Ring opening of the isoxazole ring of SYN549544 and SYN549543 yielding SYN549553 and SYN549554, respectively.

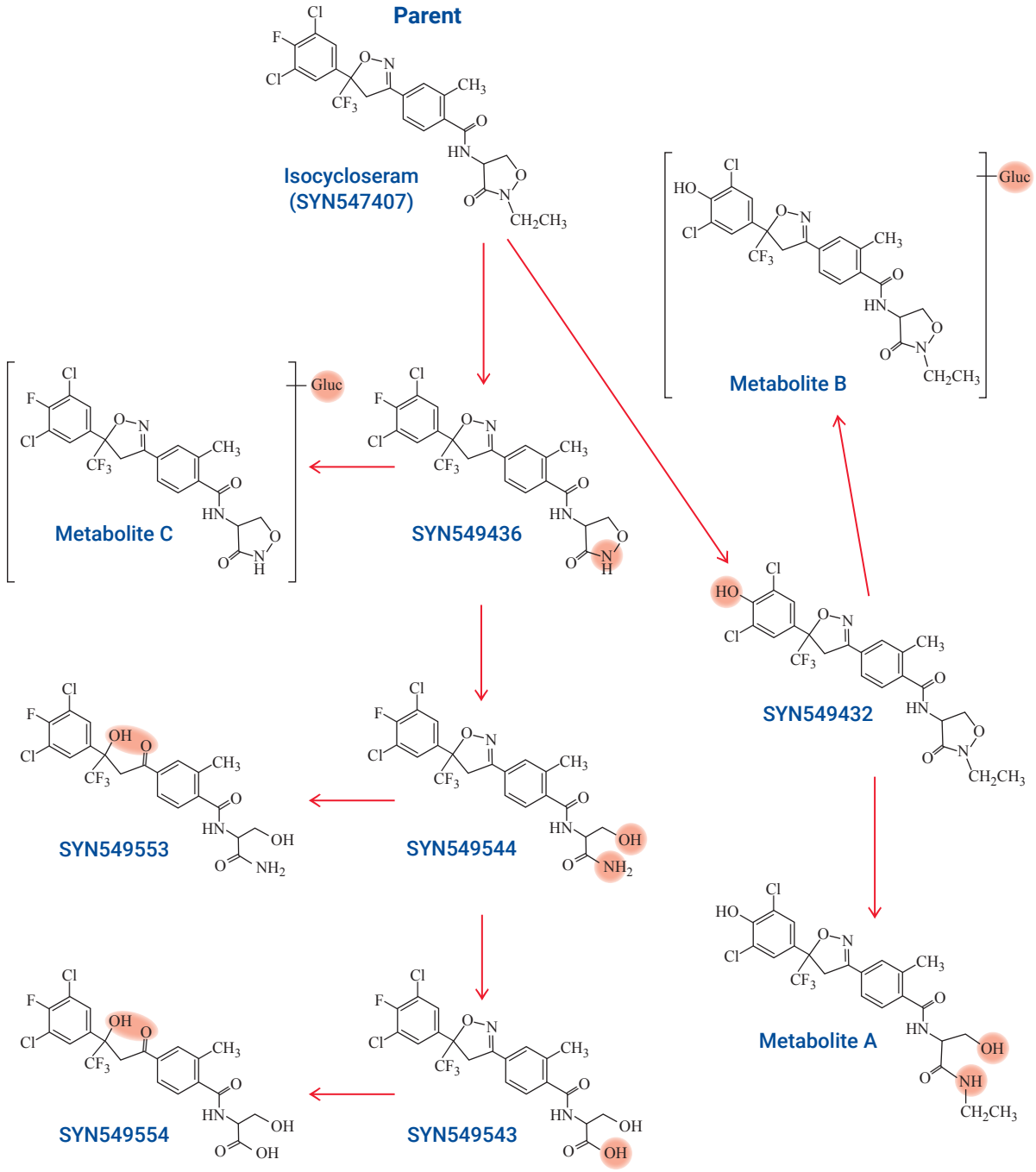
- Oxidative defluorination of SYN547407 resulted in the formation of SYN549432 and Metabolite A.
- Cleavage of the isoxazole and oxazolidinone rings giving SYN549433 and SYN550602, followed by oxidative defluorination to form metabolites SYN549551 and Metabolite D.
- Reduction of the cleaved isoxazole ring of SYN550602 to form SYN550455.
- Loss of the oxazolidinone ring to form SYN549431 followed by cleavage of the isoxazole ring to form SYN549549.

In phase II reactions, metabolites SYN549432 and SYN549436 underwent glucuronic acid conjugation. The postulated biotransformation of isocycloseram, based on its identified metabolites in the rat, is presented in Fig. 2a and b.

To conclude, metabolism of isocycloseram was extensive with unchanged parent occurring as a minor component at amounts less than 3.5% of the administered dose in excreta when doses of up to 10 mg/kg bw had been administered. A multitude of metabolites was detected and identified, of which most occurred in relatively small amounts in the various matrices. Since the majority of administered dose was excreted via bile into faeces, bile samples were the most important for characterization of metabolites. Based on metabolite characterization in bile, SYN549543 may be considered the major metabolite of isocycloseram in the rat. Metabolite SYN549436 and its glucuronide (metabolite C), taken together, may be considered a significant metabolite at least in females. In addition, SYN549436 was, in both sexes, the most abundant circulating metabolite in plasma, accounting for up to 80% of the TRR. On balance, there was sufficient evidence to regard SYN549436 also as a major rat metabolite from isocycloseram.

In general, the metabolite profile was similar across all samples, irrespective of dose, single or repeated administration, position of radiolabel or sex.

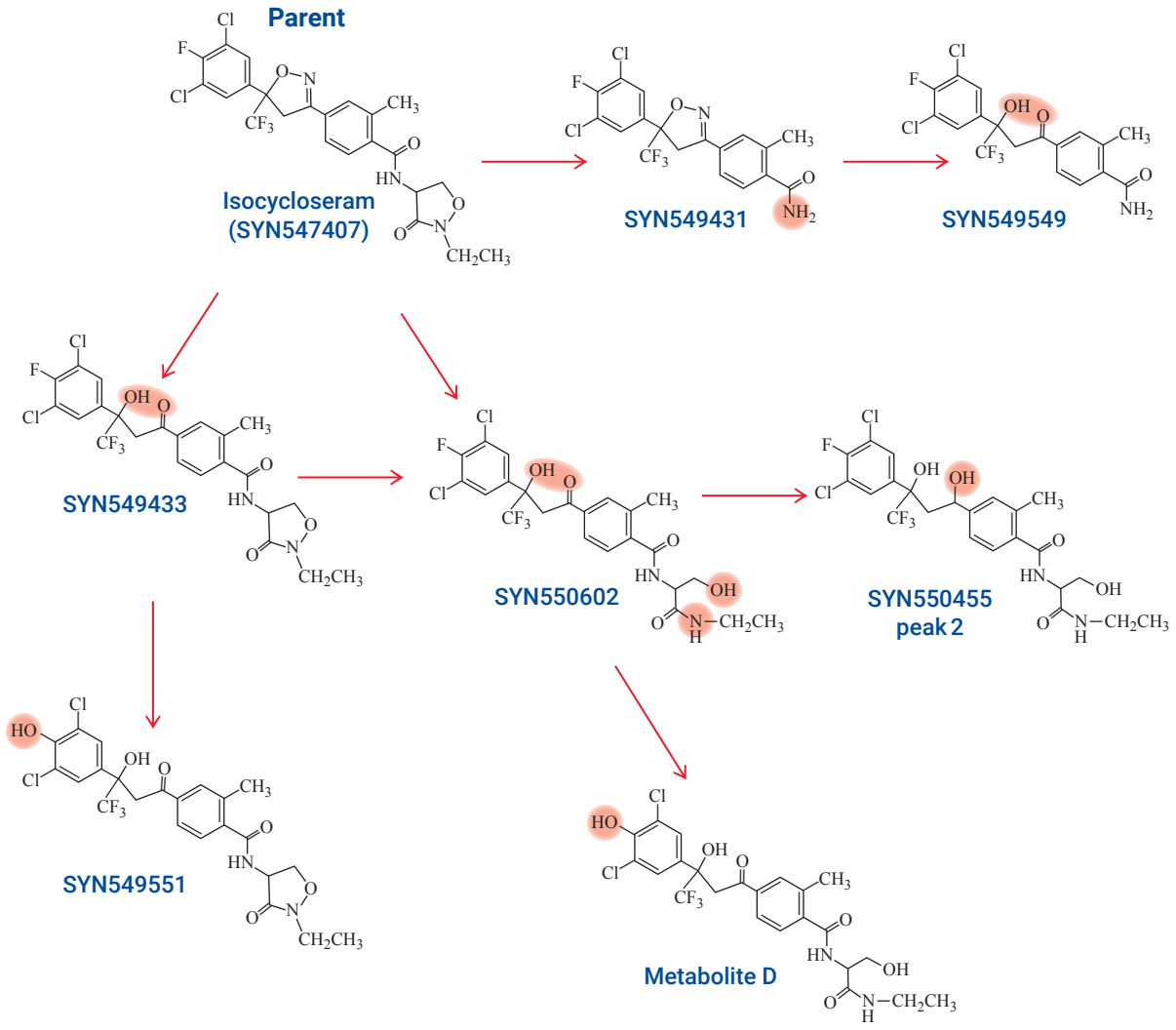
*Figures 2a and 2b. Metabolic pathways of isocycloseram in the rat*




highlights where the molcule has changed as a result of the immediately prior transformation

[ ] indicates tentative identification only

Source: Real Sesma, 2018




Source: Real Sesma, 2018

To a limited extent, phase I metabolism of isocycloseram was also investigated in vitro. The fate of isocycloseram radiolabelled in the three different positions (see Fig. 1) was followed after incubation with liver microsomes of human or Wistar rat origin. Mixed samples of microsomes from males and females were purchased from a commercial supplier. Their metabolic competence was proven by means of appropriate control substance (for example testosterone and 7-ethoxycoumarin). Stability of isocycloseram in the incubation medium was also demonstrated prior to the main experiment.

In the in vitro assay, 10 µM radiolabelled isocycloseram was incubated for 60 minutes with the microsomal suspension (0.5 mg/mL) in a potassium phosphate buffer containing magnesium chloride. Following a pre-incubation phase of five minutes, an appropriate NADPH-regenerating system was added to give final concentrations of 1 mM NADP, 5 mM glucose-6-phosphate (G6P) and 1 unit/mL of glucose-6-phosphate dehydrogenase (G6PDH). In an additional experiment without that system, it was shown that virtually no NADPH-independent metabolism occurred.

After the reaction was stopped by cooling and the addition of acetonitrile, radioactivity in supernatants and pellets was measured by LSC. Parent compound and metabolites were separated by high-performance liquid chromatography (HPLC) and the various peaks distinguished by their different retention times. Quantification was performed by calculating for each peak the respective percentage of total radioactivity recovered. Those peaks corresponding to parent compound and to the metabolites of the positive control substances were confirmed by co-chromatography with either unlabelled isocycloseram or the respective reference items. This method, however, did not permit the comparison

and assignment of in vitro metabolites of isocycloseram to those found in vivo, as the metabolites were not specifically identified.

For both rat and human microsomes, recovery of radioactivity was around 100% and more than 98% was located in the supernatants. Metabolism was much more limited than in vivo since, after incubation for one hour, parent isocycloseram still accounted for ca 80% with human microsomes and 86%–87% with rat microsomes. In addition to the parent, up to 10 additional peaks were observed. Qualitatively, biotransformation by human and rat microsomes was similar but there were some quantitative differences. In both cases, P6 was the main metabolite accounting for 15%–16% in human samples and for ca 8% where rat microsomes were employed. Two metabolites (P3 and P4) were not observed with rat microsomes, but found at trace levels of up to 0.2% with human microsomes. However, because of their low abundance, they cannot be considered unique human metabolites of concern. Since there were no differences in metabolism when the test compound was radiolabelled at different sites, cleavage of the molecule apparently had not taken place under the chosen test conditions.

On balance, the metabolism of isocycloseram by human and rat microsomes was similar but the information obtained from this study is rather limited. Use of hepatocytes from various species would have been preferable, as well as more variation in incubation times and test item concentrations (Thibaut, 2019).

### 1.3 Effects on enzymes and other biochemical parameters

Summarized in this subsection are those measurements of isocycloseram concentrations in blood that were performed as part of apical toxicological studies with oral administration. For the only dermal study providing toxicokinetic data, see above

Following daily gavage administration of isocycloseram to pregnant rats for 14 days in a range-finding study, dose levels of 3.5, 7.5 or 15 mg/kg bw per day led to mean blood concentrations of 241, 660, and 1700 ng/mL, respectively, and were thus roughly proportional to dose (Britton, 2015).

Systemic exposure to isocycloseram was also verified in pregnant rabbits. Following five or six daily doses of 30 mg/kg bw, blood concentrations of 2960–3300 ng/mL were determined. Lower doses of 7.5 and 15 mg/kg bw per day resulted in mean blood levels of 400 and 1170 ng/mL respectively, but these were measured on gestation day 28 and thus are not really comparable to those observed on days 11 and 12 in the high-dose group (Blunt, 2015).

In a one-generation gavage study, blood was sampled from male and female rats six times (from predose to 24 hours post dose) on study day 29, that is, after four weeks of daily administration but before mating. The results suggested a proportional increase with dose of isocycloseram concentrations in blood for female rats, whereas the increase was less than proportional for males (Penn, 2018).

Blood concentrations measured in this latter reproduction study and in further oral short-term studies are summarized in Table 13. For better comparability, mean daily intakes are given for dietary studies instead of nominal concentrations. It is worth noting that only the active compound was measured in these studies; no metabolites were determined. Sampling was performed at different time points but the figures in the table reflect the concentrations that were measured on the sampling day that was closest to study termination. An exception is the 28-day study in rats for which the measurement on day 9 was used since, afterwards, no data from high-dose females were available because of mortality and premature termination.

*Table 13. Mean blood concentrations of isocycloseram at selected time points in oral short-term studies, (results are $C_{max}$ expressed in ng/mL [dose levels in mg/kg bw per day])*

| Study | Sample day | Males | | | Females | | |
|---|---|---|---|---|---|---|---|
| | | Low dose | Mid dose[a] | High dose | Low dose | Mid dose[a] | High dose |
| 4-week, mouse (Dymarkowska, 2015) | Day 29 | 282 [17.4] | 865 [55.9] | 4428 [172] | 603 [20.9] | 817 [60.5] | 3832 [176] |
| 90-day, mouse (Laidlaw, 2019a) | Day 85 | 98.5 [8.0] | 738 [48.8] | 1740 [117] | 114 [9.9] | 819 [51.6] | 2160 [140] |
| 4-week, rat (Dymarkowska, 2017) | Day 9 | 154 [4.3] | 1109 [16.3] | 2820 [37] | 216 [4.5] | 4012 [50.1] | 5590 [46[b]] |
| One-generation, rat (Penn, 2018) | Day 29 | 501 [7.5] | 696 [15] | 1330 [45] | 284 [3.5] | 640 [7.5] | 1130 [15] |
| 90-day, rat (Laidlaw, 2019b) | Day 85 | 63.1 [3.9] | 359 [11.2] | 959 [22] | 146 [4.4] | 914 [13.4] | 2410 [24] |
| 4-week, dog (Robertson, 2019a) | Day 28 | 953 [10] | 2450 [50] | ND | 1020 [10] | 2280 [35] | 4030[c] [70] |
| 90-day, dog (Robertson, 2019b) | Week 13 | 904 [5] | 2930 [15] | 3350 [25] | 808 [5] | 2940 [15] | 3250 [25] |

ND: No data due to premature termination;

[a] If more dose groups were employed, data for the low intermediate is given here;

[b] Prematurely terminated on day 11;

[c] Data for one animal available only.

A clear increase with dose was observed in all species and studies that was often not linear but subproportional or supraproportional. Remarkable interindividual differences in blood levels were common, in particular in dogs. Sometimes, the comparison was complicated by nominal dose levels that were different for males and females in the same study (for example Dymarkowska, 2017; Penn, 2018; Robertson, 2019a).

## 2. Toxicological studies

### 2.1 Acute toxicity

#### (a) Lethal doses

In an acute oral toxicity study (up-and-down procedure, OECD TG 425), female Wistar rats (Crl:WI) were given single doses of isocycloseram (purity 96.9%, Batch no. SMU5IP15011) in 0.5% aqueous CMC containing 0.1% Tween. After dosing, animals were observed at 30 minutes, 1, 2, 3, 4, and 6 hours, then once each day for 14 days. As a starting dose, 1750 mg/kg bw was given to one animal and no clinical signs of toxicity or mortality were observed. Thus, three animals were dosed with 5000 mg/kg bw. There were no mortalities and no clinical signs of toxicity recorded. All animals gained weight throughout the observation period and there were no gross changes at necropsy. The acute oral $LD_{50}$ for the test item was greater than 5000 mg/kg bw in female rats (Tarcai, 2016a).

A second study tested the acute oral toxicity of isocycloseram (purity 96.9%, Batch no. SMU5IP15011) in female Sprague Dawley rats (SPF:SD). At first, and up-and-down procedure was employed with one dose group of 5000 mg/kg bw in corn oil and three animals. After dosing clinical observations were performed at 30 minutes, four hours, and then once each day for 21 days. One animal showed low spontaneous movement five days after treatment and died on the sixth day. Delayed mortality occurred in the other two animals on the fourth and fifth days after administration, respectively. Thus, acute oral toxicity testing was changed to Horn's method using four dose levels (464, 1000, 2150 and 4650 mg/kg bw in corn oil) with five animals in each dose group. No mortality and no clinical signs of toxicity were recorded at 464 and 1000 mg/kg bw. All animals in these dose groups gained weight

throughout the observation period. Delayed mortality occurred in 2/5 animals at 2150 mg/kg bw on the third and fourth day after treatment, no abnormalities preceding death. No clinical signs of toxicity were recorded for the other animals until the end of the observation period. At 4650 mg/kg bw delayed mortality occurred in 2/5 animals. One animal died on the third day with no prior abnormalities. The other animal showed blood red secretion around the nose and mouth on days 3 and 4 and emaciation and perineal staining on day 4 post treatment; the animal died the same day. In two animals, secretion around nose and mouth were observed on the days 4–6 post treatment. Another rat appeared emaciated on days 5 and 6 post treatment. In these three animals clinical signs of toxicity then disappeared until the end of the observation period. The mean body weights at 2150 and 4640 mg/kg bw were reduced on day 7 compared to day 0, but thereafter body weight increased. No gross changes were observed at necropsy for any dose group. The acute oral $LD_{50}$ for isocycloseram for female rats was 4569 mg/kg bw (Xue, 2021a).

In an acute dermal toxicity study (OECD TG 402), five Wistar rats (Crl:WI) of each sex were given a limit dose of isocycloseram (purity 96.9%, Batch no. SMU5IP15011) dampened with water at 5000 mg/kg bw. The test item was applied semi-occlusively for 24 hours to intact, shaved skin. Clinical observations were performed after 1 and 4 hours after dosing and daily for 14 days thereafter. There were no mortalities and no signs of systemic toxicity or irritation. All animals gained weight throughout the study and there were no changes at gross necropsy. The acute dermal $LD_{50}$ for isocycloseram in male and female rats was greater than 5000 mg/kg bw (Tarcai, 2016b).

In a second study, the acute dermal toxicity of isocycloseram (purity 96.9%, Batch no. SMU5IP15011) was tested in five male and five female Sprague Dawley rats (SPF:SD). The test item was dampened with corn oil and applied semi-occlusively for 24 hours to intact, shaved skin at 5000 mg/kg bw. Clinical observations were performed once daily for 14 days. There were no mortalities or signs of systemic toxicity. All animals gained weight throughout the study and there were no changes at gross necropsy. The acute dermal $LD_{50}$ for isocycloseram in male and female rats was greater than 5000 mg/kg bw (Xue, 2021b).

In an acute inhalation toxicity study (OECD TG 403), Wistar (Crl:WI) rats were given a maximum achievable concentration of isocycloseram (purity 96.9%, Batch no. SMU5IP15011), nose-only as a dust for four hours. The particle size distribution of the test item was determined regularly during the exposure period. A sighting study was performed with two males and two females at a mean achieved concentration (MAC) of 4.38 mg/L with a mass median aerodynamic diameter (MMAD) of 3.11 µm and a geometric standard deviation (GSD) of 2.37. In the main study, five males and five females were exposed at a MAC of 4.62 mg/L with a MMAD of 3.38 µm and a GSD of 2.34. Clinical observations were performed at hourly intervals during exposure, one hour after treatment and daily for 14 days thereafter. One female in the main study was found dead on day 7 and was cannibalized. A specific cause of death was not established, but necropsy revealed collapsed, dark/red discoloured lungs. During the sighting and the main study, treated animals exhibited clinical signs including slightly laboured respiration (day 0), ruffled fur or red–brown staining (as chromodacryorrhea) and fur staining on the head (cranium) due to the test item (days 0–2). No clinical signs were observed three days after exposure. All animals showed slight to moderate body weight loss in the first three days of the observation period, thereafter, body weight gain returned to its normal range. No changes at necropsy were noted in the surviving animals. The acute inhalation $LC_{50}$ for isocycloseram in male and female rats was greater than 4.62 mg/L (Rosos-Matting, 2017).

Isocycloseram was therefore of low acute oral, dermal and inhalation toxicity in rats (Table 14).

***Table 14. Summary of acute toxicity studies in rats with isocycloseram***

| Species | Strain | Sex | Route | Purity | Result | Reference |
|---|---|---|---|---|---|---|
| Rat | Wistar | Female | Oral | 96.9% | $LD_{50}$ > 5000 mg/kg bw | Tarcai, 2016a |
| Rat | SD | Female | Oral | 96.9% | $LD_{50}$ = 4569 mg/kg bw | Xue, 2021a |
| Rat | Wistar | Male & Female | Dermal | 96.9% | $LD_{50}$ > 5000 mg/kg bw | Tarcai, 2016b |
| Rat | SD | Male & Female | Dermal | 96.9% | $LD_{50}$ > 5000 mg/kg bw | Xue, 2021b |
| Rat | Wistar | Male & Female | Inhalation | 96.9% | $LC_{50}$ > 4.62 mg/L | Rosos-Matting, 2017 |

bw: Body weight; $LC_{50}$: Median lethal concentration; $LD_{50}$: Median lethal dose

**(b) Dermal irritation**

In a dermal irritation study (OECD TG 404) in New Zealand White rabbits, 0.5 g of isocycloseram (purity 98.4%, Batch no. MEQ-02794-09) was moistened with water (pH 5.5) and topical, semi-occlusive application made to the intact, shaved flanks of three male rabbits, remaining there for four hours. Clinical observations were performed at 1, 24, 48 and 72 hours after patch removal. No local dermal signs (erythema, oedema) were observed in any of the treated animals at any time point. The primary irritation index was 0.00. No clinical signs of systemic toxicity were observed and no mortality occurred. Isocycloseram was considered to be not irritating to the skin of rabbits (Matting, 2015a).

In a second study, the dermal irritation of isocycloseram (purity 96.9% , Batch no. SMU5IP15011) was tested on three male Japanese White rabbits. The test item was moistened with corn oil and 0.5 g was applied semi-occlusively to the intact, shaved skin for four hours. Clinical observations were performed at 1, 24, 48 and 72 hours after patch removal. No local dermal signs (erythema, oedema) were observed in any of the treated animals at any time point. The primary irritation index was 0.00. No mortality occurred and the body weight of the animals increased normally. Isocycloseram was considered to be not irritating to the skin of rabbits (Xue, 2021c).

**(c) Ocular irritation**

The potential for eye irritation was studied in vitro as well as in two classical in vivo studies. First, in the in vitro study (OECD TG 438), the irritant effect of isocycloseram (purity 98%, Batch no S02F921772W) was evaluated in isolated chicken eye (ICE) evaluations. This assay can be used as a screening tool for corrosivity/severe irritancy to avoid unacceptable effects in vivo. Following the zero reference measurements, the eye was positioned horizontally and 30 mg of test item applied onto the centre of the cornea of three eyes, covering the entire surface of the cornea. As a positive control three eyes were treated with 30 g imidazole and the negative control eye was treated with 30 µL sodium chloride solution. After an exposure period of 10 seconds, the cornea was rinsed thoroughly with 20 mL isotonic saline. When some test item or imidazole remained on the surface of the cornea despite this rinsing, additional gentle rinsing with 20 mL of saline was performed. Evaluations of corneal effects were performed pretreatment and at approximately 30, 75, 120, 180 and 240 minutes after rinsing. Corneal thickness and corneal opacity were measured at all time points. Fluorescein retention was measured on two occasions, at base line (immediately after rinsing) and approximately 30 minutes later. Other observations indicating eye damage, such as loss of epithelium, were also taken into account. Results from corneal swelling, opacity and fluorescein retention were evaluated separately to generate an ICE class for each end-point, ranging each from class I (no effects) to class IV (severe effects).

According to the study author, the negative control and positive control results were in good correlation with historical data. For the positive control, the mean values for maximum corneal swelling up to 75 and 240 minutes were 8.7% and 16.9% respectively (class II), the mean maximum corneal opacity change and mean fluorescein retention change were 3.83 and 3.83 (class IV). No corneal swelling, corneal opacity or fluorescein retention was observed for the control eye. For the eyes treated with isocycloseram, the mean values for maximum corneal swelling up to 75 and 240 minutes were 0.0%, and the mean maximum corneal opacity change and mean fluorescein retention change were 0.0 . Thus isocycloseram can be assigned to ICE class I. Furthermore, one corneal surface (1/3) was cleared 120 minutes after the post treatment rinse and two corneal surfaces (2/3) were not cleared 240 minutes after the post-treatment rinse.

Based on this in vitro eye irritation study in isolated chicken eyes, isocycloseram was not classified as a severe irritant but not classified as a nonirritant due to the observation that 2/3 corneal surfaces were not cleared 240 minutes after the post treatment rinse (Váliczkó, 2014).

In an eye irritation study (OECD TG 405), 0.1 mg of isocycloseram (purity 98.4%, Batch no. MEQ-02794-09) was instilled into the conjunctival sac of the left eyes of three male New Zealand White rabbits. The untreated contralateral eyes served as controls. Scoring of irritation effects were performed at 1, 24, 48 and 72 hours after test material instillation. For evaluation, the system of Draize as modified in 1977 was used. Conjunctival redness (score 2) was seen in all rabbits and chemosis (score 1) was seen in one rabbit at one hour after treatment. No corneal or iridial effects were observed. The treated eyes were rinsed with physiological saline solution at one hour after treatment,

as the test item remained in the eye sac. At 24, 48 and 72 hours after the application, no clinical signs and no conjunctival, corneal or iridial effects were observed in any animal. Fluorescein staining was negative in all animals during the observation period. No initial pain reaction or pain reaction at any time point was observed. The control eyes of animals were symptom-free during the study. Isocycloseram was graded as a minimal irritant to the rabbit eye (Matting, 2015b).

In a second study, the ocular irritation of isocycloseram (purity 96.9%, Batch no. SMU5IP15011) was tested in three male Japanese White rabbits by installation of 0.1 mg of the test item into the conjunctival sac of each right eye. Left eyes were untreated and served as controls. Clinical observations were performed at 1, 24, 48 and 72 hours after test material installation. Ocular reactions (corneal opacity, iridial effects, conjunctivae and chemosis) were assessed according to the numerical scoring system in GB/T 15670.8-2017. One hour after treatment, two rabbits had slight redness (hyperaemic blood vessels) in the conjunctivae (1 on a 0–3 scale), slight chemosis of the conjunctivae (1 on a 0–4 scale) and conjunctival secretions. These conjunctival findings did not persist and no signs of eye irritation were seen at 24, 48 or 72 hours after application. No corneal or iridial eye reactions were observed at any time point. As all signs of eye irritation had completely recovered within 24 hours, isocycloseram was classified as a slight irritant to eyes according to the grading criteria by GB/T 15670.8-2017 (Xue, 2021d).

In addition to the studies on skin and eye irritation, the potential of isocycloseram for irritation of the airways was investigated in an in vitro model using cells of human origin that resemble the structure and function of human tracheobronchial epithelium (MucilAir™). Exposure, for 24 hours, was to six concentrations of the test compound in physiological saline ranging from 31.72 to 317.17 mg/L.

Parameters that might indicate a positive effect in a study of this type are a reduction in transepithelial electrical resistance (TEER), an increase in lactate dehydrogenase (LDH) release, or a diminished metabolism of resazurin. In addition, histological evidence of cell damage such as lower viability or a decrease in cell membrane or monolayer integrity might point to a positive response.

No signs of respiratory irritation were observed in this study with isocycloseram up to the highest concentrations. The positive control substance (sodium dodecyl sulfate at 4 mM) caused at least a strong increase in LDH release and a markedly lower TEER, but apparently no cell damage (Wallace, 2022).

Overall, isocycloseram is minimally irritant to rabbits' eyes.

**(d) Dermal sensitization**

In a dermal sensitization study using a local lymph node assay (LLNA; OECD TG 429), CBA/CA female mice were exposed to isocycloseram (purity 96.9%, Batch no. SMU5IP15011) . In the preliminary screening study, the dorsal surface of each ear of one mouse was treated daily with 25 µL of 50% test item (w/w) in acetone/olive oil mixture (ratio 4:1) for three consecutive days. No signs of systemic toxicity, visible local skin irritation or any increase in mean ear thickness were noted. Based on this result, isocycloseram at concentrations of 10%, 25% and 50% of the test item, diluted as in the preliminary screening, were selected for the main study. Groups of five animals were tested with either vehicle only, the test item or a positive control (a 25% solution of α-hexylcinnamaldehyde; carrier as for the test item) by applying 25 µL to the dorsal surfaces of each animal's ears for three consecutive days. Sensitization potential was evaluated by methyl-[$^3$H]-thymidine incorporation five days after the first topical application.

One animal treated with the test item at a concentration of 50% and one animal treated with the test item at a concentration of 25% were found dead on day 4. No previous clinical signs had been noted in these animals. No clinical signs were noted in the surviving animals of any group during the study. The number of responses (radioactive disintegrations per minute) and the stimulation index (SI) increased with increasing concentration. The stimulation index (SI) value at a concentration of 50% was above 3 and the EC3 (the amount of test substance needed to induce a three-fold increase in cell proliferation in a LLNA) was calculated to be 41.2%. The positive control elicited the expected response (SI value of 4.2). Isocycloseram was a dermal sensitizer according to this assay (Pooles, 2016).

In a second study, the dermal sensitization potential of isocycloseram (purity 96.9%, Batch no. SMU5IP15011) was tested in 20 male albino (Dunkin-Hartley) Guinea pigs

using the method of Buehler. For the control group, ten animals were used. Approximately 24 hours before each induction and before the final challenge application, the left flank of each Guinea pig (4–6 $cm^2$) was clipped free of hair. In the induction phase, animals were treated semi-occlusively on the prepared skin area with either 0.4 mL of a 50% preparation of isocycloseram in corn oil, or vehicle only (naïve control group) for six hours, once a week, for three weeks. After six hours the dressings were removed and the application area was cleaned with cotton wool. Two weeks after application of the third induction dose a challenge dose of 0.4 mL of test item was administered in the same manner to all animals. The challenge sites were assessed for irritation/sensitization (erythema and oedema) approximately 24 and 48 hours following challenge, and scored according to GB/T 15670.9-2017.

No signs of skin irritation, systemic toxicity or mortalities were observed in the induction and challenge phase. Scoring for erythema and eschar formation (0–4 scale) and oedema (0–3 scale) 24 or 48 hours following challenge in test or control animals resulted in a maximum score of 0 (0–7 scale) for all animals. Hence, there were no signs of skin sensitization, and isocycloseram was considered non-sensitizing to Guinea pigs. However, the negative results should be regarded with caution, since according to the method, the concentration of test chemical used for each induction exposure should be the highest to cause mild irritation, and in this study no irritation at all was observed in animals (Xue, 2021e).

According to the Guidance on the Application of CLP Criteria, the LLNA is the animal method of first choice, because mouse LLNA measures the responses provoked during the induction of sensitization, whereas the Buehler test measures challenge-induced elicitation reactions in previously sensitized animals (ECHA, 2017). The Buehler test has been shown to be less sensitive.

Overall it was concluded that isocycloseram is sensitizing.

## 2.2 Short-term studies of toxicity

### (a) Oral administration

***Mouse***

In a 28-day toxicity study (OECD TG 407), groups of five Crl:CD-1 mice received isocycloseram (purity 98%, Batch no. MEQ-02958-03) in the diet at concentrations of 0, 100, 300, 700 or 1000 ppm (equal to 0, 17.4, 55.9, 132 and 172 mg/kg bw per day for males, 0, 20.9, 60.5, 142 and 176 mg/kg bw per day for females). Animals were inspected daily for mortality, moribundity and clinical signs of toxicity. Clinical observations, body weights and food consumption were recorded for all animals at predetermined intervals from pretrial until study completion. Blood samples were collected for haematology and clinical chemistry investigations prior to termination. In addition, toxicokinetic samples were collected on days 2, 9, 16 and the day of termination. After completion of 28 days of treatment all surviving animals were killed and subjected to a detailed necropsy, where selected organ were weighed and tissues preserved and histological slides prepared. Histopathological evaluation was carried out for tissues from control and high-dose animals and gross lesions in all groups, and in addition the livers from all dose groups (except control). The adrenals, duodenum and spleen from animals from the high dose group were evaluated. In the medium dose group, the spleen and duodenum were examined from all animals, and the adrenals were examined from females only. In the low-dose group only the spleen from females and the duodenum from males were examined.

Bioanalytical evaluation showed that in general, animals were continuously exposed to measurable concentrations of isocycloseram at all dietary inclusion levels. No mortality occurred and there were no notable treatment-related clinical observations. A group mean body weight loss was observed for males and females receiving 700 and 1000 ppm during the first week of treatment. Body weight performance in these animals then recovered. By the end of the 28-day treatment period slightly lower group mean body weights (by 6%) and overall lower group mean weight gains (by 49%) were observed compared to controls for the males receiving 1000 ppm. For the females receiving 1000 ppm, there were higher group mean body weights (by 7%) and overall higher group mean weight gains (by 104%) compared to controls. In animals receiving 700 ppm the group mean body weights and overall group mean weight gains were similar to controls in both males and females. Food consumption was slightly lower in males and females receiving 700 or 1000 ppm compared with controls at the beginning

of treatment. Thereafter, food consumption in both groups was comparable to controls. Body weight, cumulative body weight change and food consumption in animals receiving diet at 100 or 300 ppm were unaffected by treatment.

Reticulocyte parameters (percentage reticulocytes and absolute count) were increased compared to controls in males and females at 300, 700 and 1000 ppm, with statistical significance achieved only in males at 1000 ppm (increased by 4.7% and 52% respectively) and females at 300 ppm (by 4.7% and 94% respectively). The increase was dose-dependent with males, but less obviously so for females. With regard to the observed effects on spleen weight and histopathology, these effects were considered at 300 ppm and above. Higher group mean white blood cell values compared with controls were seen in males and females at 300, 700 and 1000 ppm, but were not considered adverse since statistical significance was achieved. There were no other notable intergroup differences in haematology parameters that were considered treatment-related.

Results on selected clinical chemistry parameters are shown in Table 15. Statistically significant higher total protein levels compared to controls were noted in males at 700 and 1000 ppm and females at 300, 700 and 1000 ppm. Higher globulin levels compared to controls were noted in males at all dose levels and females at 700 and 1000 ppm. Albumin concentration were unaffected by treatment at all dose levels tested, and as a result, a lower albumin:globulin ratio was noted in males at all dose levels and females at 1000 ppm, compared to controls. Due to the small difference and lack of histopathological correlation the changes noted for males at 100 ppm were considered not to be toxicologically significant by the study authors. Low cholesterol levels compared to controls were noted in males at 300, 700 and 1000 ppm; there was no indication of such an effect in females. High phosphate levels compared to controls were noted in females at 1000 ppm. Overall, treatment-related changes in clinical chemistry were noted at 300 ppm and above, and these correlated with increased liver weights and the findings of liver hypertrophy.

***Table 15. Intergroup comparison of selected clinical chemistry and haematology parameters in a 28-day mouse study with isocycloseram; (mean ± standard deviation, [animals examined])***

| | Dietary concentration (ppm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Males | | | | | Females | | | | |
| **Parameter** | **0** | **100** | **300** | **700** | **1000** | **0** | **100** | **300** | **700** | **1000** |
| Total protein (g/L) | 48 ± 1 [3] | 56 ± 6 [4] | 56 ± 4 [4] | 59* ± 4 [3] | 63** ± 3 [5] | 52 ± 1 [4] | 56 ± 2 [3] | 58* ± 4 [5] | 63** ± 1 [4] | 65** ± 4 [5] |
| Globulin (g/L) | 15 ± 2 [3] | 22* ± 5 [4] | 24** ± 4 [4] | 28** ± 1 [3] | 30** ± 2 [5] | 18 ± 1 [4] | 21 ± 3 [3] | 22 ± 5 [5] | 26** ± 2 [4] | 29** ± 3 [5] |
| Albumin:globulin ratio | 2.2 ± 0.4 [3] | 1.6** ± 0.3 [4] | 1.4** ± 0.3 [4] | 1.2** ± 0.2 [3] | 1.1** ± 0.1 [5] | 1.9 ± 0.2 [4] | 1.7 ± 0.3 [3] | 1.7 ± 0.5 [5] | 1.4 ± 0.1 [4] | 1.3** ± 0.1 [5] |
| Cholesterol (mmol/L) | 2.6 ± 0.2 [4] | 2.6 ± 0.7 [4] | 1.7* ± 0.5 [4] | 1.8 ± 0.3 [3] | 1.2** ± 0.3 [5] | 1.6 ± 0.2 [4] | 1.9 ± 0.5 [3] | 1.6 ± 0.3 [5] | 1.3 ± 0.4 [4] | 1.9 ± 1.0 [5] |
| Phosphate (mmol/L) | 2.59 ± 0.20 [3] | 2.12 ± 0.10 [3] | 2.69 ± 0.54 [4] | 2.78 ± 0.35 [3] | 2.98 ± 0.60 [5] | 2.37 ± 0.47 [4] | 2.74 ± 0.08 [3] | 2.83 ± 0.67 [4] | 3.27 ± 0.40 [2] | 3.60** ± 0.15 [4] |
| Reticulocytes (percentage) | 2.9 ± 0.2 [5] | 3.0 ± 0.5 [4] | 3.7 ± 0.9 [5] | 4.2 1.4 [5] | 4.7* ± 1.1 [4] | 2.3 ± 0.8 [5] | 2.9 ± 0.7 [5] | 4.7* ± 1.9 [3] | 3.3 0.9 [4] | 4.1 ± 1.1 [5] |
| Reticulocyte count ($\times 10^9$/L) | 272 ± 15 [5] | 264 ± 58 [4] | 349 ± 68 [5] | 389 ± 121 [5] | 413* ± 70 [4] | 208 ± 80 [5] | 273 ± 70 [5] | 404* ± 169 [3] | 310 ± 75 [4] | 363 ± 88 [4] |

| | Dietary concentration (ppm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Males | | | | | Females | | | | |
| **Parameter** | **0** | **100** | **300** | **700** | **1000** | **0** | **100** | **300** | **700** | **1000** |
| White blood cell count ($\times 10^9$/L) | 6.78 ± 3.23 [5] | 6.64 ± 1.79 [4] | 7.56 ± 3.06 [5] | 8.53 2.88 [5] | 7.35 ± 2.48 [4] | 6.55 ± 3.47 [5] | 5.45 ± 0.63 [5] | 7.61 ± 2.37 [3] | 8.30 1.26 [4] | 8.71 ± 3.53 [5] |

Source: Dymarkowska, 2015

Different from control group mean at statistical significance: * $p < 0.05$, ** $p < 0.01$

There were test substance-related findings in the liver. Hepatocyte hypertrophy was observed at 300, 700 and 1000 ppm in males and females. The zonal distribution of this was mainly centrilobular, extending to diffuse on occasions. These findings correlate with gross observations, showing prominent lobular architecture at 100 (1/5), 300 (3/5), 700 (2/5) and 1000 ppm (3/5) in males and 300 ppm (1/5) in females, as well as higher liver weights. Pale discolouration was observed in one male at 100 ppm and one female at each 300 and 1000 ppm. There were no changes in liver function tests, but some changes in clinical chemistry. The effects on liver were considered adverse at 300 ppm and above.

Treatment-related findings were also observed in the adrenal gland (hypertrophy of the zona fasciculata) of females at 700 and 1000 ppm, and males at 1000 ppm. The duodenum was affected (epithelial vacuolation) in males and females at 700 and 1000 ppm, and in males at 300 ppm.

***Table 16. Intergroup comparison of selected organ weights in a 28-day mouse study with isocycloseram; (mean weight ± standard deviation shown)***

| | Dietary concentration (ppm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Males | | | | | Females | | | | |
| **Parameter** | **0** | **100** | **300** | **700** | **1000** | **0** | **100** | **300** | **700** | **1000** |
| Number of mice examined | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| Liver weight; absolute (g) | 1.92 ± 0.20 | 2.23* ± 0.14 | 2.36** ± 0.20 | 2.35** ± 0.21 | 2.38** ± 0.09 | 1.48 ± 0.14 | 1.59 ± 0.09 | 1.83 ± 0.45 | 1.73 ± 0.19 | 2.17* ± 0.60 |
| Liver weight; relative[a] (%) | 5.53 ± 0.45 | 6.12 ± 0.24 | 6.52* ± 0.35 | 6.76* ± 0.47 | 7.41* ± 0.29 | 5.91 ± 0.38 | 5.88 ± 0.34 | 6.86 ± 1.45 | 6.86 ± 0.29 | 8.09* ± 1.75 |
| Adrenal weight; absolute (g) | 0.0048 ± 0.0027 | 0.0043 ± 0.0015 | 0.0075 ± 0.0041 | 0.0059 ± 0.0009 | 0.0066 ± 0.0005 | 0.0109 ± 0.0013 | 0.0102 ± 0.0048 | 0.0099 ± 0.0016 | 0.0132 ± 0.0039 | 0.0135 ± 0.0054 |
| Adrenal weight; relative[a] (%) | 0.0138 ± 0.0078 | 0.0118 ± 0.0033 | 0.0205 ± 0.0103 | 0.0169 ± 0.0028 | 0.0204 ± 0.0022 | 0.043 ± 0.0061 | 0.0377 ± 0.0178 | 0.0373 ± 0.0071 | 0.0530 ± 0.0178 | 0.0514 ± 0.0219 |
| Spleen weight; absolute (g) | 0.089 ± 0.022 | 0.100 ± 0.019 | 0.126 ± 0.015 | 0.124 ± 0.040 | 0.134 ± 0.034 | 0.101 ± 0.020 | 0.103 ± 0.018 | 0.149 ± 0.051 | 0.159 ± 0.091 | 0.141 ± 0.043 |
| Spleen weight; relative[a] (%) | 0.259 ± 0.064 | 0.275 ± 0.054 | 0.349 ± 0.025 | 0.354 ± 0.101 | 0.416* ± 0.101 | 0.404 ± 0.065 | 0.378 ± 0.051 | 0.556 ± 0.170 | 0.620 ± 0.321 | 0.525 ± 0.127 |

[a] Organ weight relative to body weight;

Source: Dymarkowska, 2015

Different from control group mean at statistical significance: * $p < 0.05$, ** $p < 0.01$

Extramedullary haematopoiesis was more severe than in controls in the spleens of males and females at 700 and 1000 ppm, and also in females at 300 ppm. This correlated with the higher spleen weights noted particularly in females.

There were no treatment-related gross or microscopic findings at 100 ppm. Other findings observed were considered incidental, of a nature commonly observed in this strain and age of mouse, and/or were of similar incidence and severity in control and treated animals.

*Table 17. Test substance-related microscopic findings in a 28-day mouse study with isocycloseram*

| | | Dietary concentration of SYN547407 (ppm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | | Females | | | | |
| **Parameter** | | **0** | **100** | **300** | **700** | **1000** | **0** | **100** | **300** | **700** | **1000** |
| Liver (number examined) | | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| Hepatocyte hypertrophy | total | 0 | 0 | 4 | 5 | 5 | 0 | 0 | 2 | 4 | 5 |
| | minimal | 0 | 0 | 3 | 3 | 3 | 0 | 0 | 1 | 3 | 3 |
| | mild | 0 | 0 | 1 | 2 | 2 | 0 | 0 | 1 | 1 | 2 |
| Adrenal (number examined) | | 5 | 0 | 0 | 5 | 5 | 5 | 0 | 5 | 5 | 5 |
| Hypertrophy, zona fasciculata | total | 1 | - | - | 0 | 4 | 0 | - | 0 | 4 | 5 |
| | minimal | 1 | - | - | 0 | 4 | 0 | - | 0 | 2 | 0 |
| | mild | 0 | - | - | 0 | 0 | 0 | - | 0 | 2 | 5 |
| Duodenum (number examined) | | 5 | 5 | 5 | 5 | 5 | 5 | 0 | 5 | 5 | 5 |
| Epithelial vacuolation | total | 0 | 0 | 1 | 2 | 1 | 0 | - | 0 | 2 | 2 |
| | minimal | 0 | 0 | 0 | 2 | 1 | 0 | - | 0 | 1 | 1 |
| | mild | 0 | 0 | 1 | 0 | 0 | 0 | - | 0 | 1 | 1 |
| Spleen (number examined) | | 5 | 0 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| Extramedullary haemopoeisis | total | 5 | - | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| | minimal | 5 | - | 3 | 1 | 0 | 4 | 3 | 1 | 1 | 1 |
| | mild | 0 | - | 2 | 4 | 5 | 1 | 2 | 4 | 3 | 3 |
| | moderate | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |

Source: Dymarkowska, 2015

The NOAEL for oral toxicity of isocycloseram in the 28-day study in mice was 100 ppm (equal to 17.4 mg/kg bw per day) based on histopathological changes in liver, spleen and duodenum at the LOAEL of 300 ppm (equal to 55.9 mg/kg bw per day) (Dymarkowska, 2015).

In a 90-day toxicity study (OECD 408), groups of ten Crl:CD-1 mice received isocycloseram (purity 98.4%, Batch No. MEQ–02794-09) in the diet at concentrations of 0, 50, 300 or 700 ppm (equal to 0, 8.0, 48.8 and 117 mg/kg bw per day for males, 0, 9.9, 51.6 and 140 mg/kg bw per day for females). Animals were inspected daily for mortality, moribundity and clinical signs of toxicity. Clinical observations, body weights, food consumption and achieved dosage were assessed on all animals at predetermined intervals from pretreatment until study completion. Blood samples were collected for haematology and clinical chemistry investigation prior to termination. In addition, toxicokinetic samples were collected for days 2, 28 and 85. After completion of 13 weeks of treatment all surviving animals were killed and subjected to a detailed necropsy, where selected organ were weighed and tissues were preserved and histological slides prepared. Histopathological evaluation was carried out for tissues from control and high-dose animals and any unscheduled deaths. Gross lesions, duodenum, liver, spleen, adrenal glands and thymus were also evaluated for low- and mid-dose animals. In addition, microvesicular vacuolation in the liver was examined using Oil Red O staining.

Systemic exposure to isocycloseram was measured in the blood; results are shown in Table 13. No treatment-related mortality occurred and there were no notable treatment-related clinical observations. Body weight loss was observed for males and females in all groups (including controls) during the first week of treatment. Loss was higher for the high-dose group, reaching statistical significance in both sexes. Thereafter body weight performance recovered by day 7. From day 7 onwards the body weight of males receiving 700 ppm was slightly lower than controls, such that by day 91 the overall gain was lower than controls by 29%. Group mean body weight and body weight gain in males and females at 50 and 300 ppm, and females at 700 ppm, were considered to be unaffected by treatment. Overall food consumption in males and females receiving 50, 300 or 700 ppm was considered to be similar to that of the controls.

A statistically significant lowering of haemoglobin (Hb; by 8.1%), and haematocrit (Ht; by 7.8%) were noted in males and lower red blood counts (statistically significant) were noted for males (by 7.8%) and females (by 7.7%) receiving 700 ppm: also statistically significant was a higher red blood cell distribution width at this dose level (by 16% in males only). Higher reticulocyte counts compared to controls were noted in males (by 32%) and females (by 11%) at 700 ppm but without statistical significance. With regard to the observed effects on spleen weight (at 700 ppm) and histopathology (at 300 ppm), these effects were considered treatment-related. A higher white blood cell count was noted in males and females receiving 300 or 700 ppm, but statistical significance was only achieved in females at 700 ppm. At this dose, also, a higher thymus weight was noted in females. There were no other changes in haematology parameters considered to be related to dietary exposure to isocycloseram.

Activities measured for aspartate transaminase (AST; males and females), alanine transaminase (ALT; males and females) and alkaline phosphatase (ALP; females) were slightly increased in the highest dose group compared to controls. Lower triglycerides at 700 ppm and cholesterol levels at 300 and 700 ppm were also noted, as well as some changes in electrolyte levels. Higher total protein and globulin levels were noted at 300 and 700 ppm compared to controls. Albumin concentrations were unaffected by treatment at all dose levels tested, and as a result lower albumin:globulin ratios were also observed. Overall, adverse changes in clinical chemistry parameters were observed at 300 ppm and above.

***Table 18. Intergroup comparison of selected clinical chemistry parameters in a 90-day mouse study with isocycloseram (mean values ± standard deviation shown)***

| | Dietary concentration (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Parameter** | **0** | **50** | **300** | **700** | **0** | **50** | **300** | **700** |
| Animals (No. examined) | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| AST (U/L) | 50 ± 16 | 56 ± 20 | 84 ± 43 | 93* ± 57 | 54 ± 8 | 64 ± 17 | 79 ± 42 | 148 ± 203 |
| ALT (U/L) | 32 ± 8 | 35 ± 11 | 61 ± 31 | 72** ± 42 | 28 ± 7 | 30 ± 11 | 50 ± 60 | 83 ± 124 |
| ALP (U/L) | 42 ± 7 | 44 ± 17 | 39 ± 10 | 49 ± 14 | 56 ± 14 | 59 ± 17 | 61 ± 12 | 74* ± 13 |
| Triglycerides (mmol/L) | 1.31 ± 0.41 | 1.44 ± 0.54 | 1.02 ± 0.65 | 0.83 ± 0.36 | 1.05 ± 0.40 | 1.31 ± 0.71 | 0.68 [a] ± 0.34 | 0.44* ± 0.37 |
| Total protein (g/L) | 51 ± 1 | 53 ± 2 | 57** ± 2 | 57** ± 3 | 50 ± 2 | 51 ± 2 | 57** [a] ± 4 | 60** ± 3 |
| Globulin (g/L) | 18 ± 2 | 19 ± 2 | 24** ± 1 | 24** ± 2 | 13 ± 2 | 15 ± 2 | 21** [a] ± 3 | 23** ± 3 |
| Albumin:globulin ratio | 1.9 ± 0.3 | 1.8 ± 0.2 | 1.4** ± 0.1 | 1.4** ± 0.1 | 2.8 ± 0.4 | 2.4* ± 0.3 | 1.8** [a] ± 0.3 | 1.7** ± 0.3 |
| Cholesterol (mmol/L) | 3.4 ± 0.7 | 3.1 ± 0.5 | 2.0** ± 0.5 | 1.3** ± 0.3 | 2.2 ± 0.6 | 2.5 ± 0.8 | 1.7 [a] ± 0.4 | 1.7 ± 0.6 |
| Phosphate (mmol/L) | 2.19 ± 0.36 | 2.32 ± 0.69 | 2.14 ± 0.21 | 2.97** ± 0.77 | 1.90 ± 0.34 | 2.11 ± 0.45 | 2.09 [a] ± 0.21 | 2.61** [a] ± 0.71 |
| Chloride (mmol/L) | 112 ± 1 | 111 ± 2 | 111 ± 1 | 111 ± 2 | 112 ± 1 | 112 ± 2 | 111 ± 1 | 109** ± 2 |
| Calcium (mmol/L) | 2.43 ± 0.07 | 2.37 ± 0.09 | 2.31** ± 0.06 | 2.26** ± 0.06 | 2.39 ± 0.07 | 2.38 ± 0.10 | 2.38 [a] ± 0.06 | 2.32 [a] ± 0.08 |

[a] Only 9 animals were examined; Source: Laidlaw, 2019a

ALT: Alanine transaminase; ALP: Alkaline phosphatase; AST: Aspartate transaminase

Different from control group mean at statistical significance: * $p < 0.05$, ** $p < 0.01$

Higher liver (males and females), adrenal (females), spleen (males and females), and thymus (females) weights were observed when compared to controls, in the case of both absolute and relative organ weights. These increases were statistically significant predominantly at 700 ppm. In females, the changes in liver weight were also statistically significant at 300 ppm.

***Table 19. Intergroup comparison of selected organ weights in a 90-day mouse study with isocycloseram (mean values ± standard deviation shown)***

| | Dietary concentration (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Parameter** | **0** | **50** | **300** | **700** | **0** | **50** | **300** | **700** |
| Liver (examined) | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Liver weight; absolute (g) | 2.04 ± 0.27 | 2.19 ± 0.25 | 2.22 ± 0.17 | 2.38** ± 0.23 | 1.63 ± 0.22 | 1.65 ± 0.27 | 2.11** ± 0.25 | 2.28** ± 0.28 |
| Liver weight; relative[a] (%) | 5.30 ± 0.75 | 5.45 ± 0.48 | 5.84 ± 0.31 | 6.28** ± 0.36 | 5.39 ± 0.47 | 5.68 ± 0.71 | 6.66** ± 0.54 | 7.17** ± 0.58 |
| Adrenal (examined) | 8 | 10 | 8 | 10 | 10 | 9 | 10 | 10 |
| Adrenal weight; absolute (g) | 0.0048 ± 0.0020 | 0.0054 ± 0.0011 | 0.0056 ± 0.0016 | 0.0057 ± 0.0018 | 0.0102 ± 0.0025 | 0.0094 ± 0.0019 | 0.0116 ± 0.0039 | 0.0147* ± 0.0042 |
| Adrenal weight; relative[a] (%) | 0.0114 ± 0.0061 | 0.0137 ± 0.0035 | 0.0150 ± 0.0043 | 0.0150 ± 0.0044 | 0.0339 ± 0.0082 | 0.0328 ± 0.0062 | 0.0369 ± 0.0130 | 0.0463* ± 0.0127 |
| Spleen (examined) | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Spleen weight; absolute (g) | 0.103 ± 0.030 | 0.108 ± 0.014 | 0.150 ± 0.082 | 0.165* ± 0.036 | 0.132 ± 0.042 | 0.105 ± 0.030 | 0.160 ± 0.059 | 0.192* ± 0.060 |
| Spleen weight; relative[a] (%) | 0.268 ± 0.086 | 0.271 ± 0.040 | 0.395 ± 0.223 | 0.438* ± 0.107 | 0.438 ± 0.135 | 0.361 ± 0.090 | 0.499 ± 0.159 | 0.599* ± 0.149 |
| Thymus (examined) | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Thymus weight; absolute (g) | 0.034 ± 0.015 | 0.034 ± 0.010 | 0.036 ± 0.014 | 0.033 ± 0.007 | 0.038 ± 0.008 | 0.031 ± 0.007 | 0.044 ± 0.013 | 0.051* ± 0.012 |
| Thymus weight; relative[a] (%) | 0.085 ± 0.032 | 0.086 ± 0.031 | 0.094 ± 0.036 | 0.087 ± 0.017 | 0.126 ± 0.029 | 0.106 ± 0.027 | 0.139 ± 0.040 | 0.161 ± 0.037 |

[a] Organ weight relative to body weight; Source: Laidlaw, 2019a

Different from control group mean at statistical significance: * $p < 0.05$, ** $p < 0.01$

There were test substance-related histopathological findings in the liver. Minimal to mild centrilobular hypertrophy was observed at 700 ppm in males and females. Moreover, minimal to mild microvesicular vacuolation was observed in 5/10 males (statistically significant) and 3/10 females (not significant) at 700 ppm. These findings correlate with gross observations that show increased incidence of prominent lobular architecture at 50 (2/10), 300 (2/10) and 700 ppm (4/10) in males and at all dose levels (1/10) in females. Microscopic findings were also noted in adrenal glands (vacuolation of the zona fasciculata in females at 700 ppm), duodenum (epithelial cell vacuolation in females at 700ppm), spleen (increased erythropoiesis and increased lymphoid cellularity in both sexes at 300 ppm) and thymus (increased lymphoid cortical cellularity in females at 700 ppm). These results are summarized in Table 20, below.

*Table 20. Test substance-related microscopic findings in a 90-day mouse study with isocycloseram*

| | | Dietary concentration of SYN547407 (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | Females | | | |
| **Finding** | | **0** | **50** | **300** | **700** | **0** | **50** | **300** | **700** |
| **Animals (number examined)** | | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| **Liver** | | | | | | | | | |
| Centrilobular hypertrophy | total | 0 | 0 | 0 | 7** | 0 | 0 | 0 | 5* |
| | minimal | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 3 |
| | mild | 0 | 0 | 0 | 5* | 0 | 0 | 0 | 2 |
| | total | 0 | 0 | 0 | 5* | 0 | 0 | 0 | 3 |
| Microvesicular vacuolation | minimal | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 1 |
| | mild | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 2 |
| **Adrenal** | | | | | | | | | |
| Vacuolation, zona glomerulosa | total | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| | minimal | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| Vacuolation, zona fasciculata | total | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 3 |
| | minimal | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| | mild | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| **Duodenum** | | | | | | | | | |
| Vacuolation, epithelial cells | total | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| | minimal | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| **Spleen** | | | | | | | | | |
| Increased erythropoiesis | total | 0 | 0 | 3 | 2 | 1 | 3 | 3 | 3 |
| | minimal | 0 | 0 | 2 | 1 | 1 | 0 | 1 | 2 |
| | mild | 0 | 0 | 1 | 1 | 0 | 2 | 1 | 0 |
| | moderate | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| Increased lymphoid cellularity | total | 0 | 0 | 3 | 4 | 0 | 0 | 2 | 4 |
| | minimal | 0 | 0 | 2 | 0 | 0 | 0 | 2 | 2 |
| | mild | 0 | 0 | 1 | 4 | 0 | 0 | 0 | 2 |
| **Thymus** | | | | | | | | | |
| Increased cellularity, lymphoid, cortex | total | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| | minimal | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| | mild | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |

Different from control group mean at statistical significance: * $p < 0.05$, ** $p < 0.01$; Source: Laidlaw, 2019a

Overall, higher reticulocyte counts and red blood cell mass (males only) compared to controls were seen at 300 ppm although statistical significance was not achieved. Lower cholesterol and calcium levels compared to controls were seen at 300ppm. When compared to controls, higher weights of adrenal gland (females), liver (males and females), spleen (males and females) and thymus (females) were observed at 700 ppm. Macroscopic findings were observed in the liver, including prominent lobular architecture in both sexes at 700 ppm. Microscopic findings seen in the adrenal gland consisted of vacuolation of the zona glomerulosa and zona fasciculata, in males and females respectively, at 300 ppm. Microscopic findings in the spleen were increased erythropoiesis and increased lymphoid cellularity in males and females at 300 ppm.

At 50 ppm macroscopic findings were observed in the liver (prominent lobular architecture) in males and females. The prominent lobular architecture was not associated with increased liver weights

or microscopic findings, so it was considered not to be adverse. Microscopic findings were seen in the spleen consisting of increased erythropoiesis in females. This was not however associated with increased spleen weight or changes in red blood cell parameters at this dose level, so it was considered not to be adverse.

The NOAEL for oral toxicity of isocycloseram in a 90-day study in mice was 50 ppm (equal to 8.0 mg/kg bw per day), based histopathological changes in adrenals and spleen (both sexes) and supported by changes in clinical chemistry parameters at the LOAEL of 300 ppm (equal to 48.8 mg/kg bw per day) (Laidlaw, 2019a).

***Rat***

In a 28-day toxicity study (OECD TG 407), groups of five Han Wistar rats (Crl:WI(Han)) received isocycloseram (purity 98%, MEQ-02958-03) in the diet of males at concentrations of 0, 50, 200, 350 or 500 ppm (equal to 0, 4.3, 16.3, 26.8 and 37.0 mg/kg bw per day) and females at 0, 50 and 700 ppm (equal to 0, 4.5 and 50.1 mg/kg bw per day). Two female groups fed diet at 800 and 1000 ppm (equal to 48.9 and 46 mg/kg bw per day) were terminated prematurely on day 11 of the study due to mortality (1/5 animals on day 11 at 800 ppm, 2/5 animals on day 10 at 1000 ppm) and unscheduled termination (2/5 animals on day 9 at 800 ppm) due to severity of the clinical signs observed. These doses were considered to be in excess of a maximum tolerated dose. Following a request from the sponsor, an evaluation of unscheduled deaths from female groups at 800 and 1000 ppm was not performed and the 700 ppm was treated as the high-dose group for females. The dose level selection was based on individual 14-day dose range-finding studies; data were not provided.

Animals were inspected daily for mortality, moribundity and clinical signs of toxicity. Clinical observations, body weights, food consumption were assessed for all animals at predetermined intervals from pretrial until study completion. Urine samples were collected during week 4 for analysis. Blood samples were collected on days 2, 9, 16 and prior to termination for bioanalytical evaluation. Blood samples were collected for haematology, coagulation and clinical chemistry investigations prior to termination. After completion of 28 days of treatment, all surviving animals were euthanized and subjected to a detailed necropsy, where selected organs were weighed and tissues preserved and histological slides prepared. Histopathological evaluation was undertaken for all control and high-dose animals (500 ppm in males and 700 ppm in females) and target tissues from males and females in the low-dose (50 ppm) and from the two intermediate male groups (200 and 350 ppm).

There were no prior clinical signs recorded for any of the animals found dead. Group mean body weights, cumulative body weight changes and food consumption were lower in females receiving 800 or 1000 ppm. At 800 ppm, clinical signs were observed in 2/5 animals; signs included muscles spasms, reduced activity, laboured respiration and subdued behaviour or convulsions, nervous and aggressive behaviour, piloerection, hunched body posture and excess salivation. Animals receiving diet at 1000 ppm showed non-specific clinical signs, such as piloerection and a body weight loss. One female receiving diet at 700 ppm underwent unscheduled termination on day 12 due to hunched body posture, laboured respiration, body weight loss and piloerection. Three surviving females receiving 700 ppm were noted with piloerection from day 12, 13 or 17 until the end of the dosing. There were no adverse clinical signs related to the treatment noted in the males fed 50, 200, 350 or 500 ppm, or females at 50 ppm.

Systemic exposure to isocycloseram was measured in the blood; results are given in Table 13. Females receiving 700 ppm and males at 350 and 500 ppm had statistically significant lower mean body weights and cumulative body weights throughout the treatment period when compared with controls. Food consumption was slightly lower compared to controls in males receiving diet at 500 ppm and in females at 700 ppm. Body weight, body weight gain and food consumption at 200 ppm (males only) and 50 ppm (males and females) were considered to be similar to those of the control group.

There was a higher white blood cell count compared to controls (statistically significant) in females at 700 ppm. This was characterized by higher counts of lymphocytes, monocytes, basophils and large, unclassified cells. No other haematology or coagulation parameters were considered to be related to treatment.

In males, urea and creatinine levels were higher at 500 ppm and phosphate levels were lower; both differences from controls were statistically significant. In females, urea was higher at 700 ppm (statistically significant) compared to the control animals. Lower triglyceride and cholesterol levels were observed in males receiving 500 ppm (not statistically significant) and in females at 700 ppm (statistically significant) when compared to controls. Lower triglyceride levels were also observed at 50 ppm in females (statistically significant). Total protein and albumin concentrations were lower in females receiving 700 ppm. Some minor intergroup differences were observed in a few parameters (globulin:albumin ratios were higher and globulin lower in males at 500 ppm) but they were considered to be due to biological variability since the mean values were within historical control data (HCD) ranges. No other clinical chemistry parameters were considered to be related to treatment.

***Table 21. Intergroup comparison of selected clinical chemistry parameters in a 28-day rat study with isocycloseram (mean values ± standard deviation shown)***

| | **Dietary concentration (ppm)** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Males** | | | | | **Females** | | | | |
| **Parameter** | **0** | **50** | **200** | **350** | **500** | **0** | **50** | **700** | **800** | **1000** |
| Number of animals | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 0 | 0 |
| Urea, (mmol/L) | 5.6 ± 0.3 | 5.8 ± 0.3 | 5.7 ± 0.5 | 6.3 ± 0.7 | 7.4** ± 0.5 | 6.2 ± 0.6 | 5.8 ± 0.8 | 7.6* ± 1.1 | - | - |
| Creatinine, (µmol/L) | 31 ± 3 | 33 ± 2 | 31 ± 4 | 33 ± 4 | 36* ± 2 | 32 ± 2 | 36 ± 4 | 38 ± 5 | - | - |
| Phosphate, (mmol/L) | 1.80 ± 0.14 | 1.81 ± 0.27 | 1.99 ± 0.17 | 1.76 ± 0.26 | 1.36* ± 0.28 | 1.51 ± 0.09 | 1.72 ± 0.25 | 1.38 ± 0.27 | - | - |
| Triglyceride, (mmol/L) | 1.76 ± 0.31 | 1.65 ± 0.31 | 1.52 ± 0.35 | 1.32 ± 0.32 | 1.17 ± 0.43 | 1.82 ± 0.35 | 0.87* ± 0.26 | 0.58** ± 0.28 | - | - |
| Cholesterol, (mmol/L) | 1.6 ± 0.2 | 1.7 ± 0.4 | 1.6 ± 0.4 | 1.4 ± 0.2 | 1.2 ± 0.2 | 1.4 ± 0.2 | 1.3 ± 0.2 | 0.8** ± 0.1 | - | - |
| Globulin:albumin ratio | 2.2 ± 0.2 | 2.6 ± 0.7 | 2.5 ± 0.2 | 2.6 ± 0.3 | 2.9* ± 0.2 | 3.1 ± 0.4 | 2.9 ± 0.4 | 3.2 ± 0.6 | - | - |
| Globulin, (g/L) | 19 ± 1 | 17 ± 3 | 18 ± 1 | 17 ± 1 | 16* ± 1 | 15 ± 1 | 16 ± 2 | 14 ± 2 | - | - |

Different from control group mean at statistical significance: * $p < 0.05$, ** $p < 0.01$ Source: Dymarkowska, 2017

Specific gravity was noted to be lower compared to controls in females at 700 ppm, achieving statistical significance. This was considered to result from the low urine volumes collected for control group females. There were no other notable intergroup differences in urinalysis parameters that were considered related to treatment.

Higher adrenal weights were observed in the treated animals compared with controls, and these followed a dose dependent pattern for both absolute and relative organ weights. Statistically significance was noted at 200, 350 and 500 ppm for males and at 700 ppm for females. No other test item-related organ weight changes were noted. There were other, isolated organ weights that differed from their respective controls, but no patterns, trends, or correlating data to suggest that these values were toxicologically relevant.

*Table 22. Intergroup comparison of selected organ weights in a 28-day rat study with isocycloseram (mean values ± standard deviation shown)*

| | Dietary concentration (ppm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Males | | | | | Females | | | | |
| **Parameter** | **0** | **50** | **200** | **350** | **500** | **0** | **50** | **700** | **800** | **1000** |
| No animals | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 0 | 0 |
| Adrenal weight; absolute (g) | 0.0582 ± 0.0126 | 0.0658 ± 0.0057 | 0.0802* ± 0.0129 | 0.0755 ± 0.0120 | 0.0911** ± 0.0050 | 0.0845 ± 0.0110 | 0.0811 ± 0.0101 | 0.1453** ± 0.0194 | - | - |
| Adrenal weight; relative[a] (%) | 0.0172 ± 0.0033 | 0.0190 ± 0.0016 | 0.0233** ± 0.0037 | 0.0249** ± 0.0026 | 0.0341** ± 0.0022 | 0.0379 ± 0.0077 | 0.0392 ± 0.0036 | 0.0797** ± 0.0063 | - | - |
| Liver weight; absolute (g) | 12.87 ± 1.71 | 12.46 ± 1.47 | 13.28 ± 1.16 | 11.78 ± 0.90 | 10.47* ± 0.88 | 8.73 ± 0.55 | 7.59 ± 1.12 | 7.35 ± 1.02 | - | - |
| Liver weight; relative[a] (%) | 3.91 ± 0.35 | 3.59 ± 0.33 | 3.86 ± 0.32 | 3.89 ± 0.25 | 3.91 ± 0.16 | 3.89 ± 0.31 | 3.65 ± 0.17 | 4.04 ± 0.45 | - | - |
| Spleen weight; absolute (g) | 0.53 ± 0.08 | 0.53 ± 0.07 | 0.57 ± 0.07 | 0.58 ± 0.09 | 0.50 ± 0.06 | 0.45 ± 0.02 | 0.43 ± 0.04 | 0.36** ± 0.04 | - | - |
| Spleen weight; relative[a] (%) | 0.16 ± 0.03 | 0.15 ± 0.03 | 0.17 ± 0.02 | 0.19 ± 0.04 | 0.19 ± 0.03 | 0.20 ± 0.02 | 0.21 ± 0.02 | 0.20 ± 0.02 | - | - |
| Kidney weight; absolute (g) | 2.16 ± 0.22 | 2.23 ± 0.20 | 2.40 ± 0.19 | 2.27 ± 0.30 | 2.03 ± 0.08 | 1.55 ± 0.07 | 1.43 ± 0.13 | 1.37 ± 0.14 | - | - |
| Kidney weight; relative[a] (%) | 0.66 ± 0.05 | 0.64 ± 0.02 | 0.70 ± 0.08 | 0.75* ± 0.03 | 0.76** ± 0.03 | 0.69 ± 0.05 | 0.69 ± 0.06 | 0.76 ± 0.05 | - | - |

[a] Organ weight relative to body weight; Source: Dymarkowska, 2017

Different from control group mean at statistical significance: * $p < 0.05$, ** $p < 0.01$

In the females at 800 and 1000 ppm (terminated prematurely due to excessive toxicity), enlargement of the adrenal gland was observed in 3/5 females from both groups at necropsy. Pale discolouration of the adrenal gland was also observed in 2/5 females at 1000 ppm. Dark discolouration was observed in several tissues across the unscheduled deaths. This finding was considered to be due to congestion related to the agonal process. At necropsy of those animals surviving until scheduled termination, the gross findings observed were considered incidental, of the nature commonly seen in this strain and age of rats, and/or were of similar incidence in control and treated animals, and therefore were considered not to be related to treatment.

Microscopic findings were observed in the adrenal gland (hypertrophy of the zona fasciculata) of males receiving diet at 200, 350 and 500 ppm, and females receiving 700 ppm. Hypertrophy of the zona fasciculata was characterized by increased cell size with or without cytoplasmic vacuolation. The microscopic findings observed in the adrenal gland correlated with higher adrenal weights in high-dose animals compared to controls. Microscopic findings were observed in the kidney (tubular vacuolation in females receiving 700 ppm and in the liver (hepatocyte vacuolation diffuse and/or centrilobular) of males receiving 500 ppm and females 700 ppm in the diet. Other microscopic findings observed were considered incidental, of a nature commonly seen in this strain and age of rats, and/or were of similar incidence and severity in control and treated animals, and therefore, not considered treatment related.

*Table 23. Test substance-related microscopic findings in a 28-day rat study with isocycloseram*

| | | Dietary concentration (ppm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | | Females | | | | |
| **Parameter** | | **0** | **50** | **200** | **350** | **500** | **0** | **50** | **700** | **800** | **1000** |
| **Adrenal** (number examined) | | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 0 | 0 |
| Hypertrophy, zona fasciculata | total | 0 | 0 | 2 | 4* | 5** | 0 | 0 | 4** | – | – |
| | minimal | 0 | 0 | 2 | 4* | 5** | 0 | 0 | 2 | – | – |
| | mild | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | – | – |

| | | Dietary concentration (ppm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | | Females | | | | |
| **Parameter** | | **0** | **50** | **200** | **350** | **500** | **0** | **50** | **700** | **800** | **1000** |
| **Liver** (number examined) | | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 0 | 0 |
| Hepatocyte vacuolation, diffuse | total | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | - |
| | mild | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | - |
| Hepatocyte vacuolation, centrilobular | total | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | - | - |
| | mild | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | - | - |
| | moderate | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | - | - |
| **Kidney** (number examined) | | 5 | - | - | - | 5 | 5 | 5 | 4 | - | - |
| Tubular vacuolation, cortical | total | 0 | - | - | - | 0 | 0 | 0 | 2 | - | - |
| | minimal | 0 | - | - | - | 0 | 0 | 0 | 2 | - | - |

Different from control group mean at statistical significance: * $p < 0.05$, ** $p < 0.01$; Source: Dymarkowska, 2017

Overall, adverse findings were observed in males receiving daily administrations at 200 ppm and above, and in females receiving 700 ppm, both for at least 28 consecutive days

The NOAEL for oral toxicity of isocycloseram in a 28-day study in rats was 50 ppm (equal to 4.3 mg/kg bw per day) was based on histopathological findings in the adrenals of males at the LOAEL of 200 ppm (equal to 16.3 mg/kg bw per day). In females, the dietary NOAEL was the same, but the wide margin to the next dose level of 700 ppm (equal to 50.1 mg/kg bw per day) must be taken into account, as this was level at which were noted clinical signs, reductions in body weight gain and food consumption, alterations in a few haematological and clinical chemistry parameters and microscopic findings in adrenals, liver, and kidneys. Dietary administration of isocycloseram to female rats at concentrations of 800 ppm and above was not tolerated (Dymarkowska, 2017).

Isocycloseram, its two isomers SYN548089 and SYN548090, and a racemic mixture of these three test articles (SYN548285) were examined for toxicity in a comparative dietary, 28-day study in groups of five male and five female Han:Wistar rats. All test substances were fed at nominal dose levels of 100, 200 or 400 ppm to male animals and of 100, 300 or 600 ppm to females. A single control group of the same size and composition was administered a test article-free diet. The study design was compliant with OECD TG 407 and similar to the 28-day study by Dymarkowska (2017) described above.

In this monograph only results for technical isocycloseram (SYN547407) are briefly reported. It is worth noting, however, that the toxicological findings with isocycloseram and racemate SYN548285 were broadly similar. Isomer SYN548089 produced similar effects in females and did not cause any significant effects in male rats, whereas SYN548090 appeared less toxic. It may be concluded that the other isomers of isocycloseram were of no higher toxicity.

The achieved mean daily doses of isocycloseram were 7.5, 15.0, and 25.4 mg/kg bw per day in males, 8.2, 22.4 and 42.6 mg/kg bw per day in females. There were no premature deaths or clinical signs in any animal that could be attributed to administration of this test compound. However, body weight gain was significantly lower in top-dose males and in females receiving 300 or 600 ppm resulting in a lower terminal mean body weight. A similar, but less pronounced and non-significant effect, was seen in mid-dose males. Food consumption was reduced, in parallel, in high-dose males and in mid- and high-dose females.

Whereas haematology did not reveal what were presumably treatment-related changes, some alterations in clinical chemistry parameters were noted comprising higher urea levels in males receiving 200 and 400 ppm and, at the high dose, also in females. In addition, triglycerides were reduced in mid- and high-dose females.

Based on necropsy findings, organ weight changes and microscopic observations, the adrenals, the liver, the duodenum and jejunum were identified as toxicological target organs of isocycloseram, whereas effects on kidneys and female reproductive organs were more equivocal. The adrenal glands were enlarged in two high-dose females and their absolute organ weight was significantly increased in

both sexes at the mid and high doses, indicating a dose response. Microscopically, hypertrophy of the zona fasciculata was evident in the same male and female groups.

In the liver, vacuolation was observed in both sexes at the two upper dose levels. In the intestines, abnormal contents was accompanied, in both sexes, by epithelial vacuolation in the duodenum at the two upper dose levels and in the jejunum in the high-dose group. Kidney weights were increased in all treated male groups as well as ovary weights in mid- and high-dose females, but these changes were not corroborated by any histopathological findings. Atrophy of the uterus and vaginal epithelium was observed in one high-dose female and thus was of questionable toxicological relevance.

The NOAEL in this study was the lowest dose of 100 ppm (equal to 7.5 mg/kg bw per day) (Laidlaw & Robertson, 2023).

In a 90-day toxicity study (OECD TG 408), groups of 10 Han Wistar rats (Crl:WI(Han)) received isocycloseram (purity 98.4%, Batch No. MEQ-02794-09) in the diet at concentrations of 0, 50, 150 or 300 ppm (equal 0, 3.9, 11.2 or 22.0 mg/kg bw per day for males, 0, 4.4, 13.4 or 24.0 mg/kg bw per day for females). Animals were inspected daily for mortality, moribundity and clinical signs of toxicity. Clinical observations, body weights and food consumption were assessed for all animals at predetermined intervals from pretrial until study completion. Detailed functional observations were performed on all animals during weeks 11/12. Blood samples were collected for haematology, coagulation and clinical chemistry investigation prior to termination. Urinalysis was conducted during week 13. In addition, toxicokinetic samples were collected on days 2, 28 and 85. After completion of 13 weeks of treatment, all animals were euthanized and subjected to a detailed necropsy, where selected organs were weighed and tissues were preserved and histological slides prepared. Histopathological evaluation was carried out for all tissues from control and high-dose animals. In addition, histopathological evaluation was conducted on gross lesions and adrenal glands from all low- and mid-dose animals and testes and epididymides from low- and mid-dose males.

Systemic exposure to isocycloseram was measured in the blood; results are given in Table 13. No treatment-related mortality occurred and no adverse clinical signs or ophthalmic findings were recorded.

Compared with the controls, statistically significant reductions in mean body weight (by 12%) and cumulative body weight change (by 21%) were recorded in males receiving 300 ppm, . Cumulative body weight change was also lower in females at 300 ppm (by 15%; not statistically significant). Food consumption was lower in males and females at 300 ppm throughout the treatment period compared with controls, with statistical significance achieved at some time points.

In the functional observation battery (FOB) the only finding of note was a slightly lower fore-grip strength in males receiving 300 ppm. Given the small size of this change it was considered a reflection of the treatment-related reduction in mean body weight noted for this group (approximately 12% lower than controls) rather than a direct effect of treatment. There were no other treatment-related intergroup differences in FOB parameters or in motor activity scores for males or females following dietary administration of isocycloseram at levels up to 300 ppm.

Higher white blood cell counts (statistically significant), mostly consisting of lymphocytes, were noted in males at 150 ppm (by 31%) and 300 ppm (by 32%), when compared to controls. Statistically significant increases in counts of higher white blood cell (by 79%), lymphocytes (by 88%), monocytes (by 57%), basophils (by 100%) and large unclassified cells counts (by 100%) were noted in females at 300 ppm, when compared to the control values. With regard to an observed lowering of thymus weight at 300 ppm (both sexes, not statistically significant) and a higher incidence of tubular cystic hyperplasia at 300 ppm in males (2/10, but no changes in females at 300 ppm), these changes were considered to be adverse.

Lower cholesterol levels compared to controls were noted in males at 300 ppm. Higher potassium levels and higher phosphate levels (statistically significant) were noted in males and females at 300 ppm when compared to their controls. Lower calcium levels were observed in males and females receiving 300 ppm. Given the small magnitude of these changes, and in the absence of any associated microscopic changes or alterations in other clinical chemistry parameters indicative of target organ toxicity, these findings were not considered to be adverse. There were no notable intergroup differences in urinalysis parameters that were considered to be treatment-related.

***Table 24. Intergroup comparison of selected clinical chemistry parameters in a 90-day rat study with isocycloseram (mean values ± standard deviation shown)***

| | Dietary concentration (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Parameter** | **0** | **50** | **150** | **300** | **0** | **50** | **150** | **300** |
| Number of animals | 10 | 10 | 9 | 10 | 10 | 10 | 10 | 10 |
| Total protein, (g/L) | 62 ± 1 | 61 ± 2 | 60 ± 1 | 61 ± 2 | 64 ± 3 | 63 ± 3 | 62 ± 2 | 60* ± 2 |
| Cholesterol, (mmol/L) | 1.9 ± 0.3 | 1.7 ± 0.2 | 1.8 ± 0.3 | 1.5** ± 0.2 | 1.5 ± 0.3 | 1.4 ± 0.4 | 1.5 ± 0.4 | 1.3 ± 0.2 |
| Potassium, (mmol/L) | 4.4 ± 0.2 | 4.4 ± 0.3 | 4.4 ± 0.3 | 4.7 ± 0.6 | 3.6 ± 0.3 | 3.7 ± 0.2 | 3.7 ± 0.2 | 3.9* ± 0.2 |
| Phosphate, (mmol/L) | 1.33 ± 0.18 | 1.38 ± 0.23 | 1.45 ± 0.11 | 1.59* ± 0.24 | 1.35 ± 0.19 | 1.34 ± 0.17 | 1.42 ± 0.18 | 1.69** ± 0.25 |
| Calcium, (mmol/L) | 2.62 ± 0.06 | 2.55* ± 0.06 | 2.56 ± 0.04 | 2.49** ± 0.06 | 2.63 ± 0.10 | 2.59 ± 0.08 | 2.53* ± 0.08 | 2.53* ± 0.08 |

Different from control group mean at statistical significance: * $p < 0.05$, ** $p < 0.01$; Source: Laidlaw, 2019b

Statistically significant higher absolute and relative adrenal gland weights compared to controls were observed in females receiving 300 ppm. Adrenal gland weights were also higher in females receiving 150 ppm and in males receiving 150 and 300 ppm, although statistical significance was not attained. Lower absolute testis and epididymis weights were noted in one male at 300 ppm. However on a relative basis epididymis and testis weights were comparable to controls. Higher relative kidney weights (statistically significant) were observed in males and females at 150 and 300 ppm. In females at 300 ppm, relative ovary weights were statistically higher than for controls. There were no microscopic correlates for these weight differences, thus they were not considered adverse.

***Table 25. Intergroup comparison of selected organ weights in a 90-day rat study with isocycloseram (mean values ± standard deviation shown)***

| | Dietary concentration (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Parameter** | **0** | **50** | **150** | **300** | **0** | **50** | **150** | **300** |
| Number of animals | **10** | **10** | **10** | **10** | **10** | **10** | **10** | **9** |
| Liver weight; absolute (g) | 14.12 ± 1.37 | 13.42 ± 1.58 | 14.00 ± 1.99 | 12.64 ± 1.26 | 8.16 ± 0.57 | 8.48 ± 0.97 | 8.56 ± 0.92 | 8.21 ± 0.82 |
| Liver weight; relative[a] (%) | 3.30 ± 0.24 | 3.20 ± 0.22 | 3.35 ± 0.30 | 3.34 ± 0.16 | 3.42 ± 0.27 | 3.39 ± 0.19 | 3.61 ± 0.25 | 3.62 ± 0.25 |
| Adrenal weight; absolute (g) | 0.0593 ± 0.0154 | 0.0570 ± 0.0079 | 0.0647 ± 0.0128 | 0.0624 ± 0.0076 | 0.0713 ± 0.0087 | 0.0794 ± 0.0125 | 0.0845 ± 0.0170 | 0.0986** ± 0.0157 |
| Adrenal weight; relative[a] (%) | 0.0139 ± 0.0034 | 0.0136 ± 0.0016 | 0.0155 ± 0.0030 | 0.0165 ± 0.0019 | 0.0299 ± 0.0040 | 0.0319 ± 0.0053 | 0.0361 ± 0.0084 | 0.0433** ± 0.0061 |
| Kidney weight; absolute (g) | 2.42 ± 0.22 | 2.48 ± 0.14 | 2.57 ± 0.11 | 2.51 ± 0.24 | 1.55 ± 0.13 | 1.66 ± 0.20 | 1.64 ± 0.09 | 1.58 ± 0.10 |
| Kidney weight; relative[a] (%) | 0.57 ± 0.06 | 0.59 ± 0.03 | 0.62* ± 0.05 | 0.66** ± 0.03 | 0.65 ± 0.03 | 0.66 ± 0.05 | 0.69* ± 0.03 | 0.70* ± 0.05 |
| Thymus weight; absolute (g) | 0.450 ± 0.106 | 0.443 ± 0.139 | 0.460 ± 0.138 | 0.372 ± 0.072 | 0.349 ± 0.079 | 0.356 ± 0.054 | 0.385 ± 0.061 | 0.317 ± 0.040 |
| Thymus weight; relative[a] (%) | 0.105 ± 0.026 | 0.106 ± 0.033 | 0.110 ± 0.031 | 0.099 ± 0.019 | 0.145 ± 0.030 | 0.143 ± 0.019 | 0.162 ± 0.021 | 0.140 ± 0.020 |

| | Dietary concentration (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Parameter** | **0** | **50** | **150** | **300** | **0** | **50** | **150** | **300** |
| Epididymis weight; absolute (g) | 1.3344 ± 0.2322 | 1.3675 ± 0.1664 | 1.3862 ± 0.1710 | 1.1868 ± 0.1477 | - | - | - | - |
| Epididymis weight; relative[a] (%) | 0.3120 ± 0.0557 | 0.3264 ± 0.0352 | 0.3331 ± 0.0407 | 0.3146 ± 0.0377 | - | - | - | - |
| Testes weight; absolute (g) | 3.70 ± 0.35 | 3.70 ± 0.28 | 3.79 ± 0.22 | 3.46 ± 0.69 | - | - | - | - |
| Testes weight; relative[a] (%) | 0.864 ± 0.073 | 0.885 ± 0.080 | 0.911 ± 0.072 | 0.912 ± 0.154 | - | - | - | - |
| Ovaries weight; absolute (g) | - | - | - | - | 0.099 ± 0.008 | 0.107 ± 0.018 | 0.115 ± 0.017 | 0.120 ± 0.030 |
| Ovaries weight; relative[a] (%) | - | - | - | - | 0.042 ± 0.004 | 0.043 ± 0.008 | 0.049 ± 0.007 | 0.053* ± 0.013 |

[a] Organ weight relative to body weight; Source: Laidlaw, 2019b

Different from control group mean at statistical significance: * $p < 0.05$, ** $p < 0.01$

A treatment-related change in the gross pathology was noted; abnormal content (pale yellow fluid) in the duodenum and/or jejunum of 2/10 females at 300 ppm. Other gross findings observed were of similar incidence in control and treated animals, and/or of a nature commonly observed in this strain and age of rats and/or related to differences among estrous cyclicity. These findings were therefore considered incidental and not treatment-related.

In the adrenal gland zona fasciculata there was minimal vacuolation in males and minimal hypertrophy in females at 300 ppm. Hypertrophy correlated with higher adrenal gland weights in females. In males at 150 or 300 ppm there was minimal to marked tubular degeneration in the testes, minimal or mild cellular debris and minimal or moderately reduced sperm in the epididymis, showing a dose-related increase in incidence and severity. These findings were bilateral in all except one case of minimal tubular degeneration of the testis. Other microscopic findings observed were of similar incidence in control and treated animals, and/or of a nature commonly observed in this strain and age of rats. These latter findings were therefore considered incidental and not treatment-related.

***Table 26. Test substance-related microscopic findings in a 90-day rat study with isocycloseram***

| | | Dietary concentration (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | Females | | | |
| **Parameter** | | **0** | **50** | **150** | **300** | **0** | **50** | **150** | **300** |
| Adrenals (number examined) | | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Vacuolation, zona fasciculata | total | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 |
| | minimal | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 |
| Hypertrophy, zona fasciculata, | total | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| | minimal | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| Testes (number examined) | | 10 | 10 | 10 | 10 | - | - | - | - |
| Tubular degeneration | total | 1 | 0 | 3 | 10** | - | - | - | - |
| | minimal | 1 | 0 | 2 | 5 | - | - | - | - |
| | mild | 0 | 0 | 1 | 3 | - | - | - | - |
| | moderate | 0 | 0 | 0 | 1 | - | - | - | - |
| | marked | 0 | 0 | 0 | 1 | - | - | - | - |

| | | Dietary concentration (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | Females | | | |
| **Parameter** | | **0** | **50** | **150** | **300** | **0** | **50** | **150** | **300** |
| Epididymis (number examined) | | 10 | 10 | 10 | 10 | - | - | - | - |
| Cellular debris, luminal | total | 0 | 0 | 2 | 8** | - | - | - | - |
| | minimal | 0 | 0 | 2 | 2 | - | - | - | - |
| | mild | 0 | 0 | 0 | 6 | - | - | - | - |
| Reduced sperm, luminal | total | 0 | 0 | 1 | 3 | - | - | - | - |
| | minimal | 0 | 0 | 1 | 2 | - | - | - | - |
| | moderate | 0 | 0 | 0 | 1 | - | - | - | - |

Different from control group mean at statistical significance: * $p < 0.05$, ** $p < 0.01$; Source: Laidlaw, 2019b

The NOAEL for oral toxicity of isocycloseram in a 90-day study in rats was 50 ppm in males (equal to 3.9 mg/kg bw per day) based on histopathological findings in testes and epididymides at the LOAEL of 150 ppm in (equal to 11.2 mg/kg bw per day). In females the NOAEL was 150 ppm (equal to 13.4 mg/kg bw per day), based on higher adrenal gland weights and histopathological changes in the adrenal gland at the LOAEL of 300 ppm (equal to 24 mg/kg bw per day) (Laidlaw, 2019b).

***Dog***

In a range-finding experiment for a subsequent 28-day study, one male and one female beagle dog received isocycloseram by oral capsules at escalating doses of 10, 20, 50, 100, 250 and 500 mg/kg bw. Each of these doses was administered for four consecutive days followed by a treatment-free interval of 10 days minimum and, before the maximum dose, of 18 days. (In parallel, there were similar investigations on two other compounds of which the results are not reported here since they are irrelevant for the assessment of isocycloseram.)

The animals were monitored for clinical signs, body weight and food consumption. On the last day of each dose escalation blood samples were drawn for examination of haematological and clinical chemistry parameters. Also blood concentrations of parent isocycloseram were repeatedly determined. On day 93, the animals were killed and necropsied. Specified tissues were collected but not examined under the microscope.

Both dogs survived the complete escalation study. Clinical signs were observed at 100 mg/kg bw per day and above, at which dose the female displayed reddening of the eyes but recovered during the first two days of the subsequent treatment-free interval. At 250 mg/kg bw per day, the same sign was apparent in both animals. In addition, the male dog was observed to regurgitate some food. The maximum dose of 500 mg/kg bw per day was not tolerated since, besides regurgitation, the male dog was quiet, displayed twitch movements of the head and limbs and fell over but was able to regain its stance. In the female, reddened and partially closed eyes were observed.

Body weight losses were noted in the female at 100 mg/kg bw, in the male at 250 mg/kg bw and in both animals at 500 mg/kg bw. Apart from the female that received 500 mg/kg bw, body weight was regained in the treatment-free intervals. Lower food consumption was observed during the treatment intervals in the female at between 20 and 500 mg/kg bw per day and in the male at 250 and 500 mg/kg bw per day.

Clinical pathology did not reveal conclusive evidence of treatment-related changes, but there might have been a decrease in haemoglobin at 100 mg/kg bw and above, along with lower urea, cholesterol and phosphate concentrations, even though findings were inconsistent with regard to sex and the particular dose level at which they were first observed. Blood levels of isocycloseram increased with dose in the male dog but this increase was less than linear. In the female there was no clear relation between dose and blood concentration; for example the blood level at 24 hours after first administration of 100 mg/bw was twice the level after a dose of 500 mg/kg bw.

Gross pathological examination at necropsy did not reveal any remarkable findings. The conclusion of the study author was that maximum dose levels for subsequent studies in dogs might be 250 mg/kg bw in males and 100 mg/kg bw in females (Robertson, 2019a).

In a 28-day oral toxicity study (OECD TG 409), isocycloseram (purity 98.4%, Batch No. S02F921772W) was administered via capsule to groups of three beagle dogs at dose levels of 0, 10, 50 or 150/80 mg/kg bw per day for males and at 0, 10, 35 or 70 mg/kg bw per day for females. Males receiving 150 mg/kg per day were dosed for four days before the test-article was withdrawn due to the extent of body weight loss and inappetence. Following a 10 day treatment-free period, dosing recommenced on day 15 at 80 mg/kg per day for males. despite this change, however, excessive toxicity was observed, and 2/3 males were prematurely euthanized on days 17 and 18. Two females receiving 70 mg/kg bw per day showed body weight loss and inappetence, consequently these animals were not dosed after day 9 and day 14, respectively. These two animals were prematurely euthanized on day 18. In addition, one of the three males at 50 mg/kg bw per day was not dosed from days 10–21 due to body weight loss and prolonged inappetence.

Animals were inspected daily for mortality, moribundity and clinical signs of toxicity. Body weights and food consumption were measured and recorded at predetermined intervals. Blood samples for clinical pathology parameters (haematology, coagulation and clinical chemistry) and urine samples were collected during pretreatment and at week 4, with the exception of two males and two females from the high-dose group where blood collection occurred on day 18 (prior to termination). Blood samples were also collected on days 1, 7, 14, 18 and 28 for bioanalysis and toxicokinetic evaluation. All animals killed on days 17, 18 or 29 were subjected to a detailed necropsy, with selected organs being weighed. Tissues from all animals were subjected to a comprehensive histopathological evaluation.

One male receiving 80 mg/kg bw per day was prematurely killed on day 17 due to poor overall condition. At necropsy, its adrenal glands appeared pale, the liver was pale with prominent architecture and one dark focus, and the lung had multiple dark foci. Microscopically, there was minimal increased apoptosis in the cortex of the thymus. The cause of the moribund status was not determined microscopically. High-dose animals (2/3 males and 2/3 females) were prematurely killed on day 18 of treatment due to body weight loss and low food consumption. At necropsy, there were no macroscopic or microscopic changes observed in any of these animals.

Systemic exposure to isocycloseram generally increased with increasing dose on days 1 and 28. Treatment-related, adverse clinical observations were seen in high-dose males; one dog showed abnormal head movements followed by falling over on its right-hand side and then standing again, whilst another exhibited mild salivation, was moderately unsteady on all limbs and displayed moderately red ears. These clinical signs alongside the body weight loss and low food consumption led to a cessation of treatment at 150 mg/kg bw day in the high-dose male animals. Marked reductions in body weight were observed in high-dose animals during the period in which they were dosed, which correlated with low food consumption. In high-dose males the severity of body weight loss during the first four days of dosing at 150 mg/kg bw per day was marked. Body weights and food consumption recovered when these animals were removed from dosing, with the exception of one female where no effect on body weight or food consumption had been observed in the first place.

For high-dose females (70 mg/kg bw per day), although the degree and severity of signs observed were not as pronounced as those observed for males, regurgitation of food was seen on occasions throughout the treatment period. These signs, coupled with body weight losses and low food consumption lasting several days, led to the cessation of treatment and premature euthanasia for 2/3 females on day 18 of the study.

In addition, in the mid-dose group one of the three males was not dosed from day 10–21 due to body weight loss and low food consumption. Females at 35 mg/kg bw per day had a lower overall body weight gain (statistically significant) compared to controls. There was no treatment-related effect on body weight at 10 mg/kg bw per day in males or females. There were no differences in food consumption compared to controls for males at 10 mg/kg bw per day or for females at 10 or 35 mg/kg bw per day.

There were no treatment-related differences in haematological, coagulation, blood chemistry or urinalysis parameters. Any differences were within the range of the overall control group data, or were

judged to be individual changes, or due to biological variation or lacked a true dose–effect relationship and were therefore not considered to be related to treatment.

No test item-related gross findings were noted at day 18 or 29. The gross findings observed were considered incidental, of a nature commonly observed in this strain and age of dog and/or were of similar incidence in control and treated animals and therefore not related to treatment

There was a slight increase in liver weight compared to controls in females (by 14%) treated at 35 mg/kg per day. This difference was not statistically significant and there was no microscopic correlation. In the absence of histopathological changes and changes in the clinical chemistry, the liver weight increase was not considered adverse. No other test item-related organ weight changes or test item-related microscopic findings were noted. There were some isolated organ weight changes and microscopic findings that differed from their respective controls, but these showed no patterns or trends, and there was no correlating data to suggest the observations were toxicologically relevant. Thus, they were considered incidental and/or related to differences in sexual maturity and not treatment-related.

The NOAEL for oral toxicity of isocycloseram in a 28-day study in dog was 10 mg/kg bw per day in males and females, based on body weight loss in both sexes at the LOAEL of 50 mg/kg bw per day in males and 35 mg/kg bw per day in females. Treatment with isocycloseram at or above 80 mg/kg per day in males and 70 mg/kg per day in females was not tolerated (Robertson, 2019b).

In a 90-day oral toxicity study (OECD TG 409), isocycloseram (purity 96.9%, Batch No SMU5IP15011) was administered via capsule to groups of four beagle dogs (approximately 8–9 months old) at dose levels of 0, 5, 15 or 35/25 mg/kg bw per day. High-dose animals received 35 mg/kg per day for 12 days before dosing was suspended due to the extent of body weight loss. Following a treatment-free period of seven days for three males and three females and nine days for one male and one female, dosing recommenced on day 20 and day 22, respectively, at 25 mg/kg bw per day.

Animals were inspected daily for mortality, moribundity and clinical signs of toxicity. Body weights and food consumption were measured and recorded at predetermined intervals. Blood and urine samples were collected from animals at two intervals during pretreatment and weeks 6 and 13 for haematology, coagulation, clinical chemistry parameters and urinalysis. All animals were killed on day 92/93 and subjected to a detailed necropsy examination, with selected organs being weighed. Tissues from all animals were subjected to comprehensive histopathological evaluation.

Systemic exposure to isocycloseram was measured in the blood; results are given in Table 13. No mortalities occurred and there were generally no adverse clinical observations recorded for any animal receiving isocycloseram. Transient incidences of liquid and/or soft faeces were observed in animals that received 35/25 mg/kg bw per day; this was however considered related to the administration of wetted/tinned food as a consequence of inappetence.

For animals that received 35 mg/kg bw per day a marked reduction in body weight and food consumption was noted during the first 13 days of treatment. Upon cessation of dosing, body weights and food consumption recovered for those affected animals and following a seven day treatment-free period 3/4 males and females were returned to dosing at 25 mg/kg per day on day 20. Two of the more markedly affected individual animals were given a nine day treatment-free period before dosing recommenced at 25 mg/kg bw per day on day 22. Following resumption of treatment the body weight and body weight gain of most individuals improved, with the exception of one male and one female. These two animals lost body weight on the resumption of treatment. Following approximately two weeks of treatment at 25 mg/kg bw per day their body weights and food consumption slowly improved, the male having a low overall body weight gain across the whole study and the female eventually being comparable to controls.

Females dosed at 15 mg/kg bw per day showed a lower body weight gain than controls over the treatment period, and this correlated with occasional intervals of lower food consumption. However, given the absence of other correlating effects in any other parameters measured, and given the recovery of body weights in the high-dose animals dosed at 25 mg/kg bw per day for 70 days these body weight findings were considered not to be adverse. Body weights and food consumption were unaffected in males or females that received 5 mg/kg bw per day and males that received 15 mg/kg bw per day.

*Table 27. Intergroup comparison of mean body weight in a 90-day dog study with isocycloseram for select time points, (mean values ± standard deviation in kg shown)*

| | Dose level (mg/kg bw per day) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| Day/period | 0 | 5 | 15 | 35/25 | 0 | 5 | 15 | 35/25 |
| Number of dogs | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| −1 | 10.40 ± 1.10 | 10.15 ± 0.99 | 10.30 ± 0.96 | 10.10 ± 0.94 | 8.75 ± 0.76 | 8.85 ± 0.53 | 8.33 ± 0.70 | 8.33 ± 0.67 |
| 7 | 10.68 ± 1.31 | 10.48 ± 1.03 | 10.63 ± 0.86 | 9.98 ± 1.24 | 8.95 ± 0.86 | 8.88 ± 0.57 | 8.38 ± 0.62 | 8.10 ± 0.42 |
| 14 | 10.73 ± 1.27 | 10.55 ± 1.07 | 10.70 ± 1.06 | 9.68 ± 1.03 | 9.00 ± 0.90 | 8.93 ± 0.62 | 8.43 ± 0.68 | 8.08 ± 0.57 |
| 28 | 11.00 ± 1.31 | 10.83 ± 1.16 | 10.65 ± 1.14 | 10.00 ± 1.30 | 9.20 ± 1.07 | 8.88 ± 0.38 | 8.33 ± 0.67 | 8.58 ± 0.95 |
| 56 | 11.20 ± 1.45 | 11.18 ± 0.99 | 10.85 ± 1.28 | 10.68 ± 1.20 | 9.25 ± 1.07 | 9.00 ± 0.62 | 8.10 ± 0.66 | 9.15 ± 0.89 |
| 92 | 11.45 ± 1.42 | 11.40 ± 1.01 | 11.03 ± 1.32 | 10.98 ± 0.96 | 9.45 ± 0.95 | 9.10 ± 0.64 | 8.40 ± 0.68 | 9.25 ± 0.83 |
| −1 to 7 | 0.28 ± 0.22 | 0.33 ± 0.17 | 0.33 ± 0.17 | -0.13 ± 0.39 | 0.20 ± 0.14 | 0.03 ± 0.05 | 0.05 ± 0.19 | -0.23* ± 0.26 |
| 7–14 | 0.05 ± 0.13 | 0.08 ± 0.05 | 0.08 ± 0.21 | -0.30 ± 0.56 | 0.05 ± 0.13 | 0.05 ± 0.06 | 0.05 ± 0.10 | -0.03 ± 0.50 |
| −1 to 91 | 0.95 ± 0.48 | 1.28 ± 0.40 | 0.73 ± 0.41 | 0.85 ± 0.54 | 0.68 ± 0.39 | 0.25 ± 0.24 | 0.00* ± 0.34 | 1.00 ± 0.24 |

Different from control group mean at statistical significance: * $p < 0.05$; Source: Robertson, 2019c

There were no findings during the ophthalmoscopic examinations that could be considered treatment related. There were no toxicologically significant treatment-related differences in haematological, coagulation, blood chemistry or urinalysis parameters. A number of inter-group differences occurred between treated groups and the control, some of which attained statistical significance. For example, the mean corpuscular haemoglobin concentration (MCHC) was statistically higher in all treated, male groups compared to controls at weeks 6 and 13. However, there was no clear dose–response relationship and no change in any other related haematological parameters. Also, in high-dose females red blood cell count was significantly lower than controls at weeks 6 and 13, which was consistent with a lower red blood cell count recorded for these animals pretreatment. In addition, in high-dose females at Week 13, haemoglobin concentration and haematocrit were statistically lower than for controls, and mean corpuscular haemoglobin (MCH) and MCHC were statistically higher than control values. To a statistically significant extent, globulin concentrations were lower (and consequently the A:G ratio was higher) in mid- and high-dose females at week 13. There were no other toxicologically significant changes in clinical chemistry parameters. Overall. these differences were minor, occurred on one occasion only (lacked a frequency-based relationship), were evident during pretrial or lacked a dose–response relationship. Consequently, these differences were attributable to biological variation and were considered not to be treatment related.

There were no treatment-related organ weight changes, gross pathology or histopathology changes.

Overall, oral administration of 35 mg/kg bw per day of isocycloseram was not tolerated and resulted in a reduction in body weight and food consumption. Following the resumption of treatment at 25 mg/kg bw per day, most animals showed improved body weight and body weight gain, except for one male which had an overall lower body weight gain across the entire study.

The NOAEL for oral toxicity of isocycloseram in a 90-day study in dog was 15 mg/kg bw per day in males and females, based on body weight loss and lower food consumption in both sexes at the LOAEL of 35/25 mg/kg bw per day (Robertson, 2019c).

**(b) Dermal application**

***Rat***

In a 28-day percutaneous toxicity study (OECD TG 410), groups of 10 Han Wistar rats (RccHan™:WIST) received isocycloseram (purity 96.9%, Batch No. SMU5IP15011) at concentrations of 0, 100, 300 or 1000 mg/kg bw per day for six hours per day with semi-occlusion to areas of intact shaved skin for five days per week, with the exception of the final week when they were dosed daily. Animals were inspected daily for mortality, moribundity and clinical signs of toxicity. Also, dosing sites were inspected daily for signs of irritation. During the study, toxicokinetics, clinical condition, detailed physical examination, body weight, food consumption, and ophthalmic examination were all undertaken at predetermined intervals At termination, haematology (peripheral blood), blood chemistry, organ weights, macropathology and histopathology investigations were performed.

Systemic exposure to isocycloseram was measured in the blood, results are given in Table 13. No treatment-related mortalities occurred and the behaviour and appearance of the animals was unaffected. Intermittent eschar formation occurred at the dermal application site of one male and two females given 100 mg/kg bw per day, two males and one female given 300 mg/kg bw per day and one male given 1000 mg/kg bw per day, and erythema was recorded in one female given 100 mg/kg bw per day, one male given 300 mg/k bw per day and one male and one female given 1000 mg/kg bw per day. The low incidences of these findings and lack of any dose–response relationship indicated that they were incidental.

The body weight gains of females given 1000 mg/kg bw per day were lower (by 35%; statistically significant) compared to controls. The effect was particularly marked during the final two weeks when there was a 48% suppression of gain compared to controls. There was also a reduction in body weight gain in the final two weeks in females given 300 mg/kg bw per day. There was no clear effect of treatment upon the body weights of males. At 1000 mg/kg bw per day there was reduction by 42% in body weight gain compared to controls during the first two weeks, but in contrast, the body weight gains of these animals were higher than controls in week 3 and 4. The overall (days 1–28) weight gains were similar to controls at all doses. Food consumption was lower (statistically significant) compared to pretreatment values and controls, in females given 300 mg/kg bw per day (by 11%) or 1000 mg/kg bw per day (by 15%), with males given 1000 mg/kg bw per day showing a similar finding but only in the first two weeks of treatment. There were no treatment-related effects on body weight or food consumption at 100 mg/kg bw per day in either sex, nor at 300 mg/kg bw per day in males. There were no treatment-relating ophthalmic findings.

Statistically significant, higher neutrophil and lymphocyte counts were seen at 1000 mg/kg bw per day in males and at 300 and 1000 mg/kg bw per day in females, but there was no clear dose–response relationship in either sex. There were also minor increases in eosinophil and basophil counts in males and females at 1000 mg/kg bw per day and monocyte counts in males at 1000 mg/kg bw per day. As a consequence of these findings the total leucocyte counts of females at 300 mg/kg bw per day and of males and females at 1000 mg/kg bw per day were higher than for controls. Platelet counts were high, when compared to controls at all doses in females, and at 1000 mg/kg bw per day in males. Due to the observed histopathological changes (vacuolation), these effects were considered adverse. All other differences from controls, including those attaining statistical significance, were considered due to natural biological variation and were not therefore related to treatment.

***Table 28. Intergroup comparison of selected haematological findings in a 28-day percutaneous rat study with isocycloseram, (mean values ± standard deviation shown)***

| | Dose level (mg/kg bw per day) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Parameter** | **0** | **100** | **300** | **1000** | **0** | **100** | **300** | **1000** |
| Number of rats | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Neutrophils ($\times 10^9$/L) | 1.09 ± 0.332 | 1.08 ± 0.607 | 1.70 ± 0.743 | 1.88* ± 0.647 | 0.63 ± 0.275 | 0.72 ± 0.220 | 1.37** ± 0.703 | 1.42** ± 0.692 |
| Lymphocytes ($\times 10^9$/L) | 4.58 ± 1.076 | 6.53* ± 2.390 | 5.61 ± 1.522 | 6.58* ± 1.023 | 3.39 ± 0.690 | 4.22 ± 1.247 | 5.67** ± 1.324 | 5.38** ± 1.573 |

| | Dose level (mg/kg bw per day) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Parameter** | **0** | **100** | **300** | **1000** | **0** | **100** | **300** | **1000** |
| Eosinophils ($\times 10^9$/L) | 0.07 ± 0.026 | 0.08 ± 0.021 | 0.08 ± 0.026 | 0.11* ± 0.054 | 0.08 ± 0.021 | 0.06 ± 0.036 | 0.06 ± 0.025 | 0.10 ± 0.111 |
| Basophils ($\times 10^9$/L) | 0.01 ± 0.006 | 0.02 ± 0.007 | 0.02 ± 0.012 | 0.03** ± 0.012 | 0.01 ± 0.005 | 0.01 ± 0.008 | 0.02** ± 0.010 | 0.02* ± 0.012 |
| Monocytes ($\times 10^9$/L) | 0.09 ± 0.029 | 0.10 ± 0.041 | 0.09 ± 0.039 | 0.15** ± 0.052 | 0.07 ± 0.022 | 0.07 ± 0.032 | 0.09 ± 0.033 | 0.07 ± 0.047 |
| Total white blood cells ($\times 10^9$/L) | 5.87 ± 1.218 | 7.88 ± 2.850 | 7.55 ± 2.091 | 8.81** ± 1.563 | 4.18 ± 0.669 | 5.12 ± 1.434 | 7.26** ± 1.613 | 7.01** ± 2.331 |
| Platelets ($\times 10^9$/L) | 874 ± 115.9 | 836 ± 98.4 | 898 ± 71.1 | 1056** ± 100.5 | 784 ± 75.4 | 902 ± 149.4 | 1011* ± 146.1 | 1166** ± 455.5 |

Different from control group mean at statistical significance: * $p < 0.05$, ** $p < 0.01$; Source: Cooper, 2019

Differences in clinical chemistry parameters were noted mainly for the high-dose group in females. including slightly higher ALP, higher plasma urea concentrations significant reduction in plasma cholesterol and triglyceride concentrations. Similar findings were not observed in males. By contrast, variations in plasma electrolyte levels were observed in males, including lower chloride and higher potassium concentrations. In the absence of any corroborative histopathological findings in the liver and kidney which could account for these changes they are considered to be incidental and not related to treatment. All other differences from controls, including those attaining statistical significance, were minor, lacked any evidence of a dose–response relationship and consequently, were attributed to normal biological variation.

***Table 29. Intergroup comparison of selected clinical chemistry findings in a 28-day percutaneous rat study with isocycloseram, (mean values ± standard deviation shown)***

| | Dose level (mg/kg bw per day) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Parameter** | **0** | **100** | **300** | **1000** | **0** | **100** | **300** | **1000** |
| Number of rats | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Alkaline phosphatase, (U/L) | 86 ± 15.4 | 80 ± 16.5 | 91 ± 13.9 | 84 ± 13.0 | 42 ± 9.2 | 51 ± 9.6 | 55* ± 12.9 | 57** ± 11.8 |
| Alanine transaminase, (U/L) | 49 ± 6.8 | 55 ± 18.3 | 58 ± 14.7 | 59 ± 9.5 | 43 ± 7.7 | 40 ± 6.5 | 50 ± 9.5 | 65** ± 12.5 |
| Urea (mmol/L) | 7.31 ± 0.746 | 7.60 ± 0.606 | 8.00 ± 0.779 | 7.50 ± 0.982 | 7.48 ± 0.948 | 7.46 ± 1.178 | 8.29 ± 1.286 | 9.82** ± 1.681 |
| Cholesterol (mmol/L) | 2.06 ± 0.456 | 2.18 ± 0.264 | 1.98 ± 0.278 | 2.16 ± 0.190 | 2.20 ± 0.293 | 2.01 ± 0.191 | 1.87 ± 0.491 | 1.62** ± 0.477 |
| Triglycerides (mmol/L) | 0.32 ± 0.086 | 0.42 ± 0.207 | 0.36 ± 0.061 | 0.31 ± 0.117 | 0.45 ± 0.148 | 0.38 ± 0.110 | 0.34 ± 0.086 | 0.25** ± 0.072 |
| Chlorine (mmol/L) | 101 ± 1.3 | 99 ± 1.2 | 98** ± 1.4 | 97** ± 1.6 | 100 ± 1.0 | 100 ± 1.1 | 99 ± 1.3 | 100 ± 1.4 |

Different from control group mean at statistical significance: * $p < 0.05$, ** $p < 0.01$; Source: Cooper, 2019

There was seen to be dose-related, non-significant increase compared to controls in absolute (14%) and relative (11%) adrenal weights in males administered 100 mg/kg bw per day and a statistically significant increase in these weights in males and females given 300 or 1000 mg/kg bw per day. In addition, statistically significant, slightly higher absolute liver weights were recorded in males given 300 mg/kg bw per day and in males and females given 1000 mg/kg bw per day, and higher absolute and relative kidney weights were recorded for males given 1000 mg/kg bw per day. All other intergroup

differences from controls were minor, lacked a dose–response relationship or were confined to one sex and were therefore attributed to normal biological variation.

***Table 30. Intergroup comparison of selected organ weights in a 28-day percutaneous rat study with isocycloseram, (mean values ± standard deviation shown)***

| | Dose level (mg/kg bw per day) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Parameter** | **0** | **100** | **300** | **1000** | **0** | **100** | **300** | **1000** |
| Number of rats | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Adrenal weight; absolute (g) | 0.0764 ± 0.0115 | 0.0869 ± 0.0149 | 0.0973** ± 0.0205 | 0.1020** ± 0.0167 | 0.0889 ± 0.0163 | 0.0911 ± 0.0130 | 0.1009* ± 0.0116 | 0.1347** ± 0.0205 |
| Adrenal weight; relative[a] (%) | 0.0243 ± 0.0035 | 0.0268 ± 0.0047 | 0.0315** ± 0.0055 | 0.0335** ± 0.0043 | 0.0399 ± 0.0060 | 0.0421 ± 0.0054 | 0.0492* ± 0.0055 | 0.0659** ± 0.0096 |
| Liver weight; absolute (g) | 8.2115 ± 0.9749 | 9.0948 ± 1.3595 | 8.6764* ± 0.7106 | 8.9169** ± 0.8765 | 7.0126 ± 0.9114 | 6.5622 ± 0.8562 | 6.5228 ± 0.6288 | 7.0211* ± 0.9998 |
| Liver weight; relative[a] (%) | 2.61 ± 0.26 | 2.80 ± 0.26 | 2.82 ± 0.12 | 2.93** ± 0.08 | 3.15 ± 0.27 | 3.02 ± 0.26 | 3.18 ± 0.20 | 3.42 ± 0.29 |
| Kidney weight; absolute (g) | 2.0170 ± 0.1793 | 2.0588 ± 0.2390 | 2.0467 ± 0.1551 | 2.1402** ± 0.1631 | 1.4102 ± 0.0759 | 1.4266 ± 0.1504 | 1.3672 ± 0.1241 | 1.3603 ± 0.1236 |
| Kidney weight; relative[a] (%) | 0.641 ± 0.045 | 0.634 ± 0.043 | 0.665 ± 0.036 | 0.705** ± 0.032 | 0.636 ± 0.041 | 0.658 ± 0.036 | 0.667 ± 0.045 | 0.665 ± 0.035 |

[a] Organ weight relative to body weight; Source: Cooper, 2019

Different from control group mean at statistical significance: * $p < 0.05$, ** $p < 0.01$)

Treatment-related histopathological findings were seen in the small intestine (duodenum and jejunum) and in the adrenal glands. Epithelial vacuolation within the duodenum was observed at 100, 300 and 1000 mg/kg per day in females, with the incidence and the extent of the finding being dose-related. Similar dose-related changes within the jejunum were also observed at 300 and 1000 mg/kg bw per day in females. In the adrenal glands, hypertrophy of the zona fasciculata, generally associated with vacuolation, was observed at 300 or 1000 mg/kg bw per day in both sexes, with the incidence and extent of the hypertrophy being dose-related. The morphological changes within this organ correlated with a statistically significant increase in mean absolute organ weight at 300 and 1000 mg/kg bw per day in both sexes.

***Table 31. Test substance-related microscopic findings in a 28-day percutaneous rat study with isocycloseram***

| | | Dose level (mg/kg bw per day) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | Females | | | |
| **Parameter** | | **0** | **100** | **300** | **1000** | **0** | **100** | **300** | **1000** |
| **Adrenal** (number examined) | | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Hypertrophy, Cortical, Zona Fasciculata | total | 0 | 0 | 4 | 7 | 0 | 0 | 3 | 9 |
| | minimal | 0 | 0 | 4 | 0 | 0 | 0 | 3 | 2 |
| | slight | 0 | 0 | 0 | 7 | 0 | 0 | 0 | 7 |
| Vacuolation, Cortical, Zona Fasciculata | total | 0 | 0 | 1 | 7 | 0 | 0 | 1 | 9 |
| | minimal | 0 | 0 | 1 | 5 | 0 | 0 | 1 | 9 |
| | slight | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| **Duodenum** (number examined) | | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| | total | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 7** |
| Vacuolation, epithelial | minimal | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 4 |
| | slight | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 3 |

| | | Dose level (mg/kg bw per day) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | Females | | | |
| **Parameter** | | **0** | **100** | **300** | **1000** | **0** | **100** | **300** | **1000** |
| **Jejunum** (number examined) | | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Vacuolation, epithelial | total | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 7** |
| | minimal | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 5 |
| | slight | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 |

Different from control group mean at statistical significance: * $p < 0.05$, ** $p < 0.01$; Source: Cooper, 2019

The NOAEL for dermal toxicity of isocycloseram in a 28-day study in rat was 100 mg/kg bw per day in males and females, based on reduction in body weight gain, higher white blood cell counts, increased adrenal weights and histopathological findings in the small intestine and adrenal gland at the LOAEL of 300 mg/kg bw per day. No skin irritation was observed after repeated semi-occluded applications of up to 1000 mg/kg bw per day (Cooper, 2019).

**(c) Exposure by inhalation**

No inhalation toxicity studies employing repeated administration were submitted.

## 2.3 Long-term studies of toxicity and carcinogenicity

***Mouse***

In an 80-week carcinogenicity study (OECD TG 451), groups of 50 Crl:CD-1mice per sex received isocycloseram (purity 96.9%, Batch No. SMU5IP15011) in the diet at a concentration of 0, 15, 60 or 200 ppm (equal 1.7, 6.7 and 23.1 mg/kg bw per day for males, 1.8, 7.1 and 24.4 mg/kg bw per day for females). Animals were inspected daily for mortality, moribundity and clinical signs of toxicity and palpable masses. Body weights and food consumption were measured and recorded at predetermined intervals. Achieved dosages were calculated for each period of food consumption during the course of the study and food utilization was determined for weeks 1–4, 5–8, 9–13 and overall from weeks 1–13. Blood samples were collected from all animals on the day of termination (weeks 81/82) to evaluate haematology. All surviving animals were euthanized after at least 80 weeks of treatment and subjected to a detailed necropsy, with selected organs being weighed. All unscheduled decedents were subjected to a necropsy. Tissues from all animals were subjected to a comprehensive histopathological evaluation.

All formulated diets were found to be accurate and homogeneous. There were no treatment-related effects on mortality or clinical observations. Various neoplastic and non-neoplastic causes of death were reported. None of these were considered to be test item-related.

Body weight gain was considered to be slightly lower throughout the treatment period up to day 100 in males given 200 ppm when compared with controls, with differences in body weight gain achieving statistical significance on few occasions throughout this period Food utilization was slightly lower compared with controls over weeks 5–8 and 1–13 in males given 200 ppm, but attained statistical significance . Due to the small size of the effects, these changes were not considered adverse.

***Table 32. Intergroup comparison of body weight and cumulative body weight changes for selected time points in an 80-week mouse study with isocycloseram, (mean values ± standard deviation shown)***

| | Dose Level (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| Day/period | 0 | 15 | 60 | 200 | 0 | 15 | 60 | 200 |
| 1 | 29.61 ± 2.21 | 29.68 ± 2.06 | 30.31 ± 2.46 | 30.13 ± 2.68 | 23.94 ± 1.70 | 24.70 ± 2.21 | 25.46** ± 1.81 | 24.76 ± 2.31 |
| 8 | 31.40 ± 2.34 | 31.23 ± 2.31 | 31.87 ± 2.61 | 31.74 ± 2.92 | 24.89 ± 1.57 | 25.74 ± 2.33 | 26.46** ± 2.17 | 25.77 ± 2.19 |
| 29 | 34.32 ± 3.11 | 34.30 ± 2.74 | 35.05 ± 3.02 | 34.56 ± 3.35 | 27.70 ± 2.25 | 28.59 ± 2.71 | 29.74** ± 2.37 | 29.16* ± 2.90 |
| 92 | 40.42 ± 5.02 | 39.95 ± 4.46 | 41.33 ± 5.05 | 39.54 ± 4.78 | 32.90 ± 4.15 | 34.79 ± 4.61 | 35.00* ± 3.73 | 33.96 ± 4.37 |
| 183 | 44.37 ± 6.58 | 43.09 ± 5.85 | 45.76 ± 6.44 | 43.08 ± 5.98 | 37.11 ± 6.07 | 39.32 ± 5.96 | 39.00 ± 5.74 | 37.36 ± 5.34 |
| 365 | 47.53 ± 7.48 | 46.22 ± 6.19 | 49.07 ± 6.56 | 46.41 ± 6.88 | 41.85 ± 7.56 | 44.72 ± 7.62 | 45.77* ± 8.07 | 43.18 ± 7.07 |
| 561 | 48.97 ± 7.90 | 47.16 ± 7.21 | 50.10 ± 5.96 | 47.64 ± 7.91 | 43.70 ± 7.63 | 47.57 ± 7.72 | 48.26* ± 9.26 | 44.53 ± 6.52 |
| 1 to 8 | 1.79 ± 0.56 | 1.55 ± 0.61 | 1.57 ± 0.64 | 1.61 ± 0.88 | 0.95 ± 1.09 | 1.03 ± 1.27 | 1.00 ± 1.25 | 1.01 ± 1.20 |
| 1 to 92 | 10.81 ± 3.46 | 10.26 ± 3.19 | 11.02 ± 3.68 | 9.41 ± 2.87 | 8.93 ± 3.36 | 10.09 ± 3.61 | 9.55 ± 3.10 | 9.20 ± 3.06 |
| 1 to 561 | 19.40 ± 6.70 | 17.36 ± 5.94 | 20.38 ± 5.65 | 17.46 ± 6.21 | 19.76 ± 6.81 | 22.96 ± 6.70 | 22.87 ± 8.88 | 19.95 ± 6.03 |

Different from control group mean at statistical significance: * $p < 0.05$, ** $p < 0.01$; Source: Strepka, 2019a

There were no changes to white blood cell counts and/or related parameters that were considered test substance-related, with values that were similar for all treated groups when compared with controls. The mean white blood cell count was higher, and mean eosinophil count also higher (to a statistically significant extent) in females given 200 ppm, however review of the individual data showed that this increase was caused by high values noted for just a few animals. Most of the animals in the group produced results that were within the control group range.

Liver and kidney weights were higher in females at 200 ppm, without any macroscopic or microscopic correlation. These increases were not considered to be adverse due to their small size when compared to the control groups (different by less than 15%), and to the lack of any associated pathology. No other test substance-related organ weight changes were noted. There were individual organ weight values that were different from their respective controls, but no patterns could be discerned nor was there any correlating data to suggest these values were related to treatment with the test substance.

***Table 33. Intergroup comparison of selected organ weights in a 80-week mouse study with isocycloseram, (mean values ± standard deviation, [% difference from control])***

| Dietary Concentration (ppm) | Males | | | | Females | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 15 | 60 | 200 | 0 | 15 | 60 | 200 |
| Number of mice | 47 | 41 | 43 | 41 | 38 | 40 | 33 | 33 |
| Liver weight; absolute (g) | 2.4905 ± 0.7859 | 2.4209 ± 0.9141 | 2.3998 ± 0.3975 | 2.5721 ± 0.7716 | 1.9487 ± 0.3828 | 2.0212 ± 0.3368 | 1.8755 ± 02586 | 2.2331** ± 0.3499 |
| [% difference] | - | [−2.8%] | [−3.6%] | [+3.3%] | - | [+3.7%] | [−3.8%] | [+14.6%] |
| Liver weight; relative[a] (%) | 5.1526 ± 0.9772 | 5.2633 ± 1.8519 | 4.9332 ± 0.5036 | 5.5322 ± 1.5589 | 4.6891 ± 0.8821 | 4.4484 ± 0.7621 | 4.0882* ± 0.5553 | 5.1962** ± 0.6751 |
| [% difference] | - | [+2.1%] | [−4.2%] | [+7.3%] | - | [−5.1%] | [−13%] | [+11%] |
| Kidney weight; absolute (g) | 0.8032 ± 0.1473 | 0.7846 ± 0.1301 | 0.8341 ± 0.1436 | 0.8137 ± 0.1288 | 0.4890 ± 0.0714 | 0.4960 ± 0.0527 | 0.4877 ± 0.0594 | 0.5459** ± 0.1153 |
| [% difference] | | [−2.3%] | [+3.8%] | [+1.3%] | | [+1.4%] | [−0.3%] | [+11.6%] |
| Kidney weight; relative[a] (%) | 1.6936 ± 0.2514 | 1.7185 ± 0.2485 | 1.7214 ± 0.28264 | 1.7571 ± 0.2345 | 1.1882 ± 0.2047 | 1.1025 ± 0.1943 | 1.0760 ± 0.2218 | 1.2749 ± 0.2678 |
| [% difference] | - | [+1.5%] | [+1.6%] | [+3.7%] | - | [−7.2%] | [−9.4%] | [+7.3%] |

[a] Organ weight relative to body weight; Source: Strepka, 2019a

Different from control group mean at statistical significance: * $p < 0.05$, ** $p < 0.01$)

At necropsy, an increase in the incidence of prominent lobular architecture was observed in the livers of males exposed to isocycloseram at 60 ppm and above, however this was not considered adverse as there were no correlating histopathological findings. Other gross findings observed were considered incidental, of a nature commonly observed in this strain and age of mice, and/or were of similar incidence in controls and treated animals, and therefore were considered unrelated to treatment.

There were no test substance-related, statistically significant neoplastic or proliferative findings. All neoplastic or proliferative findings observed were considered incidental, of a nature commonly observed in this strain and age of mice, and/or were of similar incidence in controls and treated animals, and therefore were considered unrelated to treatment.

One outstanding, non-neoplastic findings attributable to the administration of isocycloseram was considered to be an increase in plasma cells in the haemolymphoreticular system in males and females at 60 ppm and above. Increased incidences of plasmacytosis/plasma cell infiltration were observed primarily in the lymph nodes, most notably the mesenteric lymph nodes. In addition, an increase in the incidence and severity of plasma cell infiltration was observed in the spleen and the thymus of females exposed to isocycloseram at 60 ppm and above, reaching statistical significance at 60 ppm ($p \leq 0.05$) when compared with concurrent controls. The latter finding was not observed in males. Plasma cell infiltration tended to be distributed throughout the body (uterus, urinary bladder, lacrimal gland, oesophagus, stomach, rectum, pancreas, lung, liver, kidney, heart, adrenal, gall bladder and/or nasal cavity) of female mice exposed at 60 ppm and above, at an incidence of up to 5/50; the plasma cell infiltrates often contained Russell bodies.

The plasma cell infiltration seen could be indicative of a local effect or an inflammatory reaction. However, based on the data provided, these hypotheses cannot be proven. Hence, the effects were considered adverse and treatment-related.

Other microscopic findings observed were considered incidental, of a nature commonly observed in this strain and age of mice, were not dose-related and/or were of similar incidence and severity in control and treated animals, and therefore were considered unrelated to treatment.

*Table 34. Plasmacytosis findings in the lymph nodes in a 80-week mouse study with isocycloseram, (Number of mice affected [as percentage of mice examined] shown)*

| Finding | Dose level (ppm of SYN547407) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| severity | 0 | 15 | 60 | 200 | 0 | 15 | 60 | 200 |
| **Mesenteric** | | | | | | | | |
| Mice examined | 50 | 50 | 50 | 48 | 49 | 47 | 49 | 50 |
| total | 0 [0%] | 1 [2%] | 3 [6%] | 21** [44%] | 1 [2%] | 0 [0%] | 8* [16%] | 24** [48%] |
| minimal | 0 | 1 | 3 | 11 | 0 | 0 | 6 | 12 |
| mild | 0 | 0 | 0 | 7 | 0 | 0 | 2 | 9 |
| moderate | 0 | 0 | 0 | 2 | 1 | 0 | 0 | 3 |
| marked | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| **Mandibular** | | | | | | | | |
| Mice examined | 47 | 50 | 49 | 47 | 48 | 49 | 50 | 49 |
| total | 0 [0%] | 3 [6%] | 0 [0%] | 1 [2%] | 2 [4%] | 0 [0%] | 13** [26%] | 7 [14%] |
| minimal | 0 | 1 | 0 | 0 | 0 | 0 | 2 | 3 |
| mild | 0 | 0 | 0 | 0 | 2 | 0 | 10 | 4 |
| moderate | 0 | 2 | 0 | 1 | 0 | 0 | 1 | 0 |

Different from control group mean at statistical significance: * $p < 0.05$, ** $p < 0.01$; Source: Strepka, 2019a

The NOAEL for toxicity of isocycloseram in a 80-week study in mice was 15 ppm (equal to 1.7 mg/kg bw per day), based on an increase in plasma cell infiltration in the mesenteric lymph node at the LOAEL of 60 ppm (6.7 mg/kg bw per day). No test substance-related neoplastic findings were noted, accordingly, the highest dose of 200 ppm (equal to 23.1 mg/kg bw per day) was the NOAEL for carcinogenicity. (Strepka, 2019a)

***Rat***

In a combined chronic toxicity and carcinogenicity study (OECD TG 453), groups of 52 Han Wistar (Crl:WI (Han)) rats per sex received isocycloseram (purity 96.9%, Batch No. 2061933-85-3) in the diet for 104 weeks at concentrations of 0, 20, 50 or 150 ppm (equal 0, 0.9, 2.3 and 7.0 mg/kg bw per day for males, 0, 1.2, 3.0 and 9.2 mg/kg bw per day for females). In addition, 12 rats of each sex were added to all groups to assess changes at 52 weeks, achieving a mean dosage equal to 0, 1.1, 2.7 and 8.1 mg/kg bw per day for males, and 0, 1.4, 3.4 and 10.3 mg/kg bw per day for females.

Animals were inspected daily for mortality, moribundity, clinical signs of toxicity and palpable masses. Body weights and food consumption were measured and recorded at predetermined intervals. Achieved dosage was determined for each period of food consumption throughout the course of the study. Food utilization was determined for weeks 1–13. Ophthalmoscopic examinations were carried out during pretreatment for all rats and for rats receiving 0 or 150 ppm in week 52 for toxicity group animals, and in week 104 for carcinogenicity group animals. Detailed functional observations and functional tests (including motor activity) were carried out during pretreatment and week 51/52. Blood and urine samples were collected from all animals at predetermined intervals throughout the course of the study to evaluate haematology, coagulation, clinical chemistry and urinalysis parameters. All animals were euthanized after at least 52 weeks (toxicity group) or 102 weeks (carcinogenicity group) of treatment and subjected to detailed necropsy, with selected organs being weighed. All unscheduled decedents were also necropsied. Tissues from all animals were subjected to a comprehensive histopathological evaluation.

All formulated diets were within the acceptable ranges for concentration and homogeneity of isocycloseram. There were no statistically significant differences in mortality between the control groups and the treated groups. One toxicity group female at 150 ppm was euthanized prematurely

due to clinical signs. However, this death was not considered to be treatment-related. The number of unscheduled deaths in the control, 20, 50 and 150 ppm carcinogenicity groups was 14/52, 13/52, 19/52 and 12/52 in males, and 18/52, 16/52, 14/52 and 19/52 in females, respectively. There was no effect on mortality and survival in all groups was acceptable for the carcinogenic assessment. There were no clinical observations or differences in the number of animals with palpable masses that were considered to be treatment-related.

Body weight gain at the end of the study was lower (by 11%) in high-dose group females (150 ppm), compared to controls. Although statistical significance was not achieved, the change was considered adverse. Dietary administration of isocycloseram was not associated with changes in food consumption, food utilization, water consumption, ophthalmoscopy findings, functional observation, motor activity, haematology, coagulation, clinical chemistry, urinalysis or organ weights.

There were no differences in motor activity that were considered treatment-related. There were incidences of statistical significance noted such as: lower basic and fine movement and x-ambulation at week 51, segment 1 (0–5 minutes) in females at 20 and 50 ppm, and basic and fine movement at week 51 segment 2 in females at 20 ppm. However, due to the lack of any dose–response relationship and consistency through the observation period or across time points these effects were considered not to be treatment-related.

The total number of white blood cells was slightly higher in females receiving 150 ppm at week 14 which was mainly attributable to a slightly higher number of lymphocytes when compared to concurrent controls. The total number of red blood cells, haemoglobin concentration (Hb) and haematocrit (Ht) were slightly lower at week 14 in females receiving 150 ppm compared to concurrent controls. The total number of white blood cells was slightly higher at week 105/106 in males receiving 50 or 150 ppm, which was mainly attributable to a the increased number of lymphocytes, neutrophils and monocytes when compared to concurrent controls. In addition, the Hb and Ht values were slightly lower at week 105/106 in males receiving 150 ppm. As all of these changes were of small in size with no correlating histopathology, and there were no similar changes at subsequent time points, they were considered not to be test item-related. Any other changes in haematology and coagulation parameters were considered to be within normal biological variation.

At week 27, calcium was slightly lower in males receiving 20 ppm and above, however no dose–effect relationship was noted and there were no similar changes at other time points. In addition, at week 27 phosphate was slightly higher in males receiving 150 ppm. Bile acids and creatinine were slightly higher at week 14 in females receiving 150 ppm compared to concurrent controls. Globulin was slightly lower and the albumin:globulin ratio was slightly higher in females receiving 150 ppm compared to concurrent controls, but only at week 27. As all these changes were small in size with no correlating histopathology, and there were no similar changes at subsequent time points, they were considered not to be treatment-related. Any other changes in clinical chemistry parameters were considered to be within normal biological variation.

There were slightly higher absolute (by 10%; statistically significant) and relative (by 5%; not significant) kidney weights in males in the receiving 150 ppm in the toxicity part of the study. As this change was small, and in the absence of any correlating clinical chemistry or histopathological changes, it was considered not to be treatment-related. No other differences were noted in organ weights after 52 weeks of treatment. At 104 weeks there were isolated organ weight values that differed from their respective controls. There were, however, no patterns, trends or correlating data to suggest these values were toxicologically relevant. Thus, the organ weight differences observed were considered incidental and not treatment-related.

In the 52-week toxicity part of the study microscopic examination showed test item-related changes in the liver (vacuolation), possibly the testes (tubular degeneration: 2/12 compared with 1/12 controls), and epididymis (cellular debris: 1/12 compared with 0/12 controls), the effects being seen in males at 150 ppm. There were no test substance-related neoplastic findings.

*Table 35. Test substance-related microscopic findings in a 52-week rat study with isocycloseram*

| | | Dietary concentration (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | Females | | | |
| **Finding** | | **0** | **20** | **50** | **150** | **0** | **20** | **50** | **150** |
| **Liver;** number examined | | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 |
| Centrilobular vacuolation | total | 1 | 1 | 2 | 10** | 0 | 0 | 0 | 0 |
| | minimal | 1 | 0 | 1 | 6 | 0 | 0 | 0 | 0 |
| | mild | 0 | 1 | 1 | 4 | 0 | 0 | 0 | 0 |
| | moderate | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Testes;** number examined | | 12 | 12 | 12 | 12 | - | - | - | - |
| Tubular degeneration | total | 1 | 0 | 0 | 2 | - | - | - | - |
| | minimal | 1 | 0 | 0 | 2 | - | - | - | - |
| **Epididymis;** number examined | | 12 | 12 | 12 | 12 | - | - | - | - |
| Cellular debris | total | 0 | 0 | 0 | 1 | - | - | - | - |
| | minimal | 0 | 0 | 0 | 1 | - | - | - | - |

Different from control group mean at statistical significance: * $p < 0.05$, ** $p < 0.01$; Source: Strepka, 2019b

In the 104-week carcinogenicity part of the study microscopic examination showed test item-related, non-neoplastic findings in the liver (vacuolation) in males dosed at 50 ppm and above, and in females dosed at 150 ppm,. Vacuolation in the liver, in the absence of any alterations in liver enzymes, organ weight or other accompanying histopathological changes was considered not to be adverse. Non-neoplastic findings were also noted in testes (tubular degeneration) and epididymis (reduced sperm, cellular debris) in males at 150 ppm. Tubular degeneration/atrophy (minimal to severe) occurs spontaneously in aged rats and the incidence and severity of tubular degeneration at 20 and 50 ppm was consistent with the concurrent control and the HCD range. In the epididymis, in addition to a reduction in sperm, an increased incidence of minimal cellular debris was also noted in males at 150 ppm, without reaching statistical significance. Both reduced sperm and cellular debris findings can be considered secondary to tubular degeneration in the testes. The concurrent control incidence of reduced sperm was considered low compared to the HCD range and the incidence of findings at 20 and 50 ppm can be considered consistent with the background incidence of this finding in this strain of rat at the laboratory at which the study was carried out.

*Table 36. Test substance-related microscopic findings in a 104-week rat study with isocycloseram*

| | | Dietary concentration (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | Females | | | |
| **Finding** | | **0** | **20** | **50** | **150** | **0** | **20** | **50** | **150** |
| **Liver;** number examined | | 52 | 52 | 52 | 52 | 52 | 52 | 52 | 52 |
| Vacuolation | total | 14 | 14 | 27* | 37** | 6 | 8 | 10 | 16* |
| | minimal | 11 | 9 | 19 | 13 | 5 | 5 | 7 | 9 |
| | mild | 0 | 3 | 7 | 10 | 1 | 2 | 3 | 5 |
| | moderate | 3 | 1 | 1 | 13 | 0 | 1 | 0 | 1 |
| | marked | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| **Testes;** number examined | | 52 | 52 | 52 | 52 | - | - | - | - |
| Tubular degeneration | total | 6 | 7 | 10 | 22** | - | - | - | - |
| | minimal | 5 | 3 | 5 | 14 | - | - | - | - |
| | mild | 1 | 2 | 2 | 3 | - | - | - | - |
| | moderate | 0 | 2 | 2 | 4 | - | - | - | - |
| | marked | 0 | 0 | 1 | 1 | - | - | - | - |

| | | Dietary concentration (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | Females | | | |
| **Finding** | | **0** | **20** | **50** | **150** | **0** | **20** | **50** | **150** |
| **Epididymis;** number examined | | 52 | 52 | 52 | 52 | - | - | - | - |
| Cellular debris | total | 1 | 2 | 1 | 6 | - | - | - | - |
| | minimal | 1 | 2 | 1 | 6 | - | - | - | - |
| Reduced sperm | total | 0 | 4 | 4 | 6* | - | - | - | - |
| | minimal | 0 | 1 | 1 | 0 | - | - | - | - |
| | mild | 0 | 2 | 2 | 5 | - | - | - | - |
| | moderate | 0 | 1 | 1 | 1 | - | - | - | - |

Different from control group mean at statistical significance: * $p < 0.05$, ** $p < 0.01$; Source: Strepka, 2019b

***Table 37. Intergroup incidence of selected microscopic findings (males) in a 104-week rat study with isocycloseram compared with historical control data mean and ranges***

| | | Mean incidence from historical control data (%) | Historical control data range (%) | Dietary concentration (ppm) Mean incidence (%) | | | |
|---|---|---|---|---|---|---|---|
| | | | | **0** | **20** | **50** | **150** |
| **Testes;** number examined | | 950 | 950 | 52 | 52 | 52 | 52 |
| Tubular degeneration | total | 12.9 | 3.8–20.0 | 11.5 | 13.5 | 19.2 | 42.3 |
| | minimal | – | 0.8–4.5 | 9.6 | 5.8 | 9.6 | 26.9 |
| | mild | – | 0–5.8 | 1.9 | 3.8 | 3.8 | 5.8 |
| | moderate | – | 0–11.0 | 0 | 3.8 | 3.8 | 7.7 |
| | marked | – | 0–11.0 | 0 | 0 | 1.9 | 1.9 |
| **Epididymis;** number examined | | 950 | 950 | 52 | 52 | 52 | 52 |
| Reduced sperm | total | 6.5 | 1.0–11.0 | 0 | 7.7 | 7.7 | 11.5 |
| | minimal | – | – | 0 | 1.9 | 1.9 | 0 |
| | mild | – | – | 0 | 3.8 | 3.8 | 9.6 |
| | moderate | – | – | 0 | 1.9 | 1.9 | 1.9 |

The NOAEL for isocycloseram in a 104-week study in rats was 50 ppm (equal to 2.3 mg/kg bw per day in males, 3.9 mg/kg bw per day in females), based on microscopic findings in testes and epididymides in males at a LOAEL of 150 ppm (7.0 mg/kg bw per day in males). No test substance-related neoplastic findings were noted and, accordingly, the carcinogenic NOAEL in this study was 150 ppm (equal to 7 mg/kg bw per day), the highest dose tested (Strepka, 2019b).

## 2.4 Genotoxicity

Isocycloseram was tested for genotoxicity in an adequate range of in vitro assays and in a single study in vivo. The results were consistently negative and are summarized in Table 38. The respective studies are briefly reported below.

***Table 38. Summary of genotoxicity studies with isocycloseram***

| Study types; genotoxicity end-points | Concentrations tested; metabolic activation | Purity | Result | Reference |
|---|---|---|---|---|
| | **In vitro** | | | |
| Bacterial mutation assay (Ames) | Plate incorporation and pre-incubation assays: 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate; ± S9 | 96.9% | Negative | Chang, 2016a |
| | Plate incorporation assay: 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate, ± S9<br>Pre-incubation assays: 33, 100, 333, 1000, 2500 and 5000 µg/plate, ± S9 | 96.1% | Negative | Chang, 2019 |
| Mammalian cell mutation assay (Mouse lymphoma assay in L5178Y cells) | Experiment I: 3.9, 7.8, 15.6, 31.3, 62.5 µg/mL (± S9)<br>Experiment II: 15.5, 31.0, 46.5, 62.0, 77.5 µg/mL (−S9) 7.8, 15.5, 31.0, 46.5, or 62.0 µg/mL (+S9) | 96.9% | Non-mutagenic | Sokolowski, 2020 |
| | Experiment I: 15, 30, 45, 60 µg/mL (± S9)<br>Experiment II: 12.5, 25, 55, and 60 µg/mL (+S9) | 96.1% | Non-mutagenic | Sokolowski, 2019 |
| Chromosome aberration assay in vitro (Human lymphocytes) | 4-hour exposure: 4.3, 13.2, 23.0 (−S9); 23.0, 40.3, 80.6 (+S9)<br>22-hour exposure (−S9): 6.4, 11.2, 19.6, 34.4 µg/mL; | 96.9% | Non-clastogenic | Chang, 2016b |
| | **In vivo** | | | |
| Micronucleus assay in rat bone marrow (oral administration) | Range-finding experiment: 2 × 2000 mg/kg bw per day with blood analysis<br>Main study: 2 × 0, 500, 1000 and 2000 mg/kg bw per day | 96.9% | Neither clastogenic nor aneugenic | Dunton, 2016 |

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*

### (a) In vitro studies

#### *Bacterial mutation assay*

In a first Ames test, isocycloseram (purity 96.9%, Batch No. SMU5IP15011) was applied to *Salmonella typhimurium* tester strains TA1535, TA1537, TA98 and TA100, as well as to *Escherichia coli* strains WP2 *uvr*A pKM101 and WP2 pKM101 at concentrations ranging from 3 to 5000 µg/plate. Plate incorporation (Experiment I) and pre-incubation (Experiment II) tests were run, both in the absence and in the presence of metabolic activation (rat liver supernatant fraction obtained by centrifuging at 9000 *g*). The test item was dissolved in DMSO which was also used alone as a negative control. In the absence of S9 mix the following positive control substances were employed: 4-nitro-*O*-phenyldiamine (for TA1537, TA98), sodium azide (for TA100 and TA1535), and methyl methane sulfonate in the case of the two *E. coli* strains. In the activation experiments, 2-aminoanthracene was used as positive control for all strains. Mutagenicity testing was performed in triplicate at all concentrations.

Weak cytotoxicity was confined to the highest concentration when applied to *E. coli* strain WP2 *uvr*A in the presence of S9 mix, since the number of revertants was reduced. Precipitation became apparent with all strains at concentrations of 1000 µg/plate and above.

No increase in revertants was observed with any of the tested concentrations of the test item in any tester strain, whereas the positive control substances gave the expected increases.

Under the conditions of this study, isocycloseram was not mutagenic to bacteria in the Ames test up to the limit concentration of 5000 µg/plate (Chang, 2016a).

In a very similar study, a different batch of isocycloseram (purity 96.1%, Batch No. 05183-TB-185) was tested in the same strains of *S. typhimurium* and *E. coli* as in Chang, 2016a. Test item concentrations were adjusted for purity and ranged from 3 to 5000 µg/plate in the plate incorporation assay (Experiment I) and from 33 to 5000 µg/plate in the pre-incubation assays (Experiments II and IIa). Experiment I and II were run both in the absence and presence of S9 mix. An additional pre-incubation trial (Experiment IIa) was performed only in tester strain TA1535 without metabolic activation. The vehicle was DMSO, which was also used as a negative control. The same positive control substances as in Chang, 2016a (see above) were applied. Mutagenicity testing was performed in triplicate.

Weak cytotoxicity to *E. coli* strain WP2 *uvr*A only was observed as before and confirmed in both the absence and presence of S9 mix, since the number of revertants was reduced. Precipitation became apparent with all strains at 333 µg/plate and above.

No increase in revertants was observed with any of the concentrations of the test item tested, whereas the positive control substances gave the expected increases. The only exception was that the positive control substance sodium azide, which failed to produce a mutagenic response in TA1535 in the pre-incubation assay without activation (Experiment II). as a result this trial was repeated.

Under the conditions of this study isocycloseram was not mutagenic to bacteria in the Ames test up to the limit concentration of 5000 µg/plate (Chang, 2019).

***Mammalian cell mutation assay***

Isocycloseram (purity 96.9%, Batch No. SMU5IP15011) was tested for its ability to induce forward mutations in mammalian cells by assessing the mutation rate at the thymidine kinase (*tk*) locus in mouse lymphoma L5178Y cells. Two independent experiments were conducted, both in the presence and absence of metabolic activation, with two parallel cultures each. The solvent was DMSO, which was also used as a negative control. Methylmethanesulfonate and cyclophosphamide (CPA) served as positive controls in the experiments without and with metabolic activation, respectively.

Based on the results of a preliminary assay, due to cytotoxicity and precipitation, only concentrations up to a maximum of 60 or 80 µg/mL could be analyzed in the main experiment. In the first experiment cells were exposed to concentrations of 3.9, 7.8, 15.6, 31.3 or 62.5 µg/mL, with and without S9 mix. In the second experiment, the following concentrations were tested in the absence of metabolic activation: 15.5, 31.0, 46.5, 62.0 or 77.5 µg/mL. With activation, slightly lower concentrations of 7.8, 15.5, 31.0, 46.5 or 62.0 µg/mL were used. In addition, there were a few more cultures included which had been exposed to lower or higher concentrations (up to 125 µg/mL in the first experiment) but these were not repeated due to precipitation or because the applied amounts of isocycloseram were too low.

The treatment intervals for both experiments in the presence and absence of metabolic activation were generally four hours. After pulse treatment, media were replaced by test substance-free culture media in both experiments and the cells were incubated for 48 hours for expression of mutant cells. This step was followed by incubation of cells in selection medium containing trifluorothymidine for 10–15 days before harvest and evaluation of two plates (cultures) per test and concentration.

In Experiment I no increase in mutant colony numbers was observed, either with or without metabolic activation, and precipitation occurred at the highest analyzed concentration of 62.5 µg/mL. This occurred with and without S9 mix, resulting in a lower relative cell growth compared to the other cultures.

In the Experiment II no evidence of mutagenicity was obtained without metabolic activation but,

this time, no precipitation was observed. Nonetheless, relative cell growth was lowest at the maximum concentration of 77.5 µg/mL. With activation, in contrast, an increase in mutant frequency over the solvent control value was observed in one of the two cultures at the highest concentration. In fact, the number of mutant colonies per million cells amounted to 355 at 62.0 µg/mL, compared to 130 µg/mL in the negative control group. At the maximum concentration some precipitation was observed. Because of a negative test for trend, and since this finding was an isolated event and not reproducible either in the parallel culture or in the previous experiment, the result was not considered biologically meaningful.

In both experiments, the positive control substance gave the expected mutagenic responses. On balance, the conclusion was drawn that isocycloseram proved negative for genotoxicity in the mouse lymphoma assay (Sokolowski, 2020).

In a similar study, another batch of isocycloseram (purity 96.1%, Batch No. 05138-TB-185) was tested for mutagenicity in the mouse lymphoma assay. Two independent experiments were performed. The test method and conditions were the same as reported above for Sokolowski, 2020, and the same solvent and positive control substances were used. However, apparently only one culture per concentration was treated.

Based on preliminary experiments, concentration of up to 80 µg/mL were tested in the first experiment, both with and without S9 mix. The following concentrations were analyzed for genotoxicity: 15, 30, 45 and 60 µg/mL. The second experiment was run in the presence of metabolic activation only. Concentrations up to 80 µg/mL were tested but those eventually analyzed were the following: 12.5, 25, 55 and 60 µg/mL. Precipitation was observed at concentrations in the region 60–80 µg/mL.

No evidence of genotoxicity was obtained with isocycloseram when tested up to concentrations at which precipitation was seen to occur. The positive control substances caused clear increases in mutant frequencies (Sokolowski, 2019).

***Chromosome aberration assay***

In an in vitro chromosome aberration test, isocycloseram (purity: 96.9%, Batch no. SMU5IP15011) was applied to peripheral human lymphocytes, with or without metabolic activation (S9 mix), in two independent experiments. The blood cells had been obtained from a healthy, 32-year-old, non-smoking male donor. The test item was dissolved in DMSO, which also served as a negative control. Positive control reagents were ethylmethane sulfonate for the experiments lacking activation, and CPA in the presence of S9 mix. It is explained in the study report that ethylmethane sulfonate was not among the positive control substances recommended in the appropriate OECD TG 473 (2014 version) but was used because of familiarity and its use in the extensive HCD data available for the performing laboratory.

Experiment I was used both as a pretest for determination of cytotoxicity and for evaluation of cytogenetic damage since all the criteria for a valid trial were met. In this experiment, two cultures were exposed for four hours in the absence and presence of S9 mix. Cells were harvested and slides (two per culture) prepared after an 18-hour recovery period (22 hours after commencement of treatment). Colcemid was added around three hours before harvest to arrest cells in metaphase. Both with and without activation, 12 test concentrations ranging from 4.3 to 5160 µg/mL were applied, adjusting for purity. Because precipitation was occurring already at comparatively low concentrations (from 23 µg/mL upwards without S9, and 80.6 µg/mL upwards with activation) cytogenetic evaluation was confined to the following concentrations: 4.3, 13.2 and 23.0 µg/mL without S9, and 23.0, 40.3 and 80.6 µg/mL with S9. For each culture 150 metaphases (300 in total per concentration) were scored for chromosome aberrations and evidence of polyploidy.

Experiment II was run under similar conditions but entirely without S9 mix. Also, the exposure time was 22 hours and cells were harvested immediately thereafter. Test item concentrations were selected on the basis of the experience from Experiment I and ranged from 2.1 to 322.5 µg/mL. Because of precipitation at 60.2 µg/mL and above, the following concentrations were used for cytogenetic evaluation: 6.4, 11.2, 19.6 and 34.4 µg/mL.

No evidence of an increase in structural chromosome aberrations, including and excluding gaps, was observed at any concentration in any of the experiments. Following four-hour exposure in the presence of S9 mix, and after the 22-hour treatment without activation, mitotic index was clearly

reduced at the highest analyzed concentrations of 80.6 µg/mL and 34.4 µg/mL, the index falling to 66% and 51%, respectively, figures that suggest some cytotoxicity. According to the study author, there was also no increase in polyploidy metaphases. Ethylmethane sulfonate and CPA gave the expected positive responses even though Experiment II had to be repeated because the cytotoxicity of the positive control substance (EMS) exceeded the acceptability criteria.

Under the conditions of this study, isocycloseram was considered negative in the in vitro chromosome aberration assay in human lymphocytes even though it could only be evaluated up to the level of cytotoxicity (Chang, 2016b).

**(a) In vivo studies**

In a bone marrow micronucleus assay in young adult rats, isocycloseram (purity: 96.9%, Batch no. SMU5IP15011) was suspended in 0.1% Tween 80 in 0.5% aqueous methylcellulose and applied by oral gavage at a volume of 10 mL/kg bw. Following a small scale dose-sighting trial with three different dose levels (500, 1250 and 2000 mg/kg bw), a range-finding experiment was performed in which three male and three female Crl:WI(Han) rats received an oral dose of 2000 mg/kg bw on two subsequent days, approximately 24 hours apart. The same dosing regimen was used in the main study in which groups of six males received either the vehicle or the test item at dose levels of 0, 500, 1000 or 2000 mg/kg bw. A concurrent positive control group of same size received an oral dose of 15 mg/kg bw of cyclophosphamide but was treated once only.

In the range-finding experiment, blood samples were taken at one hour or at four hours after the second dose and at termination to investigate blood levels of isocycloseram in order to prove exposure of the well-perfused bone marrow. All animals were killed approximately 24 hours after the second dose, or single administration of the positive control substance. Bone marrow smears were obtained from femoral bone and slides prepared on which a total of 2000 polychromatic erythrocytes (PCEs) per animal were scored for the presence of micronuclei. In addition, the ratio of PCEs to normochromatic erythrocytes (NCEs) was calculated, since its shift might indicate bone marrow toxicity.

All animals survived until scheduled termination but in the dose-sighting and range-finding experiment (but surprisingly not in the main study), rapid breathing and body weight loss were noted. The test item was detected in all blood samples from the range-finding experiment, even though it was not quantified in ng/mL; instead, peak area counts were determined. Nonetheless, this finding can be taken as proof of sufficient bone marrow exposure. In addition, since the study was performed in rats, evidence of bone marrow exposure because of high blood levels coming from the ADME studies in this species might be directly used as evidence of target tissue exposure.

There was no increase in micronucleus frequency over the negative control in any of the treatment groups whereas cyclophosphamide caused a significant increase. The PCE:NCE ratio was not affected, suggesting that isocycloseram was not toxic to bone marrow.

Under conditions of this in vivo micronucleus assay in rats with sufficient evidence of bone marrow exposure, isocycloseram was devoid of any clastogenic or aneugenic potential (Dunton, 2016).

## 2.5 Reproductive and developmental toxicity

**(a) Multigeneration studies**

In a preliminary study (not GLP), three test compounds including isocycloseram were administered via oral gavage to female rats during gestation and lactation to investigate possible effects on clinical condition, reproductive parameters and pup survival. In this monograph, only information related to, and obtained with, isocycloseram is briefly reported.

Groups of 10 time-mated Han:Wistar rats received isocycloseram (purity 97%–98%), dissolved in 0.5% aqueous CMC, at dose levels of 0, 15, 30 or 60 mg/kg bw per day in a volume of 10 mL/kg bw. The original intention was dosing from gestation day (GD) 6 until lactation day (LD) 20 but administration of the mid and high doses was discontinued from GD 16/17 or GD 9/10 and the dams were prematurely killed.

Animals were observed for mortality and clinical signs over the course of the study and body weights and food consumption were determined at regular intervals. Parturition was monitored and pups were weighed and sexed. Surviving low-dose females, as well as the pups, were killed at weaning on postnatal day (PND) 21. All parental dams plus one male and one female pup from each litter were necropsied. Adrenals were weighed and selected organs examined under the microscope.

Premature termination of the mid- and high-dose groups was due to body weight loss or at least suspended weight gain and low food intake. Piloerection, decreased activity and tremors were noted occasionally. The outstanding macroscopic finding in the dams was abnormal pale coloration of the jejunum and duodenum and thickening of the mucosa.

Low-dose dams also gained less weight than the controls and food consumption was slightly reduced during gestation whereas a compensatory higher body weight gain was observed during lactation. There were no clinical signs that could be attributed to treatment and all but one (which gave birth to no live pups) of the dams littered successfully. Litter size and reproductive indices were similar to those of the control group. In low-dose pups birth weight and body weight gain during lactation were lower than for the control group but pup survival was not altered.

Relative adrenal weight in the dams receiving 15 mg/kg bw per day was higher than for controls. Pathological findings comprised microscopic changes in the intestines of both parental dams and pups, specifically, epithelial vacuolation of the duodenum, jejunum or ileum in 4/9 parental dams and in 7/18 pups (nine and 18 being the number examined, respectively).

On balance, dose levels of 30 and 60 mg/kg bw per day were above the maximum tolerated dose (MTD). Nutritional parameters were affected and the intestines were identified as a target organ in both dams and pups (Blunt, 2014).

A one-generation study with gavage administration of isocycloseram (purity 98.4%, Batch No. MEQ-02794-09) was performed in groups of 24 male and 24 female Crl:WI(Han) rats at dose levels that differed according to sex. Male rats received isocycloseram test article as daily doses of 0, 7.5, 15 or 45 mg/kg bw with the highest dose increased to 60 mg/kg bw from study day 35 onwards. Females were administered 0, 3.5, 7.5 or 15 mg/kg bw per day because previously obtained data (Blunt, 2014; see above) suggested their higher vulnerability compared to males. The vehicle was 0.5% aqueous CMC containing 0.1% Tween 80. Treatment commenced 10 weeks before first pairing continued during the mating period (maximum length two weeks), gestation, and lactation until termination and necropsy. Male rats were killed when littering was complete. Female rats and all pups were terminated at weaning on LD 21.

All animals were examined for mortality, clinical signs and general condition. Body weight and food intake were monitored at regular intervals mostly, weekly. The usual reproductive parameters such as mating index, litter size, live birth index, pup sex ratio, viability and lactation index, together with body weight development of the pups were recorded. Sperm parameters were assessed in male rats at termination and estrous cycle was investigated in females in the premating period.

At termination, all parental animals were necropsied and gross examinations conducted. Adrenals, liver and reproductive organs were weighed. Uterus was removed and the number of implantation sites counted. In the control and high-dose groups the following organs were subject to histopathological examination: adrenals, caecum, colon, duodenum, ileum, jejunum, liver, ovaries, prostate, seminal vesicles, testes (with epididymides), uterus and vagina. Because of the findings in high-dose groups in both sexes, examination of the duodenum and jejunum was extended to all animals. In addition, in males, adrenals, liver, testes and epididymides were examined under the microscope. Pups were assessed for external appearance and, at necropsy, for gross abnormalities.

For bioanalysis of isocycloseram levels in blood, samples were taken from five rats of each sex and dose on study day 29, that is long before pairing. Relevant results are presented in Section 1.3 of this monograph, above).

With one exception, all parental animals survived until scheduled termination. One high-dose male had to be killed on day 111 because of poor clinical condition and urological signs which were all due to nephroblastoma. Since this is a malignant neoplasia of embryonic origin, the single premature

death was not treatment-related. There were no further clinical signs which could be attributed to substance administration. Body weight, its gain and food consumption were not altered at any dose level.

One low- and one mid-dose female were not pregnant but, in the absence of any dose–response relationship this finding was considered incidental. There was no effect due to the test substance on mean gestation length and all dams gave birth to live litters. On balance, fertility, reproductive performance and litter parameters were not affected up to the highest dose tested. The same holds true for estrous cyclicity.

Sperm concentration and motility were no different for control and high-dose males, but there was a significant reduction in homogenization-resistant testicular spermatid count at the top dose level. In addition, the percentage of morphologically abnormal sperm was nearly doubled at the top dose level but statistical significance was not achieved, most likely because of the strong interindividual variation as indicated by the large standard deviation in values from males receiving isocycloseram at 60 mg/kg bw per day (see Table 39).

***Table 39. Organ weight changes and testicular spermatid count in male rats in the one-generation study with isocycloseram, (mean ± standard deviation shown)***

| | Dose level (mg/kg bw per day) | | | |
|---|---|---|---|---|
| **Parameter** | **0** | **7.5** | **15** | **60[a]** |
| Terminal body weight (g) | 451.2 (± 45.2) | 477.6 (± 34.1) | 456.3 (± 42.0) | 452.1 (± 36.1) |
| Adrenal weight (mg) | 54 (± 8) | 63 (± 12)** | 61 (± 9)* | 67 (± 13)** |
| Testis weight, left (g) | 1.89 (± 0.14) | 1.91 (± 0.17) | 1.90 (± 0.13) | 1.76 (± 0.20)* |
| Testis weight, right (g) | 1.87 (± 0.13) | 1.92 (± 0.19) | 1.91 (± 0.12) | 1.75 (± 0.20)* |
| Spermatid count ($\times 10^6$/mL) | 107.80 (± 19.90) | 95.47 (± 17.79) | 98.43 (± 19.43) | 79.91 (± 20.09) ** |
| Abnormal sperm (%) | 1.13 (± 0.56) | No data | No data | 2.24 (± 2.27) |

[a] Dose of 45 mg/kg bw per day until day 35; Source: Penn, 2018

Different from control group mean using Dunnett's two-sided test at statistical significance: * $p < 0.05$, ** $p < 0.01$;()

Gross pathological examination did not reveal any abnormalities that could be attributed to isocycloseram administration. However, there were weight changes in the adrenals and testis in high-dose males (Table 39) which were in line with histopathological findings (see Table 41) and the lower spermatid count. An increase in adrenal weight was also observed in the low- and mid-dose groups but these had no histological correlate, and therefore interpretation is equivocal.

Microscopic evaluation elucidated intestinal vacuolation in both sexes (Table 40), as well as vacuolation and inflammation in the livers of males (Table 41). Testicular effects were accompanied by findings in the epididymis. These findings were considered treatment-related and adverse. At the top doses of 60 or 15 mg/kg bw per day in males and females respectively they were observed to occur with relatively high frequency, but few male animals were also affected at the next lowest dose level of 15 mg/kg bw.

***Table 40. Epithelial vacuolation of the intestines (all grades) in the one-generation study in rats with isocycloseram,***

| | | Dose level (mg/kg bw per day) | | | |
|---|---|---|---|---|---|
| **Finding** | | **0** | **7.5** | **15** | **60[a]** |
| Males: | duodenum | 0/24 | 0/24 | 2/24 | 15/24 |
| | jejunum | 0/24 | 0/24 | 0/24 | 16/24 |
| | | **0** | **3.5** | **7.5** | **15** |
| Females: | duodenum | 0/24 | 0/24 | 1/24 | 4/24 |
| | jejunum | 0/24 | 0/24 | 0/24 | 4/24 |

[a] Dose of 45 mg/kg bw per day until day 35; Source: Penn, 2018

*Table 41. Histopathological findings (all grades) in male rats in the one-generation study with isocycloseram; presumed treatment-related changes in bold (number affected/examined)*

| | Dose (mg/kg bw per day) | | | |
|---|---|---|---|---|
| **Finding** | **0** | **7.5** | **15** | **60[a]** |
| Liver, centrilobular vacuolation | 0/24 | 1/24 | **6/24** | **21/24** |
| Liver, focal infiltrates of inflammation cells | 3/24 | 4/24 | 4/24 | **8/24** |
| Adrenals (zona fasciculate), vacuolation | 0/24 | 0/24 | 0/24 | **6/24** |
| Testes, tubular degeneration | 0/24 | 1/24 | **3/24** | **18/24** |
| Epididymides, reduced sperm | 0/24 | 0/24 | 0/24 | **4/24** |
| Epididymides, exfoliated spermatogenic cells | 0/24 | 0/24 | 0/24 | **7/24** |

[a] Dose 45 mg/kg bw per day until day 35; Source: Penn, 2018

There was no apparent effect on pups and their development at any dose level.

On balance, no evidence of reproductive toxicity was obtained in this one-generation study. Accordingly, both the reproductive and the offspring NOAELs were identified at 15 mg/kg bw per day, the highest dose tested in female rats. The parental NOAEL was 7.5 mg/kg bw per day based on organ weight changes, histopathological and sperm findings. Target organs were the liver, the adrenals, testes with epididymides, and the intestines. Because of the gavage application that is unusual for a reproductive study and the availability of a recent full two-generation study (see below), the Meeting decided not to use this one-generation study for dietary risk assessment of isocycloseram (Penn, 2018).

In a two-generation feeding study, a possible impact of isocycloseram on the integrity and performance of the male and female reproductive systems, including gonadal function, estrous cycle, mating behaviour, conception, gestation, parturition, lactation and weaning, and the growth and development of the offspring were investigated in Crl:WI(Han) rats. Four groups of 24 male and 24 female rats received dietary doses that were weekly adjusted to provide dose levels of 0, 1.5, 4 or 12 mg/kg bw per day of isocycloseram (purity 96.9%, Batch no. SMU5IP15011) for 10 weeks before pairing, during the mating period, gestation and lactation, and then until necropsy. During gestation and lactation, the actual mean achieved dosages in Dosages for the F0 generation females were slightly higher than the nominal values. In the low-dose group, intakes of 2.0 and 2.5 mg/kg bw per day were calculated, 5.2 and 5.8 mg/kg bw per day for the mid dose and 14.9 and 16.9 mg/kg bw per day in high-dose dams, the values given for gestation and lactation respectively.

Four groups of 24 young rats per sex were selected from the weaned F0 generation litters to constitute the F1 generation. These animals were fed diets containing the same nominal doses of isocycloseram from weaning on PND 21 for approximately 10 weeks before pairing, during mating period, gestation and lactation and then until necropsy. Similar deviations from the nominal doses as in the F0 generation were observed. The F2 pups were kept alive until weaning and, thus, were exposed to the test substance only prenatally or via the milk but not directly in the diet. Accordingly, actual exposure cannot be known.

All animals were examined for mortality, clinical signs, effects on general condition, body weight and food intake. With the F0 and F1 parental females, the stage of the estrous cycle was recorded for 21 days before pairing. During the mating period, vaginal smears were taken daily until sperm were found in the smear to prove successful copulation. All usual reproductive indices were determined. The females were allowed to litter and rear their offspring to weaning. The day of reaching the landmarks of sexual maturation was recorded for all the F1 pups that had been selected to produce the F2 generation.

The F0 and F1 parental males were killed and necropsied once successful littering was completed, (in week 17). The testes and epididymides were removed and weighed and sperm parameters including homogenization-resistant testicular spermatid count assessed. Both F0 and F1 parental females were killed and necropsied on PND 21. In adult females of both generations, the number of implantation scars in the uterus was recorded and, in F1 dams only, ovarian follicle evaluation was performed.

In both sexes, at termination, a selection of organs were taken, weighed and fixed. Microscopic

examination covered the following organs in all parental animals from the control and high-dose groups: adrenals, duodenum, jejunum, liver, ovaries, prostate, seminal vesicles, testes (with epididymides), uterus, and vagina. Because of the observation of histological changes in the intestines of high-dose animals of both sexes, these tissues were also assessed in the low- and mid-dose groups, along with liver and testes in males. In addition, brain, kidneys, pituitary gland, spleen, and thyroids were weighed in for all parental animals but not examined under the microscope. With regard to epididymides, one per male was weighed and the weights of its cauda determined separately.

Surplus F1 pups and all F2 pups were killed at weaning and a gross examination made to identify any abnormalities. In one male and one female pup from each litter, adrenals, brain, liver, spleen and thymus were weighed, along with epididymides and testes of male offspring.

There were no test item-related deaths or clinical signs in the F0 or F1 generations nor clinical signs in the litters. One F0, high-dose dam had to be killed prematurely due to a mammary gland adenocarcinoma and one mid-dose female because of dystocia. Both cases occurred in isolation and were considered incidental. Prolonged labour was also observed in one F1 dam from the control group which was also killed prematurely.

In both generations, in all groups given the test item, group mean body weights and body weight gains in male rats were slightly higher compared to the control groups throughout dosing. In the F0 generation, this difference even gained statistical significance at termination (see Table 42). Similarly, mean body weights and body weight gains in females were also higher in the F0 generation and at the upper two dose levels in the F1 generation, but only during the pre-pairing periods. In contrast, there were no effects of treatment on body weight gain in females during the gestation or lactation periods in either generation. Body weight development amongst F1 or F2 pups was not affected, however.

There is no explanation for the scattered increases in body weight and its gain in parental animals. The differences occasionally gained statistical significance but mostly they were not clearly dose-related. There was no effect on food intake in either sex in the F0 generation, whereas for some weeks, significant increases in food consumption were noted in F1 males and females receiving 4 and/or 12 mg/kg bw per day.

Evidence of parental toxicity was obtained when organ weight changes in males and the results of histopathological examination in both sexes were taken into consideration, confirming similar findings seen in the preceding one-generation gavage study.

In F0 generation males, mean absolute adrenal weight was increased at the top dose level, mean kidney weight at the mid and top dose levels and mean liver weight in all treated groups (Table 42). These increases were considered treatment-related by the study authors even though it is worth noting that relative organ weights were not significantly altered for any of these organs. An increase in spleen weight was not dose-related and probably due to the higher mean body weight. No organ weight changes were observed in females.

A nearly identical pattern was observed in the young adult rats of the F1 generation.

***Table 42. Selected organ weights in male rats in the two-generation feeding study; (mean weight F0/mean weight F1 shown)***

| | Dietary dose (mg/kg bw per day) | | | |
|---|---|---|---|---|
| **Parameter** | **0** | **1.5** | **4** | **12** |
| Terminal body weight (g) | 447.7/449.3 | 475.1/468.0 | 492.9**/458.2 | 484.1**/465.2 |
| Adrenal weight (mg) | 53.6/61.5 | 58.5/64.7 | 59.8/75.6** | 63.4**/72.0** |
| Kidneys (g) | 2.73/2.81 | 2.88/3.01* | 3.05**/3.12** | 3.21**/3.26** |
| Liver (g) | 14.24/14.08 | 15.62*/14.81 | 16.08**/15.10 | 16.17**/15.49** |

Source: Britton & King, 2019

Different from control group using Dunnett's two-sided test at statistical significance: * $p < 0.05$, ** $p < 0.01$

The increase in liver weight was accompanied by centrilobular vacuolation of hepatocytes in five out of 24 high-dose F0 generation males compared with only one control male. A lesion of this type was also seen in four high-dose males and one mid-dose male among the F1 generation adults. In contrast, the increases in adrenal weight were not corroborated by histopathology, and kidneys were not examined under the microscope.

In F0 and F1 generation males there were microscopic findings in the testes (Table 43) even though the organ weight was unaffected. These findings were not accompanied by significant changes in sperm concentration, motility or morphology or in the homogenization-resistant spermatid count. Slight reduction in these sperm parameters in the F1 generation were confined to the highest dose level and were still within historical control ranges. In both sexes and generations, epithelial vacuolation of the small intestines was observed in the high-dose groups.

***Table 43. Histopathological findings (all grades) in adults in the two-generation study with isocycloseram in rats; (24 rats per dose group; number affected F0/F1 shown)***

| | Dietary dose (mg/kg bw per day) | | | |
|---|---|---|---|---|
| **Finding** | **0** | **1.5** | **4** | **12** |
| Testes, tubular degeneration/atrophy | 0 / 1 | 0 / 0 | 1 / 2 | 6 / 12 |
| Duodenum, epithelial vacuolation | M: 0 / 0<br>F: 0 / 0 | M: 0 / 0<br>F: 0 / 0 | M: 0 / 0<br>F: 0 / 0 | M: 6 / 5<br>F: 3 / 5 |
| Jejunum, epithelial vacuolation | M: 0 /0<br>F: 0 / 0 | M: 0 / 0<br>F: 0 / 0 | M: 0 / 0<br>F: 0 / 0 | M: 9 / 6<br>F: 12 / 7 |

M: Male; F: Female; Source: Britton & King, 2019

There was no effect from the test item on estrous cycling, mating performance or on gestation length for either generation at any dose level. A few dams were apparently not pregnant, but there was no clear pattern that might suggest an effect of treatment. At the two upper dose levels, however, there was an increase in post-implantation loss in the F0 generation. In addition, the number of stillborn F1 pups was highest at 12 mg/kg bw per day resulting in a live birth index that was by about 9% lower than for the control group. Mean pup survival between days 0 and 4 was also slightly reduced in this group.

A lower live birth index could not be confirmed for the F2 pups and an increase in post-implantation loss was confined, in the F1 dams, to the high dose group in which also the number of nonpregnant females was highest. Moreover, pup survival between days 0 and 4 was similarly, as low as in the F0 generation. However, all these differences were not statistically significant, could be attributed, to an extent, to individual dams, and were considered by the study authors to fall within the normal range. Nonetheless, at least the increased post-implantation loss at the top dose level and the lowered pup viability on the first postnatal days, both occurring at the same dose, were considered reproducible, adverse effects of treatment. The lower number of pregnant dams in the highest dose groups might be, at least partly, secondary to testicular toxicity in the sires.

***Table 44. Selected reproductive parameters in the two-generation study with isocycloseram in rats (values for F0/F1 shown)***

| | Dose group | | | |
|---|---|---|---|---|
| **Parameter** | **Control** | **Low** | **Mid** | **High** |
| Dams mated | 23/24 | 24/24 | 24/23 | 22[a]/22 |
| Dams pregnant | 22/23 | 24/23 | 23[b]/22 | 21/**20** |
| Post-implantation loss (%) | 10.3/8.7 | 10.5/8.4 | 12.0/7.1 | 12.3/13.4 |
| Live-birth index (%) | 100/98.86 | 99.68/98.84 | 99.17/99.30 | 90.59/96.53 |
| Pup viability, days 0–4 (%) | 97.70/97.73 | 98.86/95.74 | 97.98/98.94 | 93.75/92.71 |

[a] Excluding one mating pair in which the male had testicular hypoplasia and azoospermia and the dam was prematurely killed on presumed GD 6; Source: Britton & King, 2019

[b] Excluding one dam with no pups in her cage on completion of parturition, so excluded from pregnancy and post-implantation loss assessment

There were no effects on sex ratio of the pups, viability after culling, or weight gain. Regarding sexual maturation of F1 pups, balanopreputial separation, on average, was delayed by one day in the high-dose group (44.0 days compared to 42.9 days for controls, 43.0 at the low dose and 42.7 in the mid-dose groups) and the mean weight of the male pups was higher when this landmark was reached. The relevance of this findings is equivocal. In contrast, vaginal opening occurred at the same time in all groups. The mean number of ovarian primary follicles in the high-dose F1 generation was significantly lower, as was the number of small follicles (Table 45). The study authors explained these reductions as the result of large interindividual variability, but the standard deviations in the high-dose group were the lowest for any of the parameters under evaluation. Since fertility was apparently not affected, the relevance of these findings is not known.

***Table 45. Ovarian follicle evaluation in the F1 generation of a two-generation feeding study in rats, (group mean number, one ovary/animal evaluated, ± standard deviation)***

| | Dosage group | | |
|---|---|---|---|
| **Parameter** | **Control** | **4 mg/kg bw per day** | **12 mg/kg bw per day** |
| Primordial follicles | 30.0 (± 12.0) | 31.2 (± 13.0) | 23.7 (± 10.1) |
| Primary follicles | 8.2 (± 4.1) | 8.6 (± 4.3) | 5.3 (± 3.2)** |
| Small follicles | 38.1 (± 13.7) | 39.8 (± 14.9) | 28.9 (± 12.0)* |

Source: Britton & King, 2019

Different from control group using Dunnett's two-sided test at statistical significance: * $p < 0.05$, ** $p < 0.01$

There were no test item-related macroscopic findings or organ weight changes in the F1 or F2 pups which were killed at weaning. There was no effect on anogenital distance in the F2 pups.

On balance the NOAEL for parental and offspring toxicity was 4 mg/kg bw per day, based on organ weight changes and histopathological findings in testes and intestines of adult rats in both generations and supported by the more equivocal delay in male sexual maturation and decrease in ovarian follicle count in the F1 generation, all at the maximum dose level of 12 mg/kg bw per day. The offspring NOAEL was also supported by the lower pup viability on PNDs 0–4 in the high-dose groups of both generations, which could, however, also be regarded as a reproductive effect. The reproductive NOAEL was identified at 4 mg/kg bw per day because of higher post-implantation loss in high-dose groups in both generations (Britton & King, 2019).

**(b) Developmental toxicity**

***Rat***

In a non-GLP, range-finding study, groups of 10 time-mated and presumed pregnant Crl:WI(Han) rats were administered isocycloseram (purity 96–97%, Batch no. MEQ-02958-03) by oral gavage once a day from GD 6 to GD 19 at dose levels of 0 (vehicle control), 3.5, 7.5 or 15 mg/kg bw per day. The vehicle was 0.5% aqueous CMC containing 0.1% Tween 80, and the dosing volume was 10 mL/kg bw.

The dams were monitored for mortality, clinical signs, body weight and food intake. They were killed and necropsied on GD 20. The fetuses were removed from the uterus, counted, weighed, sexed and examined for external and visceral abnormalities.

All dams survived until scheduled termination and were found pregnant. Following a slight reduction in body weight gain compared to controls after first administration at the mid and the high dose, body weight gain and food intake were roughly comparable to the control and low-dose groups for the rest of the study. However, as a result of the early reductions seen at the mid and high doses , mean body weight gain over the whole administration period was lower by 9% and 20% at 7.5 and 15 mg/kg bw per day, respectively. This resulted in a significantly lower terminal body weight (adjusted for uterus weight) in high-dose dams. There were no effects of treatment on the fetuses. Because of the rather weak effects on pregnant rats, the maximum dose was retained unaltered for the subsequent main study (Britton, 2015).

In the main rat developmental study, four groups of 22 time-mated, female Crl:WI(Han) rats

were dosed from GD 6 to GD 19 once daily by oral gavage with either isocycloseram (purity 96.9%, Batch No SMU5IP15011) or the vehicle: 0.5 % (w/v) CMC with 0.1 % (v/v) Tween 80. The dose levels of 0, 3.5, 7.5 and 15 mg/kg bw per day had been selected on the basis of the preceding range-finding study by Britton (2015, see above). A constant dosing volume of 10 mL/kg bw was administered.

The animals were observed for mortality and were given a daily clinical examination. Body weights were recorded daily and food intake determined for three-day intervals over the treatment period. When the dams were killed on day 20 of (presumed) gestation, terminal body weight was determined, necropsy was performed and the internal organs examined for gross abnormalities. The progress and outcome of pregnancy were assessed and gravid uterus and placenta weights recorded. Corpora lutea were counted and distribution of implantation sites in the uterus monitored. The fetuses were removed from the uterus, weighed, sexed and examined for external, visceral, skeletal and cartilage abnormalities. Approximately half the live fetuses from every group was allocated to fixed head examination.

There were no deaths and no test item-related clinical observations that could be attributed to treatment and, in contrast to the range-finding study using the same dose levels, there were no effects of isocycloseram on body weight or food intake at any dose level. In particular this time, there was no initial body weight loss just after the first test article administration.

All dams survived to scheduled termination. Amongst the groups, 19 (control), 21 (low dose), 20 (mid dose), and 21 (high dose) animals had become pregnant. There were no remarkable gross necropsy findings in any group, no abortions and no total resorptions. Resorption rate, number of live fetuses, their weight and sex ratio were not affected, nor were the number of corpora lutea, implantation sites or placental weights.

The only outstanding developmental finding was the major abnormality of bifid sternebra(e) that was seen in two fetuses (one male, one female) from two separate litters in the top-dose group, but did not occur in any other group. Because of this pattern and since this skeletal malformation was observed only once in a historical database of 6716 fetuses from 1028 litters, and only once in a total of 63 studies performed between 2006 and 2018, a treatment-related high dose effect seemed likely.

The occurrence of bifid sternabrae might suggest a possible relationship between treatment and the significant increase in bifurcated xiphoid cartilage at the same high dose level, but this latter variation is fairly common, so any relationship remains speculative. In fact 32 of the 216 fetuses were affected compared to 19 of 199 in the control group. The number of litters in which this variation occurred was 16 and 10, respectively. An increase (not statistically significant) was also seen at the mid dose level and the percentages of fetuses showing this finding accounted for 9.7%, 9.2%, 15.0% and 14.9% in the individual groups. It should be pointed out however, that the two malformed fetuses had no bifurcated xiphoid cartilage.

With regard to all other abnormalities, there were no significant differences between the groups and no evidence of a dose–response relationship. The only possible exception was an increase in fetuses with a fused cartilaginous ventral plate of one of the cervical vertebrae. This minor variation of unknown relevance was observed in five fetuses from three litters of the high-dose group as compared to none in control and low-dose groups, and one in the mid-dose group. The fetal incidence of this variation fell just outside the HCD value.

The maternal NOAEL in this study was 15 mg/kg bw per day, the highest dose tested. The NOAEL for developmental toxicity was 7.5 mg/kg bw per day based on the occurrence of a rare skeletal anomaly (bifid sternebrae) at the maximum dose level tested, and supported by a slight increase in minor skeletal variations (Blunt & Fincher, 2019).

The sponsor provided two additional documents to explain their view that the finding of bifid sternebrae was a chance event and not related to treatment. The main arguments were:

- its isolated occurrence, that is, the absence of further major abnormalities in the actual developmental study;
- the fact that no macroscopic changes that might suggest sternal damage were observed in the two-generation study at a dose level that was only slightly lower;
- an appearance that was consistent with a normal intermediate stage of sternum development.

These arguments might be considered with regard to classification (which is beyond the remit of the JMPR evaluation) but do not challenge the developmental NOAEL for this study. Also it is not evident that the occurrence of such a finding would have been necessarily observed in the reproductive studies (De Sesso & Williams, 2019; Wolton & French, 2020).

Based on available literature (Ema et al., 2014; Kuwagara et al., 2022), the Meeting concluded that the "grey zone anomaly" of bifid sternebrae was a variation but not a malformation. However, because similar changes in humans are known and may warrant surgical correction, this variation is considered potentially adverse. Since the critical window of sensitivity in the rat may be narrow, it cannot be excluded that a single exposure will be sufficient to cause it. Therefore the respective NOAEL of 7.5 mg/kg bw per day was considered a suitable basis for deriving an ARfD, at least for women of child-bearing age.

***Rabbit***

In a non-GLP range-finding study, groups of 10 time-mated and presumed pregnant New Zealand White rabbits were administered isocycloseram (purity 96% or greater, batch no. MEQ-02958-03) by oral gavage, once a day from GD 6 to GD 27 at dose levels of 0 (vehicle control), 7.5 or 15 mg/kg bw per day. A higher dose of 30 mg/kg per day was given to a fourth group, but only until GD 11 or 12 when the does were prematurely killed because of severe weight losses and reduced food intake. The vehicle was 0.5% aqueous CMC containing 0.1% Tween 80, and the dosing volume was 2 mL/kg bw.

The dams were monitored for mortality, clinical signs, body weight and food intake. Scheduled termination was on GD 28. Does were necropsied and examined for gross pathological changes. A blood sample was drawn to assess exposure to isocycloseram (for results, see section 1.3 p 369). Selected tissues were taken and retained for possible future histopathological examination, that was apparently never performed. Gravid uterus and placenta weights were determined. The fetuses were removed from the uterus, counted, weighed, sexed and examined for external and visceral abnormalities. With the does from the top-dose group, apart from necropsy, examinations were confined to the uterus from which pregnancy status was assessed.

In high-dose females a mean body weight loss of 3.5% was noted from days 6 to 11. Group mean food intake was reduced by up to 61% compared to controls. In five out of 10 animals, liver and/or jejunum displayed an abnormal, pale colour. Apparently the prematurely terminated does from this group had all been pregnant.

In the low- and mid-dose groups, in contrast, all does survived until scheduled termination and nine (low dose) or seven (mid dose) were found pregnant, similar to the nine does in the control group. There were no clinical signs that could be attributed to treatment. Body weight, its gain and food consumption were unaffected and there were no gross pathological findings. Placenta weights, uterine and implantation parameters were not affected. There were no effects of treatment on the fetuses. On balance, dose levels up to 15 mg/kg bw per day were well tolerated, whereas 30 mg/kg bw per day was an excessively toxic dose that was not suitable for the main developmental study in rabbits (Blunt, 2015).

In the subsequent main study, four groups of 22 sexually mature timed-mated female New Zealand White rabbits were given isocycloseram (purity: 96.9%, Batch no. SMU5IP15011) or the vehicle (0.5% w/v aqueous CMC 0.1 % v/v containing Tween 80), by oral gavage from GD 6 to GD 27. The dose levels of 0, 3.5, 7.5 and 15 mg/kg bw per day had been selected on the basis of the preceding range-finding study by Blunt (2015, see above). A constant dosing volume of 2 mL/kg bw was administered.

Body weights, food intake and clinical observations were recorded. The does were killed on GD 28, a necropsy was performed and the internal organs were examined for gross abnormalities. The progress and outcome of pregnancy was assessed and maternal dead body weight, gravid uterus and placenta weights were determined. The fetuses were removed from the uterus, weighed, sexed and examined for external, visceral, skeletal and cartilage abnormalities.

There were two premature deaths in this study but these were not attributed to the effect of the test substance. One doe each from the control and high-dose groups was killed on GD 11 and 14, respectively, because of accidental gavage dosing trauma. No treatment-related maternal clinical

signs were observed and no abortions occurred. Food intake was not affected in any dose group but at 15 mg/kg bw per day, mean body weight gain over the dosing period was 9% lower than in the control group. It is worth noting, however, that this decrease was not observed during the first days of treatment but became apparent later in the study. In contrast, no effect on body weight development was noted at the low or mid dose. Based on this rather weak, but presumably treatment-related effect, the maternal a NOAEL was identified at 7.5 mg/kg bw per day even though the difference in weight gain did not achieve statistical significance.

Nineteen does were found pregnant in the control and low-dose groups, whereas 21 pregnant females were identified in the groups receiving 7.5 or 15 mg/kg bw per day. All these does had live fetuses. There were no adverse effect of treatment on the mean number of implantations, the incidences of pre- and post-implantation loss or on the mean number of live fetuses. Likewise, mean fetal and placental weights and fetal sex ratio were not affected.

External, visceral and skeletal examination of the fetuses did not reveal a significant or dose-related increase in malformations or variations. It was the case that major abnormalities were observed only in the treated groups, not in controls, but there was no dose–response relationship. In total, malformations were observed in four fetuses from two litters in the low-dose group, in nine fetuses from seven litters at the mid-dose level and in two fetuses from two litters in the top-dose group. In addition, these abnormalities were various in nature and no pattern could be recognised. Accordingly, isocycloseram was considered devoid of teratogenic potential in rabbits and the highest dose tested of 15 mg/kg bw per day was the developmental NOAEL (Pottle, 2017).

## 2.6 Special studies

### (a) Neurotoxicity

***Rat***

A preliminary (non-guideline) study was performed in male and female Han Wistar rats to select suitable dose levels for the principal acute neurotoxicity study and to establish the period of peak effects for behavioural changes. Groups of three animals per sex received a single dose by oral gavage of isocycloseram, dissolved in 0.5% aqueous CMC and 0.1% Tween. Dose levels were 300, 1000, 1500 or 2000 mg/kg bw. It is worth noting that 1500 mg/kg bw was an additional dose level that was introduced later, in view of the early findings at 2000 mg/kg bw. Following dosing, the animals were observed over a period of seven days for clinical signs. Body weight and food consumption were monitored. Neurobehavioural assessments were performed at 1, 2, 4, 6, 8 and 24 hours on the first day, following substance administration. Apparently, a control group was not included in this experiment making interpretation of this study more difficult.

All animals survived the study, but neurobehavioural observations revealed some effects at 1000 mg/kg bw and above, in particular at six and eight hours post dose. Animals appeared less active and rearing counts tended to be low. More rare findings, mostly at 1500 or 2000 mg/kg bw, included piloerection, tremors, reduced body tone, hunched posture, reduced arousal, flattened gait, partial closure of eyelids, and a reduction in body temperature.

In the same three higher dose groups, a dose-related body weight loss and lower food consumption were noted over the first three days following treatment, with some recovery thereafter.

Based on these results, the conclusion was drawn that 1000 mg/kg bw might be an appropriate maximum dose for the acute neurotoxicity study and peak effects were to be expected around seven hours after dosing (Cocker, 2016a).

In an acute neurotoxicity study (OECD TG 424), groups of 10 Han Wistar (RccHan™:WIST) of each sex received isocycloseram (purity 98.4%, Batch no. MEQ-02794-09) as a single, oral gavage dose at 0, 50, 200 or 1000 mg/kg bw. This was followed by 14-day observation period. Controls received vehicle, 0.5% (w/v) aqueous CMC and 0.1% (v/v) Tween 80. During the study clinical condition, body weight, food consumption, neurobehavioural screening observations, brain weight, macropathology and histopathology investigations were undertaken at predetermined intervals. Neurobehavioural screening observations were performed at four intervals: pretreatment; at approximately seven hours post dose on

day 1 (time of peak effect); at one week post dose; and at two weeks post dose. At study termination five animals of each sex per group were killed by intraperitoneal injection of barbiturate and the heart exposed to permit perfusion with fixative via the aorta for neuropathological examination. The remaining five animals of each sex per group were killed by carbon dioxide asphyxiation.

No mortalities occurred and no clinical signs of toxicity were observed. Statistically significant mean body weight losses over the first day following dosing (day 1–2) were noted for the 200 mg/kg bw males and 1000 mg/kg bw males and females. A further statistically significant loss of body weight over the following day (day 2–3) was noted for the 1000 mg/kg bw females. The body weight loss at 50 mg/kg bw and 200 mg/kg bw in females on day 1–2 was not considered to be related to treatment as it lacked statistical significance and a dose–response relationship. Over the first week following dose administration (days 1–8), the mean body weight gains for the 50 and 200 mg/kg bw males and females were similar to controls. In contrast, the mean day 1–8 body weight gains for the 1000 mg/kg bw males was lower than for controls (by 32%; statistically significant), and the 1000 mg/kg bw females exhibited a statistically significant mean body weight loss of 5 g, compared to a gain of 13 g for the controls. Food intake by the 1000 mg/kg bw males and females was low over the first week of the observation period, but was similar to controls during the second week of observation. By the end of the two-week observation period the 1000 mg/kg bw rats had recovered from the post dose depression in body weight gain. So overall (days 1–15), mean body weight gains by the 1000 mg/kg bw males and females were similar to those for controls, and the mean absolute body weights of all treated groups were similar to the mean control body weight.

***Table 46. Intergroup comparison of mean body weight change in an acute neurotoxicity study in rats with isocycloseram for selected time points, mean weight ± standard deviation shown (g)***

| | Dose level (mg/kg bw) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Days** | **0 (control)** | **50** | **200** | **1000** | **0 (control)** | **50** | **200** | **1000** |
| **1–2** | 3±1.4 | 0±3.4 | −6±3.5** | −12±4.0** | 0±2.4 | −4±4.5 | −4±5.3 | −10±5.4** |
| **2–3** | 6±1.4 | 9±2.2* | 8±4.2 | 2±5.9 | 1±3.7 | 5±4.1 | 2±4.3 | −4±3.6** |
| **1–8** | 30±2.9 | 30±4.3 | 28±7.1 | 20±6.7** | 13±4.1 | 14±4.0 | 16±4.7 | −5±11.9** |
| **1–15** | 55±5.2 | 59±7.0 | 55±9.6 | 53±8.2 | 26±5.0 | 27±5.9 | 29±6.0 | 23±6.6 |

Different from control group at statistical significance: * $p < 0.05$, ** $p < 0.01$; Source: Cocker, 2016b

There were no treatment-related behavioural effects reported from the in-cage, in-hand or reactivity investigations. During the arena investigations, reduced arousal, abnormal motor movements and reduced activity and rearing counts at the time of peak effect were reported on day 1 among the 1000 mg/kg bw females. Similar findings in arena investigations were also evident for the 1000 mg/kg bw females on day 8, at which time abnormal motor movements were also observed in the 1000 mg/kg bw males.

***Table 47. Summary of arena observational findings on day 1 in an acute neurotoxicity study in rats with isocycloseram, mean scores shown, some with ± standard deviation***

| | | Dose level (mg/kg bw) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | Females | | | |
| **Day** | **Grade** | **0** | **50** | **200** | **1000** | **0** | **50** | **200** | **1000** |
| Palpebral closure | **0** | 7 | 7 | 7 | 10 | 10 | 10 | 10 | 10 |
| (Graded 0–3 and U) | **1** | 2 | 2 | 3 | 0 | 0 | 0 | 0 | 0 |
| | **2** | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Posture | **S** | 9 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| | **F** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Gait | **0** | 5 | 5 | 6 | 8 | 10 | 10 | 10 | 7 |
| (Graded 0–3 and U) | **1** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| | **U** | 5 | 5 | 4 | 2 | 0 | 0 | 0 | 2 |

| | | Dose level (mg/kg bw) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | Females | | | |
| **Day** | **Grade** | **0** | **50** | **200** | **1000** | **0** | **50** | **200** | **1000** |
| Arousal | **2** | 4 | 4 | 4 | 3 | 0 | 0 | 0 | 4 |
| (Graded 0–5) | **3** | 6 | 6 | 6 | 7 | 10 | 8 | 10 | 6 |
| | **4** | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 |
| Tremor | **0** | 10 | 7 | 7 | 8 | 10 | 10 | 10 | 10 |
| (Graded 0–3) | **1** | 0 | 3 | 3 | 1 | 0 | 0 | 0 | 0 |
| | **2** | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Abnormal motor movements | **N** | 6 | 7 | 7 | 6 | 9 | 9 | 9 | 7 |
| (No/Yes) | **Y** | 4 | 3 | 3 | 4 | 1 | 1 | 1 | 3 |
| Activity count; mean | | 4.9 | 6.4 | 6.4 | 7.0 | 14.4 | 16.9 | 14.1 | 9.2 |
| standard deviation | | ± 5.1 | ± 5.1 | ± 5.3 | ± 3.2 | ± 5.3 | ± 6.0 | ± 5.3 | ± 4.7 |
| Rearing count; mean | | 2.8 | 2.2 | 3.5 | 2.4 | 7.1 | 7.5 | 7.3 | 4.7 |
| standard deviation | | ± 2.5 | ± 1.9 | ± 2.9 | ± 1.0 | ± 3.5 | ± 4.6 | ± 4.5 | ± 3.1 |
| **Ungraded observations** (number of rats) | | | | | | | | | |
| Chewing mouth movements | | 4 | 2 | 3 | 3 | 0 | 0 | 1 | 1 |
| Licking surface of arena | | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 3 |
| Tail held high | | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 3 |
| Piloerection | | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 |

F: Posture, flattened; S: Posture, sitting/standing normally; Cocker, 2016b

U: Insufficient locomotion to assess gait; Source:

Associated with these findings were reductions in motor activity; specifically, reductions in both mean high beam (rearing) and low beam (cage floor activity) scores at the time of peak effect on day 1 for the 1000 mg/kg bw males and females. These reductions were most apparent during the first portion of the one-hour assessment period. The 200 mg/kg bw males and females also exhibited reductions in mean high beam and low beam scores over the first six minutes of the one-hour assessment period on day 1. Mostly slight, but statistically significant, reductions in mean high and/or low beam scores during the initial portion of the one hour activity sessions were still apparent on day 8 for the 200 and 1000 mg/kg bw males (low beam scores) and the 1000 mg/kg bw females (high beam score). On day 15 there were no indications of any treatment-related neurobehavioural and/or motor activity findings.

***Table 48. Intergroup comparison of motor activity for selected time points in an acute neurotoxicity study in rats with isocycloseram, (percentage change from controls shown)***

| | | | | | | |
|---|---|---|---|---|---|---|
| **Days** | **50** | **200** | **1000** | **50** | **200** | **1000** |
| **Mean total (0–60 minute, entire session) high beam activity scores** | | | | | | |
| Pretreatment | +7 | −11 | −15 | −15 | +5 | −23 |
| 1 | +5 | −10 | −28 | +14 | +14 | −46 |
| 8 | +17 | +35 | +33 | −2 | +25 | −12 |
| 15 | −12 | +2 | −15 | −12 | +11 | +12 |
| **Mean high-beam activity scores during the first 6 minutes of the session** | | | | | | |
| Pretreatment | 0 | −8 | −3 | +7 | +15 | +7 |
| 1 | −6 | −27* | −40** | −6 | −33* | −66** |
| 8 | +8 | +1 | −3 | 0 | 0 | −32* |
| 15 | +1 | +1 | +4 | −3 | +9 | +11 |

| Days | 50 | 200 | 1000 | 50 | 200 | 1000 |
|---|---|---|---|---|---|---|
| **Mean total (0–60 minute, entire session) low beam activity scores** | | | | | | |
| Pretreatment | −9 | −13 | −27 | −12 | +4 | −21 |
| 1 | −1 | −1 | −12 | +17 | +23 | −13 |
| 8 | −4 | 0 | −8 | −3 | +10 | −2 |
| 15 | −12 | −9 | −15 | −15 | −9 | −19 |
| **Mean low beam activity scores during the first 6 minutes of the session** | | | | | | |
| Pretreatment | −8 | −12 | −23 | +2 | +7 | -5 |
| 1 | −21* | −31** | −42** | +3 | −8 | −34* |
| 8 | −11 | −21** | −23** | −1 | 0 | −10 |
| 15 | +5 | 0 | −7 | −9 | −12 | −13 |

Different from control group at statistical significance: * $p < 0.05$, ** $p < 0.01$; Source: Cocker, 2016b

There was no statistically significant effect of treatment on brain weights. There were no treatment-related macroscopic or neurohistopathological findings.

The NOAEL of isocycloseram for general toxicity in an acute neurotoxicity study in rats was 50 mg/kg bw, based on lower body weight gains, food consumption, and lower high/low beam activity scores (primarily during the first six minutes of the 60-minute motor activity sessions) at the LOAEL of 200 mg/kg bw in males At the end of the two week post dose observation period, all the rats had recovered.

The transient neurobehavioural findings of depressed activity were probably secondary to the generalized toxicity of isocycloseram, as evidenced by the effects on body weight gain and food consumption, rather than a specific neurological effect. Therefore, in the absence of any neurohistopathological findings, the NOAEL for neurotoxicity in this study was 1000 mg/kg bw, the highest dose tested (Cocker, 2016b).

In a 90-day neurotoxicity study groups (OECD TG 424), groups of 10 Han Wistar (RccHan™:WIST) received isocycloseram (purity 96.9%, Batch no. SMU5IP15011) in the diet at a concentrations of 0, 50, 150 or 300 ppm (equal to 0, 3.92, 13.2 and 24.8 mg/kg bw per day for males, 0, 5.49, 15.6 and 32.7 mg/kg bw per day for females). During the study the following investigations were undertaken at predetermined intervals: clinical condition, neurobehavioural screening (observations in the cage, hand and arena, reactivity investigations and motor activity), body weight, food consumption, ophthalmic examination, brain weight, macropathology and histopathology. A functional observational battery (FOB) was performed on all animals before commencement of treatment and during weeks 2, 4, 8 and 13 of treatment. At study termination all animals were euthanized and five animals of each sex per group were killed by intraperitoneal injection of barbiturate, and the heart exposed to permit perfusion with fixative via the aorta for neuropathological examination. The remaining five animals of each sex per group were killed by carbon dioxide asphyxiation.

No mortalities occurred and no clinical signs of toxicity were observed. Body weight gain compared to controls was slightly higher at 50 ppm in males and at 150 and 300 ppm in both sexes, but there was no dose–response relationship, and there was no corresponding effect on food consumption.

There were no changes in in-cage, in-hand or arena observations that were attributable to treatment, and reactivity, grip strength and motor activity were also unaffected by treatment. There were a few incidences of isolated abnormal motor movements, but these were considered transient findings that were attributed to normal biological variation. Isolated statistically significant differences in grip strength compared to controls were inconsistent between investigations, and on each occasion, the extent of the difference from controls was minimal. A statistically significant decrease in low beam (cage floor activity) breaks at the 6- and 12-minute intervals in week 8 was observed in males given 150 or 300 ppm, resulting in statistically significant lower total beam breaks in that week. These variations

were, however, reflecting trends that were discernible at the pretreatment investigation. In addition, rearing (high beam) scores (a more sensitive marker for any decrease in activity) were clearly similar to controls. Consequently, the statistically significant differences were not attributed to treatment.

There were no treatment-related ophthalmic findings. There was no effect of treatment on brain weight and there were no treatment-related macroscopic or neurohistopathological findings.

The NOAEL of isocycloseram in a 90-day neurotoxicity study in rats was 300 ppm (equivalent to 24.8 and 32.7 mg/kg bw per day in males and females respectively), the highest dose tested. In particular there was no evidence of neurotoxicity (Froud, 2019).

Neurotoxicity was also examined in the 90-day toxicity study in rats (Laidlaw, 2019b) as well as in part of a combined chronic toxicity and carcinogenicity study, after 52 weeks (Strepka, 2019b).

In the 90-day study, the only finding of note was a slightly lower fore grip strength in males receiving 300 ppm. Given the small size of this change it was considered a reflection of the treatment-related reduction in the mean body weight noted for this group (lower by ca 12% controls) rather than any direct effect of treatment. There were no other treatment-related intergroup differences in the FOB parameters or on motor activity following administration of isocycloseram in males or females at dietary inclusion levels up to 300 ppm.

In the chronic toxicity study, dietary administration of isocycloseram was not associated with any changes in functional observation or motor activity in males or females at dietary inclusion levels up to 150 ppm.

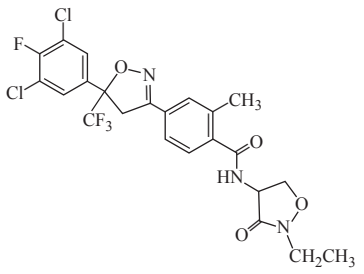
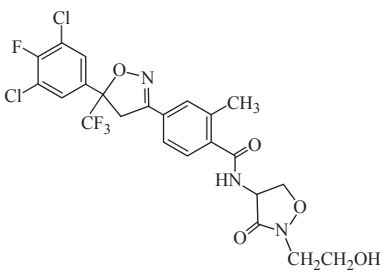
### (b) Immunotoxicity

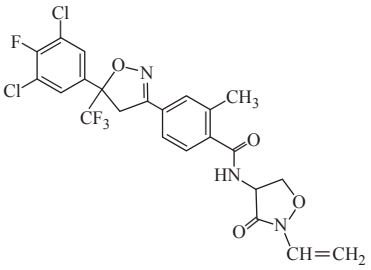
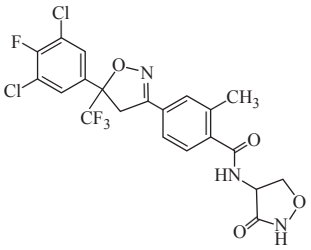
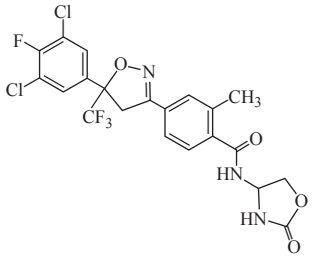
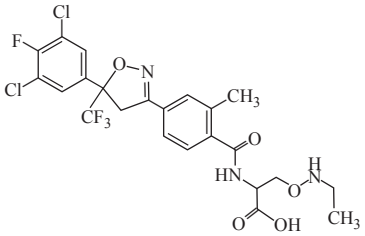
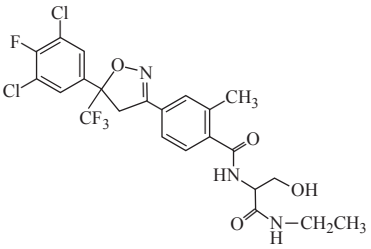
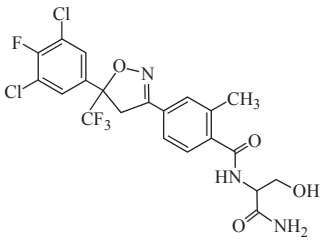
No specific study to investigate the immunotoxicity of isocycloseram was available, but no evidence of immunotoxicity was reported in routine toxicological studies with isocycloseram.

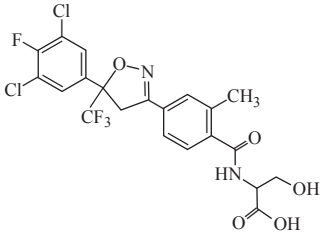
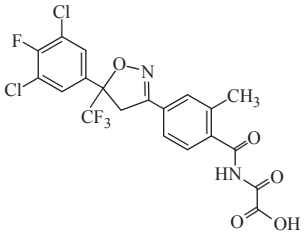
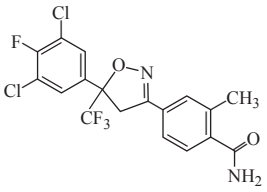
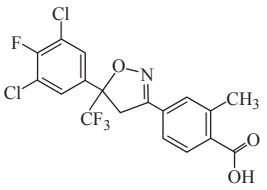
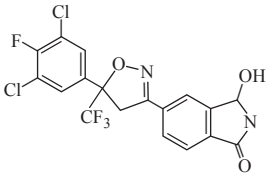
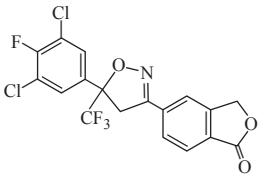
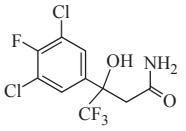
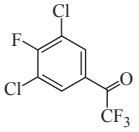
### (c) Studies on metabolites

Isocycloseram is subject to extensive biotransformation not only in the rat but also in plant and livestock matrices and processed plant commodities. In total, 16 metabolites have been identified. An overview of all these metabolites is given in Table 49, along with information on the parent compound for comparison. Metabolites present in amounts greater than 0.01 mg/kg or of more than 10% of total radioactive residues in respective studies are highlighted in bold and regarded as significant metabolites.

***Table 49. Overview on chemical structures and occurrence of dietary metabolites of isocycloseram***

| Compound | Description | Chemical structure | Detected in |
|---|---|---|---|
| **Isocycloseram** (parent) | 4-[5-(3,5-dichloro-4-fluorophenyl)-5-(trifluoromethyl)-4,5-dihydro-1,2-oxazol-3-yl]-*N*-(2-ethyl-3-oxo-1,2-oxazolidin-4-yl)-2-methylbenzamide |  | Plants (soybean, tomato, mustard greens, rice, rotated crops);<br>Livestock (goat, hen);<br>Rat (mainly in unabsorbed portion) |
| SYN550402 | 4-[5-(3,5-dichloro-4-fluoro-phenyl)-5-(trifluoromethyl)-4*H*-isoxazol-3-yl]-*N*-[2-(2-hydroxyethyl)-3-oxo-isoxazolidin-4-yl]-2-methyl-benzamide |  | Livestock (goat) |

| Compound | Description | Chemical structure | Detected in |
|---|---|---|---|
| **SYN551475** | 4-[5-(3,5-dichloro-4-fluoro-phenyl)-5-(trifluoromethyl)-4*H*-isoxazol-3-yl]-2-methyl-*N*-(3-oxo-2-vinyl-isoxazolidin-4-yl)benzamide |  | Livestock (goat, hen) |
| **SYN549436** | 4-[5-(3,5-dichloro-4-fluoro-phenyl)-5-(trifluoromethyl)-4*H*-isoxazol-3-yl]-2-methyl-*N*-(3-oxoisoxazolidin-4-yl)benzamide |  | Plants (mustard greens);<br>Livestock (goat, hen);<br>Rat (main metabolite) |
| **SYN551583** | 4-[5-(3,5-dichloro-4-fluoro-phenyl)-5-(trifluoromethyl)-4*H*-isoxazol-3-yl]-2-methyl-*N*-(2-oxooxazolidin-4-yl)benzamide |  | Livestock (goat, hen) |
| **SYN551203** | 2-[[4-[5-(3,5-dichloro-4-fluoro-phenyl)-5-(trifluoromethyl)-4*H*-isoxazol-3-yl]-2-methyl-benzoyl] amino]-3-(ethylaminooxy)propanoic acid |  | Processed plant commodities |
| SYN550737 | 4-[5-(3,5-dichloro-4-fluoro-phenyl)-5-(trifluoromethyl)-4*H*-isoxazol-3-yl]-*N*-[2-(ethylamino)-1-(hydroxymethyl)-2-oxo-ethyl]-2-methyl-benzamide |  | Livestock (goat) |
| **SYN549544** | *N*-[2-amino-1-(hydroxymethyl)-2-oxo-ethyl]-4-[5-(3,5-dichloro-4-fluoro-phenyl)-5-(trifluoromethyl)-4*H*-isoxazol-3-yl]-2-methyl-benzamide |  | Plants (rotated crops);<br>Livestock (goat, hen);<br>Rat (minor metabolite) |

| Compound | Description | Chemical structure | Detected in |
|---|---|---|---|
| SYN549543 | 2-[[4-[5-(3,5-dichloro-4-fluoro-phenyl)-5-(trifluoromethyl)-4*H*-isoxazol-3-yl]-2-methyl-benzoyl] amino]-3-hydroxy-propanoic acid |  | Plants (soybean, mustard greens, rotated crops);<br>Livestock (goat, hen);<br>Rat (major metabolite) |
| SYN551485 | 2-[[4-[5-(3,5-dichloro-4-fluoro-phenyl)-5-(trifluoromethyl)-4*H*-isoxazol-3-yl]-2-methyl-benzoyl] amino]prop-2-enoic acid |  | Processed plant commodities |
| **SYN549431** | 2-[[4-[5-(3,5-dichloro-4-fluoro-phenyl)-5-(trifluoromethyl)-4*H*-isoxazol-3-yl]-2-methyl-benzoyl] amino]-3-hydroxy-propanoic acid |  | Plants (soybean, tomato, mustard greens, rice, rotated crops);<br>Livestock (goat, hen);<br>Rat (minor metabolite) |
| SYN549107 | 4-[5-(3,5-dichloro-4-fluoro-phenyl)-5-(trifluoromethyl)-4*H*-isoxazol-3-yl]-2-methyl-benzoic acid |  | Plants (rotated crops);<br>Livestock (hen) |
| SYN551479 | 5-[5-(3,5-dichloro-4-fluoro-phenyl)-5-(trifluoromethyl)-4*H*-isoxazol-3-yl]-3-hydroxy-isoindolin-1-one |  | Livestock (hen) |
| SYN551474 | 5-[5-(3,5-dichloro-4-fluoro-phenyl)-5-(trifluoromethyl)-4*H*-isoxazol-3-yl]-3*H*-isobenzofuran-1-one |  | Livestock (goat, hen) |
| **SYN552188** | 3-(3,5-dichloro-4-fluoro-phenyl)-4,4,4-trifluoro-3-hydroxy-butanamide |  | Plants: (mustard greens, rotated crops);<br>Livestock (hen) |
| **SYN548569** (CA5697A) | 1-(3,5-dichloro-4-fluoro-phenyl)-2,2,2-trifluoro-ethanone |  | Plants: (rotated crops);<br>Livestock (goat, hen) |
| **M 741/1** | | | Livestock (goat*) |

* Identified at the Meeting by FAO group to occur in goat kidney, but not mentioned by sponsor

In contrast to the extensive toxicological database available for parent isocycloseram, toxicological data for the metabolites is extremely scarce. For only one of them (SYN548569/CA5697A) are experimental data on genotoxicity available. The relevant studies are reported in the sub-section that follows. Metabolites SYN549436 and SYN549543 were major rat metabolites with their toxicity at least partly covered by the studies with the parent compound. For all other metabolites, toxicological assessment must rely entirely on QSARs, read-across and theoretical considerations. Genotoxicity has been addressed in every case, whereas assessment of general toxicity was confined to the eight most abundant dietary metabolites.

**Metabolite SYN548569 (CA5697A) – genotoxicity testing**

In a reverse mutation (Ames) test, this metabolite (purity 99.2%, Batch No. SMU5AP15008A) was applied to *Salmonella typhimurium* tester strains TA1535, TA1537, TA98 and TA100 as well as to *Escherichia coli* strains WP2 *uvr*A pKM101 and WP2 pKM101. At first, plate incorporation assays (Experiment I) were performed in both the absence and presence of metabolic activation (hepatic S9 mix); the latter was repeated for strain TA100 (Experiment Ia) since the range of concentrations analyzed had been too low in the original trial. In parallel to this repeat experiment, a pre-incubation test (Experiment II) was run in all strains, again with and without metabolic activation. Mutagenicity testing was performed at all concentrations in triplicate.

The test item was dissolved in DMSO which was also used as the negative control substance. In Experiment I, eight concentrations ranging from 3 to 5000 µg/plate were included. Because of precipitation and cytotoxicity the maximum concentration was markedly reduced for the subsequent experiments IA and II which were run in parallel. In the repeat plate incorporation assay with TA100, seven concentrations between 1 and 1000 µg/plate were tested. In a pre-incubation assay, the test concentrations were 0.3, 1, 3, 10, 33, 100, 333, and 1000 µg/plate.

In the absence of S9 mix, the following positive control substances were employed: 4-nitro-*O*-phenyldiamine,(with TA1537 and TA98), sodium azide (with TA100 and TA1535), and methyl methane sulfonate in the case of the two *E.coli* strains. In the experiments with activation 2-aminoanthracene was used as a positive control for all strains.

Precipitation was confined to the first experiment where it occurred, in the presence of S9 mix, at 2500 and 5000 µg/plate, but was not observed in the subsequent experiments in which the concentrations were not that high. Cytotoxicity was characterized by reduced background growth of bacteria as noted with most tester strains, irrespective of metabolic activation, at concentrations of 100 µg/plate (to some extent) and certainly at 333 µg/plate and above. Strains TA1537 and TA100 of *S. typhimurium* were even more sensitive since this effect was first seen in Experiment II with metabolic activation already at much lower concentrations of 10 or 33 µg/plate. Without activation, 33 µg/plate was the LOEC for cytotoxicity towards TA1537. Where the number of revertants was considered the more appropriate measure for cytotoxicity, a severe reduction was observed with all strains, either at 100 µg/plate and above or at 333 µg/plate and above. At the high concentrations of 2500 and 5000 µg/plate in Experiment I there were no revertants of any strain growing on any plate and the number at 333 and 1000 µg/plate was seriously reduced in all experiments. These findings suggest a bacteriotoxic effect of the metabolite that was much more pronounced than in similar studies of this type conducted with the parent compound (Chang, 2016a, 2019, see above).

No increase in revertants was observed in any bacterial strain with any of the concentrations of the test item used, whereas the positive control substances gave the expected increases.

Under the conditions of this study, metabolite CA5697A was not mutagenic to bacteria in the Ames test even though meaningful evaluation at concentrations higher than 333 µg/plate was prevented by its overt toxicity to bacteria (Chang, 2020).

In an in vitro micronucleus assay, which is now the preferred method to detect both clastogenic and aneugenic properties of a test substance, metabolite C5697A (purity 99.2%, Batch No. SMU5AP15008A) was dissolved in DMSO and applied to peripheral human lymphocytes. Respective blood samples had been obtained from healthy, nonsmoking female donors aged 30–39. The assay was performed with and without metabolic activation (hepatic S9 mix). Positive control substances were mitomycin C in the absence of S9 activation and cyclophosphamide in the presence of S9 mix.

Following a 48-hour period to establish the lymphocyte cultures that were stimulated with the mitogen phytohaemagglutinine, cells were exposed to the test item for four hours, both with and without metabolic activation. This pulse treatment interval was followed by a 16-hour recovery period. At its end, cytochalasin B was added and the cells cultured for a further 20 hours before harvest, meaning harvest was 40 hours after commencement of treatment. It was noted that OECD TG 487 (adopted in 2016) allows the use of protocols with and without cytochalasin B as an inhibitor of actin polymerization and the use of this compound is even mandatory when the test is performed on human lymphocytes. However, it is usually added just after short-term treatment, not after an additional recovery period. It cannot be excluded that this modification of the study design might have had an impact on its outcome.

In each experimental group, two parallel cultures were analyzed and 1000 binucleate cells scored per culture for the presence of micronuclei. Therefore, in total 2000 binucleate cells were evaluated per concentration.

In a preliminary experiment, test substance concentrations between 13 and 2000 µg/mL were applied but severe cytotoxicity became apparent preventing the testing of concentrations above 150 µg/mL in the main experiment. However, no impact on osmolarity or pH was observed. Eventually, the maximum concentration that could be analyzed for micronucleus induction in the main trial was 35 µg/mL both with and without metabolic activation.

The results for the four concentrations of CA5697A tested and positive control substances are summarized in Table 50.

***Table 50. In vitro micronucleus assay with CA5697A – summary of results (2000 cells per conc.)***

| Test item | Micronucleus frequency (%) | Cytostasis as percentage of solvent control | Test item | Micronucleus frequency (%) | Cytostasis as percentage of solvent control |
|---|---|---|---|---|---|
| | **Without S9 mix** | | | **With S9 mix** | |
| Solvent control | 0.15 | - | Solvent control | 0.95 | - |
| CA5697A | | | CA5697A | | |
| 8.8 µg/mL | 0.45 | 7.7 | 5.0 µg/mL | 1.20 | NC |
| 15.4 µg/mL | 0.60* | 9.4 | 8.8 µg/mL | 1.60 | 11.0 |
| 26.9 µg/mL | 1.95* | 44.8 | 15.4 µg/mL | 1.65 | 35.2 |
| 35.0 µg/mL | 1.10* | 58.9 | 35.0 µg/mL | 1.45 | 56.6 |
| MMC, 0.8 µg/mL | 10.2* | 59.2 | CPA, 15 µg/mL | 4.65* | 57.6 |

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*; Source: Naumann, 2021

MMC: Mitomycin C; CPA: Cyclophosphamide; NC: Not calculated because equal to or higher than solvent control

* Statistically significant in Chi squared test at $p < 0.05$

An increase in micronucleus frequency over the solvent control was observed in all tested concentrations. Without metabolic activation the difference was statistically significant at 15.4 µg/mL and above, even though there was no clear dose–response relationship and a trend test was negative. The historical control range for binucleate cells containing micronuclei in the performing laboratory was 0.05%–1.2%; the value at 26.9 µg/mL was clearly outside this range. The 95% limit of the HCD at 1.04% was exceeded also by the percentage of micronuclei at the highest concentration. Even though some cytotoxicity was indicated by a clear increase in cytostasis at the two upper concentrations, the experiment was also concluded by the study author to be positive.

In the presence of metabolic activation statistical significance was achieved only with the positive control substance and there was no positive trend, but the 95% limit of the HCD (1.03%) was exceeded at all concentrations and, with exception of the lowest concentration, the results were outside the HCD range (0.05–1.25%). Again, an increase in cytostasis was apparent. On balance, the outcome of this experiment could also be considered positive even though the uncertainty is much higher than in the absence of S9 mix.

It is not clear if the positive findings in this study suggest a clastogenic or aneugenic mode of action. Moreover, the outcome may have been different had cytochalasin B been applied just after the treatment period. In any case, it was considered that further clarification in vivo is needed (Naumann, 2021).

An in vivo micronucleus assay was performed in male NMRI mice. The metabolite CA5697A (purity 99.2%, batch no. SMU5AP15008A) was dissolved in corn oil and administered by oral gavage at a volume of 10 mL/kg bw to groups of six (test substance) or five (vehicle and positive controls) animals. The dose levels of 0 (vehicle control), 125, 250 and 500 mg/kg bw had been selected on the basis of two pretests in which the two mice of each sex that were tested, following administration of 500 mg/kg bw, exhibited signs of toxicity such as piloerection, closed eyes, hunched posture, tiptoe gait, prostration and reduced activity. The positive control group received cyclophosphamide at 40 mg/kg bw in sterile water by oral gavage.

In the main study, the animals were killed 24 hours after dosing. Femoral bone marrow was flushed and slides prepared on which 6000 PCEs per animal were scored for the presence of micronuclei. The ratio between PCEs and NCEs was calculated. An additional group of six male mice received the high dose of 500 mg/kg bw but were terminated after 48 hours and a parallel group formed a second vehicle control group killed after 48 hours to allow direct comparison. The same procedures were used to evaluate PCEs and NCEs for these 48-hour groups.

Following administration of the high dose, the male mice displayed the same signs of toxicity as in the pretests, but for only up to four hours. Similar signs were observed in one animal receiving the low dose, but nothing was noted for mice receiving 250 mg/kg bw. A slight but regular dose-related numeric increase in micronuclei compared with controls was seen at the two upper dose levels with nearly a doubling of the micronucleus rate for the 48-hour sampling. Even though one of the criteria for a positive outcome of the study (a dose-related increase) might be considered fulfilled, the differences were rather small and did not gain statistical significance for the test item. Also, all test item treatment groups fell within the distribution of the historical negative control data (95% control limits 0.022–0.29). Looking in more detail, in studies that were run in the same laboratory between 2014 and 2020 the individual number of micronuclei ranged from 0 to 18 in a total of 473 male mice from the negative control groups. On balance, the increase in micronucleus frequency in the groups receiving 250 or 500 mg/kg bw of the metabolite was not considered biologically meaningful. In contrast, the positive control substance cyclosphosphamide caused a very clear and statistically significant increase in micronucleus frequency (see Table 51).

***Table 51. Summary of micronucleus (MN) results after single oral administration of CA5697A to male mice***

| Test group | Dose (mg/kg bw) | Sampling time (hours) | Mean number of MN per 6000 PCEs | Mean MN frequency (%) | Within group range of MN per animal | PCEs as a percentage of total RBCs |
|---|---|---|---|---|---|---|
| Vehicle control | - | 24 | 4.8 | 0.08 | 2–5 | 58.2 |
| Low dose | 125 | 24 | 4.7 | 0.08 | 2–5 | 57.9 |
| Mid dose | 250 | 24 | 7.5 | 0.13 | 3–7 | 60.1 |
| High dose | 500 | 24 | 7.8 | 0.13 | 2–8 | 61.3 |
| Positive control | 40 | 24 | 171.2* | 2.85 | 69–167 | 64.3 |
| Vehicle control | - | 48 | 4.8 | 0.08 | 2–4 | 60.9 |
| High dose | 500 | 48 | 9.0 | 0.15 | 3–9 | 57.5 |

MN: Micronucleus; PCE: Polychromatic nucleus; RBC: Red blood cell Source: Dony, 2021

* Statistically significant in the Mann-Whitney test at $p < 0.05$

The critical point for the overall assessment of this study is the question of whether or not a sufficient amount of the test substance reached the bone marrow. No plasma analysis was performed as part of this study and the PCE:NCE ratio was not affected. On the other hand, systemic exposure can be assumed because of the transient clinical signs. On balance, the study was considered negative by the Meeting (Dony, 2021).

**Assessment of other dietary metabolites of isocycloseram for genotoxicity**

The basis for assessment of these metabolites for genotoxicity and general toxicity is their grouping according to chemical similarity. Five groups were established, and each group was assessed for genotoxic concern based on structural similarity (derived from expert knowledge and physical chemistry parameters) to structures for which genotoxicity data is available, and on the QSAR alerts triggered. Three independent QSAR programs were used for this analysis including two that were based on expert knowledge rules and a third one that relies on statistical methods for the assessment of genotoxicity. This selection comprised DEREK Nexus (v. 6.1.0), Vega suite (CAESAR mutagenicity models, v. 2.1.13) and OECD QSAR Toolbox (v. 4.4). Details on the analyses run and the resulting predictions for each metabolite were submitted to JMPR as part of a supporting document (Bridgwood, 2021). Where specific OECD Toolbox alerts were triggered which could not be related to the structure of the parent or the only tested metabolite, a thorough assessment of the dataset used to underpin the respective QSAR alerts was undertaken to understand its relevance to the metabolite in question.

***Group 1***

Group 1 consists of the seven metabolites SYN549436, SYN549543, SYN549544, SYN550402, SYN550737, SYN551203 and SYN551475 which are all structurally close to parent isocycloseram. The structures for all these metabolites is shown in Table 49 above. Accordingly, their genotoxicity can be directly addressed by read-across. In fact, they all had either no (DEREK) or the same or overlapping (OECD Toolbox) QSAR alerts as the parent, and because of the comprehensive, consistent and overall negative genotoxicity database for isocycloseram, they can also be reasonably expected to have no genotoxic potential. The predictions for mutagenicity in the Ames test by CAESAR were also consistently negative for isocycloseram, as well as for the seven metabolites, but their structures could be out of the applicability domain , so these findings are of more limited value. On balance, these metabolites are not of genotoxic concern.

***Group 2***

Group 2 comprises four metabolites (SYN549431, SYN551479, SYN551474 and SYN549107) which were considered by the sponsor to be structurally very similar to each other, but different from the parent. This dissimilarity did not allow simple read-across with respect to genotoxicity. Nonetheless, for all four metabolites, no genotoxicity alerts were triggered in the DEREK or CAESAR models although for the latter, again, the structures were not in the applicability domain. Compared to the parent, metabolites SYN551479, SYN551474 and SYN549107 had the same or fewer (but overlapping) alerts in the OECD QSAR Toolbox. In contrast, SYN549431 had an additional QSAR alert that was not seen with the parent structure. This protein-binding alert, as an indication for potential for chromosome aberration, was based on the presence of a carboxylic amide in the molecule. However, there is a difference between *N*-linked and carbonyl-linked amides, and SYN549431 is of the latter type. In the training set for OASIS prediction (part of the OECD Toolbox) where the alert originated, only two compounds (2-ethoxybenzamide and 2-hydroxybenzamide) out of 29 substances in total were carbonyl-linked amides, whereas the majority were *N*-linked. For both of the carbonyl-linked examples, the in vivo micronucleus assay was negative, whereas in vitro chromosome aberration data was positive. Moreover the sponsor presented negative chromosome aberration data for four additional compounds from the OECD Toolbox containing such a structure. In three cases, these were in vitro data, but in the fourth example a negative in vivo micronucleus assay is available. In addition, the corresponding amide chromosome aberration alert in DEREK (alert 671) specifically excludes carboxylic amides. This weight of evidence approach resulted in the conclusion that the positive alert was not predictive of genotoxicity in the case of SYN549431. In summary, Group 2 metabolites were not regarded as genotoxic and no further testing is required.

***Group 3***

The single group 3 metabolite (SYN551485) is structurally different from the parent. It is not surprising therefore, that the OECD QSAR Toolbox generated a protein binding alert that was not seen with the parent compound, suggesting a potential for chromosome aberration. It was assigned to the presence of an unsaturated acid. In contrast, DEREK and CAESAR gave no alerts for genotoxicity in general, or for Ames mutagenicity, respectively, but the latter was of questionable value since the structure of this metabolite could be outside the applicability domain.

With regard to the positive alert, it could be argued (and was by the sponsor) that the respective training set comprised only two unsaturated acids, in contrast to 48 unsaturated esters and might not be entirely appropriate. For acrylic acid that is in the training set, experimental evidence of chromosome aberration in vitro is available that is contradicted however, by a negative micronucleus assay in vivo. In addition the QSAR Toolbox states that low molecular weight compounds are more toxic than those of higher molecular weight. In this respect the molecular weight of SYN551485 is 504 g/mol, a seven-fold increase over acrylic acid, which renders the alert not necessarily predictive for the genotoxicity of SYN551485. The other acid (cytembena, *cis*-β-4-methoxybenzoyl-β-bromoacrylic acid) also contains an unsaturated ketone that might be responsible for clastogenicity, which is indeed supported by positive test results. The unsaturated acid present in cytembena does not trigger a DEREK alert for genotoxicity. On this basis, the sponsor's explanation that cytembena was incorrectly present in the training set for the current alert appears plausible. Itaconic acid (2-methylene butanedioic acid), is another molecule that contains an unsaturated acid structure and is more similar to SYN551485 than either cytembena or acrylic acid but was apparently not in the training data set. Itaconic acid has proved negative for chromosome aberrations both in vitro and in vivo. Taking a weight of evidence approach, the group 3 metabolite SYN551485 was regarded not genotoxic.

***Group 4***

There is only one metabolite, SYN551583, in Group 4 . For this metabolite, the OECD QSAR Toolbox gave an in vivo alert for micronucleus formation due to the presence of a carbamate structure The respective training set of only seven substances was very small and only three compounds with the same alert had a positive experimental result, putting the validity and reliability of this prediction into question. In addition the corresponding alert in DEREK for chromosome damage of alkyl carbamates specifically excludes cyclic carbamates such as the metabolite SYN551583, and limits the acyclic variant to low molecular weight carbamates. Taking this weight of evidence approach, genotoxicity is not likely for this metabolite, although some uncertainty remains.

***Group 5***

Only two metabolites are included in group 5, SYN548569 (alternative code CA5697A) which was investigated for genotoxicity in an appropriate in vitro/in vivo test battery (see above), and SYN552188 which is structurally similar to SYN548569 but for which no experimental data is available. The same QSAR analyses as for the other groups of metabolites were run. The DEREK analysis revealed genotoxicity alerts for neither SYN548569 nor SYN552188. The same was true for predictions with regard to Ames mutagenicity by CAESAR but, as in the case of other groups, both structures could be out of the applicability domain. The OECD Toolbox revealed an additional alert for protein binding in the case of SYN548569 that was not present for the other metabolite. In contrast, for SYN552188 an alert for in vivo mutagenicity (micronucleus) was obtained that was raised for SYN548569. However, it should be noted that this structural alert is shared by the parent compound, for which negative experimental data is available (Dunton, 2016). Also, according to the sponsor, the same alert within the OECD QSAR Toolbox is poorly predictive as the training set contains 228 compounds with the alert, of which only 84 are in fact positive according to experimental data. The resulting poor predictivity score of 0.37, along with the negative experimental test result for the parent, were considered sufficient to dismiss this alert.

Experimental data is usually considered more reliable than in silico predictions. Accordingly, genotoxicity assessment of the group 5 metabolites should lean for its overall conclusions most heavily on the practically established genotoxicity of SYN548469. Reviewing the evidence from SYN548469, the Ames test for mutagenicity in bacteria was clearly negative. In contrast, a positive result was obtained in the in vitro micronucleus assay in human lymphocytes. Such evidence for either clastogenic or aneugenic properties of a test compound might be overruled by a negative in vivo micronucleus assay but only on condition that sufficient bone marrow exposure has been demonstrated. In this case, the evidence of exposure is based on transient clinical signs which were considered to provide enough evidence of systemic exposure. Accordingly, SYN548469 and, by read-across, SYN552188 can be considered negative for genotoxicity (Bridgwood, 2021).

**Assessment of dietary metabolites of isocycloseram for general toxicity**

With regard to general toxicity, considerations may be confined to the eight significant metabolites shown bold in Table 49. Of these, SYN549436, SYN549544, SYN551475 and SYN551203 belong to QSAR group 1 and occurred in amounts greater than 0.01 mg/kg or 10% of total radioactive residues either in livestock or processed plant commodities.

Metabolite SYN549436 is a major rat metabolite since it was the most abundant circulating metabolite in plasma (accounting for up to 80% of radioactivity) and was found, together with its glucuronide ("metabolite C"), at an amount of more than 10% of AD in the bile, at least in females. Therefore, it can be presumed that this metabolite is covered by studies of isocycloseram and, accordingly, the reference values as established for the parent compound are applicable.

The same holds true for SYN549544 because this is a precursor of SYN549543 which is also a major rat metabolite accounting for up to 11% of AD in bile. Hydrolysis of SYN549544 yields SYN549543. Reference values for isocycloseram are applicable.

The remaining group 1 metabolites SYN551475 and SYN551203 were not part of rat metabolism. According to the sponsor, human exposure to these metabolites will be much lower than to parent isocycloseram. If risk assessment is needed, calculated exposure might be compared to the TTC for non-genotoxic compounds, Cramer class III.

The only significant dietary metabolite from group 2 was SYN549431 which is also a minor rat metabolite. Because of its low abundance in rat metabolism and since toxicological data is missing, risk assessment should be performed by comparing expected exposure to the TTC for non-genotoxic compounds, Cramer class III.

Metabolites included in the QSAR groups 3, 4 and 5 were apparently not part of rat metabolism and the reference values as established for parent isocycloseram are not applicable.

The single metabolite in group 3 (SYN551485) occurring in processed plant commodities, was not regarded as one of the major metabolites. Because of the very low human exposure that is expected, general toxicity is not of concern. Nonetheless, if dietary risk should need evaluating in future, the TTC approach (Cramer class III) might be appropriate.

The only group 4 metabolite (SYN551583) is a significant dietary metabolite. In the absence of any toxicological data, the TTC approach (Cramer class III) should be taken for risk assessment.

Both group 5 metabolites, SYN548569 (CA5697A) and SYN552188, are significant dietary metabolites for which risk assessment is needed. In the absence of any data for general toxicity and because of the dissimilarity to the parent compound, the TTC approach (Cramer class III) should be taken for risk assessment of these non-genotoxic compounds.

## 3. Microbiological aspects

There was no information available in the public domain and no experimental data were submitted that addressed the possible impact of isocycloseram residues on the human intestinal microbiome.

## 4. Observations in humans

No robust data were available.

# Comments

## Biochemical aspects

Based on toxicokinetic investigations, a quantitatively similar elimination of radioactivity in faeces after oral and intravenous administration, and evidence of nearly complete metabolism in excreta, the oral absorption of low doses of isocycloseram of up to 3 mg/kg body weight (bw) was moderately fast and nearly complete. At 10 mg/kg bw absorption was above 80% (Hutton, 2017b, Real Sesma, 2018; Tomlinson, Hutton & O'Neill, 2015). Some saturation of oral absorption became apparent at much higher dose levels as also shown by blood level measurements in apical toxicological studies. Distribution was wide, with highest organ concentrations after 168 hours in kidneys, followed by spleen, liver and adrenals. The predominant route of elimination was via the bile, accounting for up to 50% of total radioactivity within 72 hours of dosing. Urinary excretion was very limited, less than 4% of administered dose (AD). There was evidence of significant nonbiliary excretion into the intestinal lumen which contributed to the total faecal excretion of about 90% of AD. Elimination was slow overall with 6%–10% of AD still retained in the carcass, organs and gastrointestinal (GI) tract after 168 to 192 hours, and 16% after 72 hours, but based on data from repeated administration of radiolabelled compound there was no evidence of accumulation (Hutton, 2017a).

Metabolism in the rat was extensive with a large number of metabolites identified, most of which occurred only at low levels in the various compartments. Major metabolic pathways comprised ring-opening of the isoxazole ring, ring-opening and cleavage of the oxazolidinone ring, oxidative defluorination and glucuronic acid conjugation. Metabolite SYN549543 was the most abundant in bile, accounting for up to 11% of AD after low-dose administration. Metabolite SYN549436, together with its glucuronide (metabolite C), made up around 10% of AD in the bile of female rats. In addition, it was the main circulating metabolite in plasma (Real Sesma, 2018).

In a comparative in vitro study, biotransformation of isocycloseram by human and rat liver microsomes was investigated. Human and rat metabolism appeared qualitatively similar but there were minor quantitative differences (Thibaut, 2019).

In general the impact of species, sex, repeated administration and position of the radiolabel on the toxicokinetics and metabolism of isocycloseram was low.

## Toxicological data

The acute oral median lethal dose ($LD_{50}$) of isocycloseram in the rat was greater than 4500 mg/kg bw. (Tarcai, 2016a; Xue, 2021a). The dermal $LD_{50}$ in rats was greater than 5000 mg/kg bw (Tarcai, 2016b; Xue, 2021b). The acute median lethal inhalation concentration ($LC_{50}$) was greater than 4.62 mg/L (Rosos-Matting, 2017). Isocycloseram was not irritating to the skin of rabbits (Matting, 2015a; Xue, 2019c), but was minimally to slightly irritating to rabbits' eyes (Matting, 2015b; Xue, 2019d). A positive result was observed in a local lymph node assay (LLNA; Pooles, 2016), whereas the compound proved negative in the less sensitive Buehler test in Guinea pigs (Xue, 2021e).

In repeat-dose studies of toxicity in rodents, target organs were the adrenals, testes, liver and intestines, but effects were mostly not very pronounced. In dogs the critical effect was body weight loss.

In a 28-day oral toxicity study in mice the dietary concentrations were 0, 100, 300, 700 or 1000 ppm (equal to 0, 17.4, 55.9, 132 and 172 mg/kg bw per day for males, 0, 20.9, 60.5, 142 and 176 mg/kg bw per day for females). The no-observed-adverse-effect level (NOAEL) was 100 ppm (equal to 17.4 mg/kg bw per day), based on histopathological findings in liver, spleen and duodenum as observed at the lowest-observed-adverse-effect level (LOAEL) of 300 ppm (equal to 55.9 mg/kg bw per day) (Dymarkowska, 2015).

In a 90-day study in mice, dietary concentrations of 0, 50, 300 or 700 ppm (equal to 0, 8.0, 48.8 and 117 mg/kg bw per day for males, 0, 9.9, 51.6 and 140 mg/kg bw per day for females) were administered. The NOAEL was 50 ppm (equal to 8 mg/kg bw per day), based on histopathological findings in adrenals and spleen at the next highest dose level of 300 ppm (equal to 48.8 mg/kg bw per day) and supported by alterations in clinical chemistry parameters (Laidlaw, 2019a).

In a 28-day feeding study in rats, the test substance was administered at dietary concentrations of 0, 50, 200, 350 or 500 ppm (equal to 0, 4.3, 16.3, 26.8 and 37.0 mg/kg bw per day) to male animals. Females received isocycloseram at dose levels of 0, 50 or 700 ppm (equal to 0, 4.5 and 50.1 mg/kg bw per day). Doses above 700 ppm were not tolerated and animals were terminated prematurely on day 11 due to mortality and severe clinical signs, and not further examined. The NOAEL of 50 ppm (equal to 4.3 mg/kg bw per day) was based on histopathological findings in the adrenals of males at the LOAEL of 200 ppm (equal to 16.3 mg/kg bw per day) and on clinical signs, reductions in body weight gain and food consumption, and on microscopic findings in adrenals, liver, and kidneys in females at the LOAEL of 700 ppm (50.1 mg/kg bw per day) (Dymarkowska, 2017).

In a 90-day feeding study in rats, isocycloseram was fed at dietary concentrations of 0, 50, 150 or 300 ppm (equal to 0, 3.9, 11.2 and 22.0 mg/kg bw per day for males, 0, 4.4, 13.4 and 24.0 mg/kg bw per day for females). The NOAEL was 50 ppm (equal to 3.9 mg/kg bw per day). The LOAEL was 150 ppm (equal to 11.2 mg/kg bw per day) in males, based on higher kidney weight and histopathological findings in testis and epididymis (Laidlaw, 2019b).

In a 28-day study using capsule administration, dogs received isocycloseram at dose levels of 0, 10, 50 or 150 mg/kg bw per day (later reduced to 80 mg/kg bw per day) in males, and 0, 10, 35 or 70 mg/kg bw per day in females. Toxicity in both males and females was excessive at the highest dose and resulted in premature terminations which were not further examined. The NOAEL was 10 mg/kg bw per day, based on body weight losses at higher dose levels (Robertson, 2019b).

In a 90-day feeding study in dogs, the dose levels were 0, 5, 15 and initially 35 mg/kg bw per day. The high dose caused marked body weight losses, therefore, it was reduced to 25 mg/kg bw per day. The NOAEL was 15 mg/kg bw per day, based on body weight losses and reduced food intake at the top dose of 35/25 mg/kg bw per day (Robertson, 2019c).

The overall NOAEL for dogs was 15 mg/kg bw per day, based on body weight loss and reduced food intake at higher doses.

In an 18-month carcinogenicity study in mice, isocycloseram was administered at dietary concentrations of 0, 15, 60 or 200 ppm (equal to 0 1.7, 6.7 and 23.1 mg/kg bw per day for males, 0, 1.8, 7.1 and 24.4 mg/kg bw per day for females). A NOAEL of 15 ppm (equal to 1.7 mg/kg bw per day) was identified based on increased plasma cell infiltration in the mesenteric lymph nodes at the LOAEL of 60 ppm (equal to 6.7 mg/kg bw per day). A mode of action (MOA) could not be elucidated from these findings. No increase in neoplastic lesions was seen and, accordingly, the highest dietary concentration of 200 ppm (equal to 23.1 mg/kg bw per day) was the NOAEL for carcinogenicity (Strepka, 2019a).

In a combined chronic toxicity and carcinogenicity study in rats, isocycloseram was fed over 24 months at dietary concentrations of 0, 20, 50 or 150 ppm (equal to 0, 0.9, 2.3 and 7.0 mg/kg bw per day for males, 0, 1.2, 3.0 and 9.2 mg/kg bw per day for females). The NOAEL for long-term toxicity of 50 ppm (equal to 2.3 mg/kg bw per day) was based on histopathological findings in the testes and epididymides of males at 150 ppm. The respective LOAEL was 150 ppm (equal to 7.0 mg/kg bw per day). There was no evidence of carcinogenicity and so, accordingly, the carcinogenicity NOAEL in this study was 150 ppm (equal to 7.0 mg/kg bw per day), the highest dose tested (Strepka, 2019b).

The meeting concluded that isocycloseram is not carcinogenic in mice or rats.

Isocycloseram was tested in an adequate range of studies in which no genotoxic potential was revealed.

The Meeting concluded that isocycloseram is unlikely to be genotoxic.

In view of the lack of genotoxicity, and in the absence of carcinogenicity in rats and mice, the Meeting concluded that isocycloseram is unlikely to pose a carcinogenic risk to humans.

In a two-generation reproductive toxicity study, isocycloseram was administered to rats at dietary concentrations that were adjusted weekly to provide dose levels of 0, 1.5, 4 or 12 mg/kg bw per day. The reproductive NOAEL was 4 mg/kg bw per day, based on a significant increase in post-implantation loss and impaired pup viability over the first four postnatal days (PNDs) at 12 mg/kg bw per day. The NOAEL of 4 mg/kg bw per day for both parental and offspring toxicity was based on organ weight changes (adrenals, kidney and liver) and histopathological findings in intestines and testes in adult

rats of both generations at 12 mg/kg bw per day, and further supported by a more equivocal delay in male sexual maturation and a decrease in ovarian follicle count in the F1 generation at this same dose (Britton & King, 2019).

In a developmental toxicity study in rats, isocycloseram was administered by oral gavage from gestation day (GD) 6 to GD 19 at dose levels of 0, 3.5, 7.5 or 15 mg/kg bw per day. The maternal NOAEL was 15 mg/kg bw per day, the highest dose tested. The embryo/fetal NOAEL of 7.5 mg/kg bw per day was based on the occurrence of bifid sternebrae (a rare skeletal anomaly) in two fetuses from two litters and supported by a slight overall increase in skeletal variations at 15 mg/kg bw per day. Since it is known that effects on sternal development have a relatively narrow critical sensitivity window in rodents, the resulting NOAEL of 7.5 mg/kg bw per day should for this reason be taken into consideration when deriving an acute reference dose (Blunt & Fincher, 2019).

In a developmental toxicity study in rabbits, isocycloseram was administered by oral gavage at doses of 0, 3.5, 7.5 or 15 mg/kg bw per day from GD 6 to GD 27. The maternal NOAEL was 7.5 mg/kg bw per day based on a slightly lower mean body weight gain in the does at the LOAEL of 15 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 15 mg/kg bw per day, the highest dose tested (Pottle, 2017).

The Meeting concluded that isocycloseram is not teratogenic since the occurrence of bifid sternebrae is not considered a malformation.

In an acute neurotoxicity study in rats, doses of 0, 50, 200 or 1000 mg/kg bw were administered by oral gavage. The systemic NOAEL was 50 mg/kg bw based on lower body weight gain, reduced food consumption and transiently depressed activity at higher doses. The LOAEL for these effects was 200 mg/kg bw. The NOAEL for acute neurotoxicity was 1000 mg/kg bw, the highest dose tested. No evidence of specific neurotoxic potential was observed and the highest tested dose of 1000 mg/kg bw was considered the NOAEL for neurotoxicity (Cocker, 2016b).

In a 90-day feeding study the neurotoxic potential of isocycloseram was investigated in rats that received daily dietary concentrations of 0, 50, 150 or 300 ppm (equal to 3.9, 13.2 and 24.8 mg/kg bw per day for males, 5.5, 15.6 and 32.7 mg/kg bw per day for females). The NOAELs for both systemic effects and neurotoxicity were 300 ppm (equal to 24.8 mg/kg bw per day), the highest dose tested (Froud, 2019).

The Meeting concluded that isocycloseram is not neurotoxic.

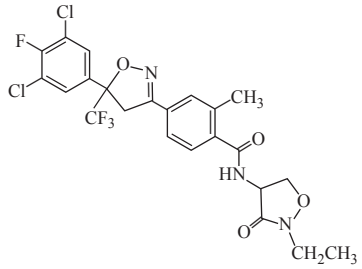
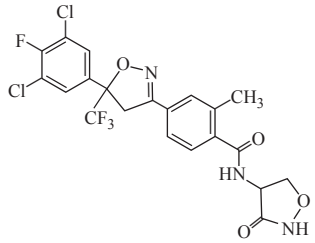
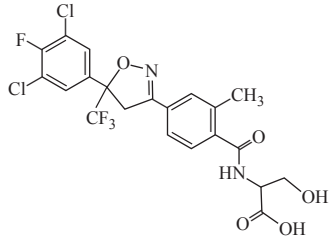
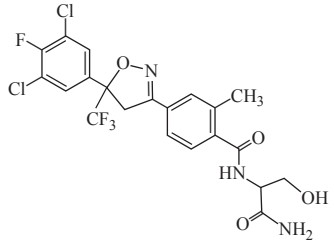
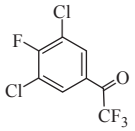
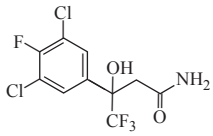
No specific study to investigate the immunotoxicity of isocycloseram was available, but no evidence of immunotoxicity was reported in routine toxicological studies with isocycloseram.

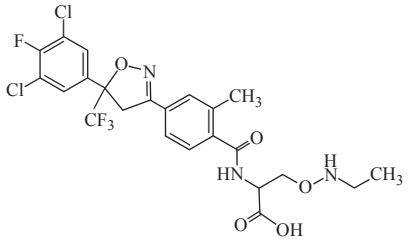
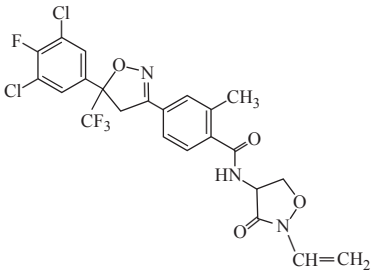
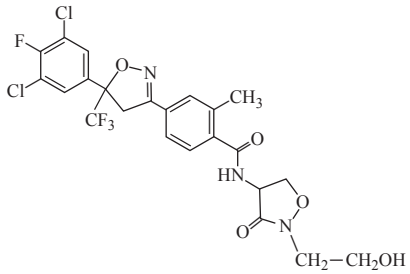
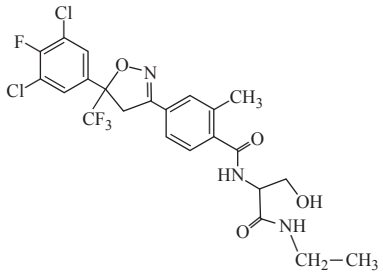
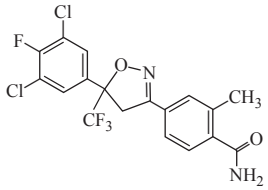
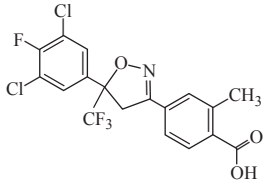
The Meeting concluded that isocycloseram is unlikely to be immunotoxic.

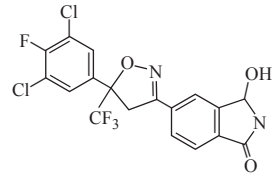
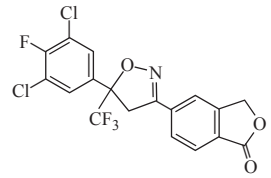
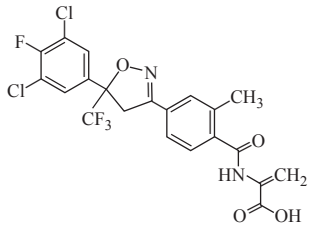
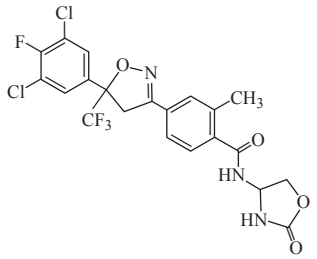
### Toxicological data on metabolites and/or degradates

Information on metabolites was scarce. A grouping concept was developed and provided by the sponsor (Bridgwood, 2021), together with predictions for genotoxicity and general toxicity based on in silico analyses. Experimental data is only available for one metabolite, SYN548569 (CA5697A), which has a very different structure from the parent, is not part of rat metabolism, but was detected in livestock matrices and rotational crops. A summary of what is known on metabolites of isocycloseram is shown in Table 52.

*Table 52. Summary on toxicological characterization of metabolites of isocycloseram in plants, livestock and processed commodities*

| Compound, code, structure | Major rat metabolite (> 10% AD) | Genotoxicity assessment (data, QSAR, read-across) | General toxicity | Toxicological reference values |
|---|---|---|---|---|
| **Isocycloseram**  | Parent | Negative, (full experimental data set) | Full data set | ADI: 0–0.02 mg/kg bw<br>ARfD: 0.08 mg/kg bw (females of child-bearing age);<br>0.5 mg/kg bw (general population) |
| **SYN549436**  | Major | Negative (QSAR, RA) | Covered by parent | Parent ADI and ARfD |
| **SYN549543**  | Major | Negative (QSAR, RA) | Covered by parent | Parent ADI and ARfD |
| **SYN549544**  | Minor | Negative (QSAR, RA) | Indirectly covered by parent (it is a precursor of SYN 549543) | Parent ADI and ARfD |
| **SYN548569** (CA5697A)  | Not found | Negative (data) | No data | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **SYN552188**  | Not found | Negative (QSAR, RA) | No data | TTC Cramer class III value: 1.5 µg/kg bw per day |

| Compound, code, structure | Major rat metabolite (>10% AD) | Genotoxicity assessment (data, QSAR, read-across) | General toxicity | Toxicological reference values |
|---|---|---|---|---|
| SYN551203  | Not found | Negative (QSAR, RA) | No data | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **SYN551475**  | Not found | Negative (QSAR, RA) | No data | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **SYN550402**  | Not found | Negative (QSAR) | No data | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **SYN550737**  | Not found | Negative (QSAR, RA) | No data | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **SYN549431**  | Minor | Negative (QSAR, RA) | No data | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **SYN549107**  | Not found | Negative (QSAR, RA) | No data | TTC Cramer class III value: 1.5 µg/kg bw per day |

| Compound, code, structure | Major rat metabolite (>10% AD) | Genotoxicity assessment (data, QSAR, read-across) | General toxicity | Toxicological reference values |
|---|---|---|---|---|
| **SYN551479**  | Not found | Negative (QSAR, RA) | No data | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **SYN551474**  | Not found | Negative (QSAR, RA) | No data | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **SYN551485**  | Not found | Negative (QSAR, RA) | No data | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **SYN551583**  | Not found | Negative (QSAR, RA) | No data | TTC Cramer class III value: 1.5 µg/kg bw per day |

TTC: Threshold of toxicological concern; RA: Read-across; AD: Administered dose
QSAR: Quantitative structural–activity relationship; ADI: Acceptable daily intake; ARfD: Acute reference dose

## Microbiological aspects

There was no information available in the public domain and no experimental data were submitted that addressed the possible impact of isocycloseram residues on the human intestinal microbiome.

## Human data

From very limited observations on the health of manufacturing personnel, no adverse effects had been reported. No information on poisoning incidents was available. As expected for a new compound, human data is very scarce.

The Meeting concluded that the existing database on isocycloseram was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

## Toxicological evaluation

The Meeting established an ADI of 0–0.02 mg/kg bw for isocycloseram based on the NOAEL of 1.7 mg/kg bw per day in the long-term study in mice and the NOAEL of 2.3 mg/kg bw per day in the two-year study in rats. A safety factor of 100 was used.

This ADI is considered applicable also to the dietary metabolites SYN549436, SYN549543 and SYN549544.

The Meeting established an ARfD for females of child bearing age of 0.08 mg/kg bw based on the embryo/fetal NOAEL of 7.5 mg/kg bw in the developmental toxicity study in rats, applying a safety factor of 100.

The Meeting established an ARfD for the general population of 0.5 mg/kg bw based on the NOAEL of 50 mg/kg bw in the acute neurotoxicity study in rats, applying a safety factor of 100.

Both ARfDs are applicable to dietary metabolites SYN549436, SYN549543 and SYN549544.

***Levels relevant to risk assessment of isocycloseram***

| Species | Study | Effect | NOAEL | LOAEL |
|---|---|---|---|---|
| Mouse | 78-week study of toxicity and carcinogenicity[a] | Toxicity | 15 ppm, equal to 1.7 mg/kg bw per day | 60 ppm, equal to 6.7 mg/kg bw per day |
| | | Carcinogenicity | 200 ppm, equal to 23.1 mg/kg bw per day[c] | - |
| Rat | Acute neurotoxicity study[b] | Neurotoxicity | 1000 mg/kg bw[c] | - |
| | | Systemic effects | 50 mg/kg bw | 200 mg/kg bw |
| | Two-year studies of toxicity and carcinogenicity[a] | Toxicity | 50 ppm, equal to 2.3 mg/kg bw per day | 150 ppm, equal to 7.0 mg/kg bw per day |
| | | Carcinogenicity | 150 ppm, equal to 7.0 mg/kg bw per day[c] | - |
| | Two-generation study of reproductive toxicity[a] | Reproductive toxicity | 4 mg/kg bw per day | 12 mg/kg bw per day |
| | | Parental toxicity | 4 mg/kg bw per day | 12 mg/kg bw per day |
| | | Offspring toxicity | 4 mg/kg bw per day | 12 mg/kg bw per day |
| | Developmental toxicity study[b] | Maternal toxicity | 15 mg/kg bw per day[c] | - |
| | | Embryo/fetal toxicity | 7.5 mg/kg bw per day | 15 mg/kg bw per day |
| Rabbit | Developmental toxicity study[b] | Maternal toxicity | 7.5 mg/kg bw per day | 15 mg/kg bw per day |
| | | Embryo/fetal toxicity | 15 mg/kg bw per day[c] | - |
| Dog | 90-day study[a] | Toxicity | 15 mg/kg bw per day | 25 mg/kg bw per day |

[a] Dietary administration

[b] Gavage administration

[c] Highest dose tested

*Acceptable daily intake (ADI)**

0–0.2 mg/kg bw

*Acute reference dose (ARfD) for females of child bearing age**

0.08 mg/kg bw

*Acute reference dose (ARfD) for the general population**

0.5 mg/kg bw

* Applies to isocycloseram and metabolites SYN549436, SYN549543 and SYN549544

## Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure.

***Critical end-points for setting guidance values for exposure to isocycloseram***

| | |
|---|---|
| ***Absorption, distribution, excretion and metabolism in mammals*** | |
| Rate and extent of oral absorption | Moderately rapid ($T_{max}$ 6–8 hours) but nearly complete at low dose of 1 mg/kg bw; above 80% at high dose of 10 mg/kg bw |
| Dermal absorption | No data |
| Distribution | Widely distributed, highest residues in kidneys, followed by spleen, liver, and adrenals |
| Potential for accumulation | No evidence of accumulation |
| Rate and extent of excretion | Mainly via faeces (up to 90% in total) but not complete within 7–8 days (6%–10% remaining in carcass and organs);<br>Significant part excreted within 72 hours via bile, (46%–50% at low dose);<br>Urinary excretion low (≤4%);<br>Exhalation negligible |
| Metabolism in animals | Extensive with a large number of metabolites mostly occurring at low levels;<br>Major rat metabolites SYN549436 and its glucuronide, and SYN549543;<br>Main pathways comprising opening of isoxazole ring, opening and cleavage of oxazolidinone ring, oxidative defluorination, and glucuronidation |
| Toxicologically significant compounds in animals and plants | Parent isocycloseram, SYN548569 (synonym CA5697A)<br>SYN549436, SYN549543 and SYN549544 |
| ***Acute toxicity*** | |
| Rat, $LD_{50}$, oral | 4500 mg/kg bw |
| Rat, $LD_{50}$, dermal | > 5000 mg/kg bw |
| Rat, $LC_{50}$, inhalation | > 4.62 mg/L (four-hour nose only exposure) |
| Rabbit, dermal irritation | Not irritating |
| Rabbit, ocular irritation | Minimally to slightly irritating |
| Dermal sensitization | Sensitizing (LLNA) |
| ***Short-term studies of toxicity*** | |
| Target/critical effect | Histological lesions in liver, spleen, adrenals, intestines, testis and epididymis in rodents; higher kidney weight in rats;<br>body weight loss in dogs |
| Lowest relevant oral NOAEL | 3.9 mg/kg bw per day (rat) |
| Lowest relevant dermal NOAEL | 100 mg/kg bw per day (rat) |
| Lowest relevant inhalation NOAEC | No data |
| ***Long-term studies of toxicity and carcinogenicity*** | |
| Target/critical effect | Plasma cell infiltration of mesenteric lymph nodes in mice;<br>histopathological findings in testes/epididymides in rats |
| Lowest relevant NOAEL | 1.7 mg/kg bw per day (mouse) |
| Carcinogenicity | Not carcinogenic in mice or rats |

| | |
|---|---|
| ***Genotoxicity*** | No genotoxic potential |
| ***Reproductive toxicity*** | |
| Target/critical effect | *Reproductive toxicity*: increase in post-implantation loss<br>*Offspring toxicity*: microscopic lesions in testes, liver, duodenum; organ weight changes; delay in male sexual maturation and lower ovarian follicle count, and decreased postnatal viability of pups<br>*Parental toxicity*: microscopic lesions in testes, and duodenum; organ weight changes |
| Lowest relevant parental NOAEL | 4 mg/kg bw per day (rat) |
| Lowest relevant offspring NOAEL | 4 mg/kg bw per day (rat) |
| Lowest relevant reproductive NOAEL | 4 mg/kg bw per day (rat) |
| ***Developmental toxicity*** | |
| Target/critical effect | *Maternal*: slightly reduced body weight gain in rabbits<br>*Developmental*: rare anomaly of bifid sternebrae and slight overall increase in skeletal variations in rat fetuses at highest dose of 15 mg/kg bw per day |
| Lowest relevant maternal NOAEL | 7.5 mg/kg bw per day (rabbit) |
| Lowest relevant embryo/fetal NOAEL | 7.5 mg/kg bw per day (rat) |
| ***Neurotoxicity*** | |
| Acute neurotoxicity NOAEL | No evidence of a specific neurotoxic potential up to highest dose of 1000 mg/kg bw, but systemic effects at 200 mg/kg bw and above (rat) |
| Subchronic neurotoxicity NOAEL | 24.8 mg/kg bw per day, highest dose tested in a 90-day study (rat)<br>No evidence of neurotoxicity or systemic effects |
| Developmental neurotoxicity NOAEL | No data, not necessary |
| ***Other toxicological studies*** | |
| Immunotoxicity | No data |
| Phototoxicity | No data |
| Mechanistic studies | No data |
| **Studies on toxicologically relevant metabolites** | |
| ***SYN548569 (CA5697A)*** | Negative Ames test but positive in vitro micronucleus assay, negative bone marrow micronucleus assay in mice |
| ***Microbiology data*** | No data submitted |
| ***Human data*** | Not available for this new compound, at least no evidence of health effects in manufacturing plant personnel |

## Summary

| | Value | Study | Safety factor |
|---|---|---|---|
| ADI | 0–0.02 mg/kg bw | 18-month study in mice, two-year study in rats | 100 |
| ARfD for females of child-bearing age | 0.08 mg/kg bw | Developmental toxicity study in rats | 100 |
| ARfD for the general population | 0.5 mg/kg bw | Acute neurotoxicity study in rats | 100 |

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# Isoflucypram (addendum)

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## Explanation

Isoflucypram (BCS-CN88460) is the International Organization for Standardization approved common name for *N*-(5-chloro-2-isopropylbenzyl)-*N*-cyclopropyl-3-(difluoromethyl)-5-fluoro-1-methyl-*1H*-pyrazole-4-carboxamide, with the Chemical Abstracts Service number 1255734-28-1. Isoflucypram is a novel broad-spectrum fungicide. It belongs in the chemical class of *N*-cyclopropyl-*N*-benzylpyrazole carboxamides and it is a succinate dehydrogenase inhibitor.

Isoflucypram was evaluated for the first time by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 2022 (JMPR, 2023), when an ADI of 0–0.06 mg/kg body weight (bw) was established for isoflucypram. An acute reference dose (ARfD) was considered unnecessary. Dietary risk assessments could not be conducted at JMPR 2022 as the Meeting was unable to recommend residue definitions for dietary risk assessment of plant and animal commodities due to genotoxic prediction for two metabolites. Additional information on these metabolites in the form of quantitative structure–activity relationship (QSAR) analyses of genotoxicity were supplied to the current Meeting by the sponsor. The information included new data on the genotoxicity of several metabolites evaluated at JMPR 2022. As the information provided may affect the dietary risk assessment, isoflucypram was evaluated at the current Meeting. There was no other new information on the toxicology of this compound.

# Evaluation for acceptable daily intake

## 1. Biochemical aspects

No new data available.

## 2. Toxicological studies

### 2.1 Genotoxicity

**(a) In silico studies**

New in silico studies of the parent were conducted alongside those of the metabolites; discussion and results are provided in Section 2.6(a), where metabolite results are reported.

### 2.5 Reproductive and developmental toxicity

No new data available.

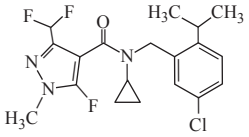
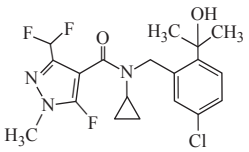
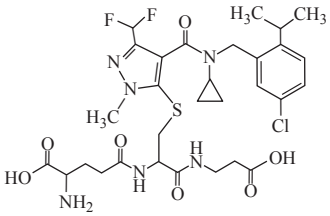
### 2.6 Special studies

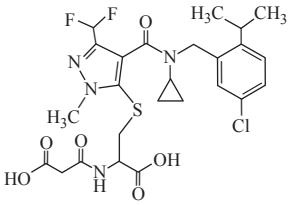
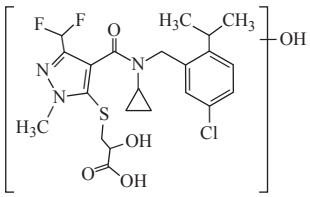
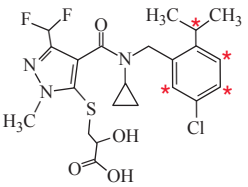
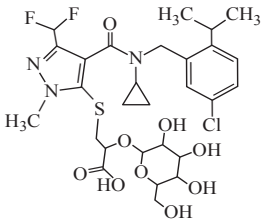
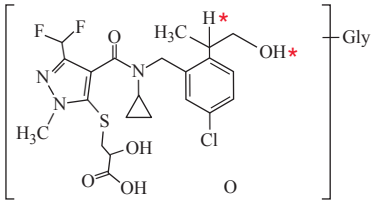
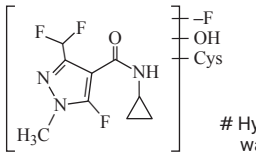
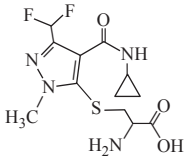
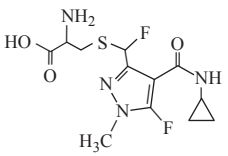
**(a) In silico studies on metabolites**

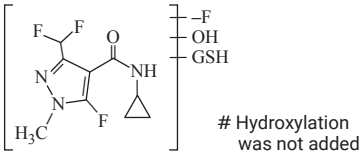
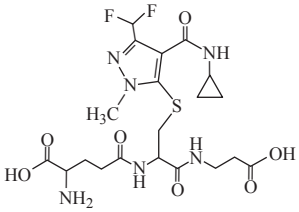
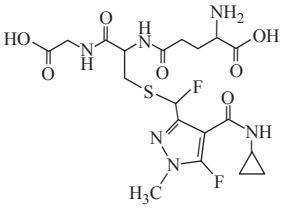
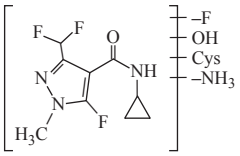
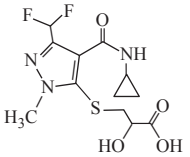
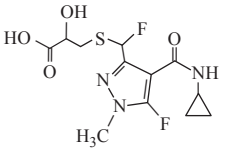
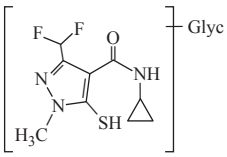
Two new in silico studies on the genotoxicity of isoflucypram and its metabolite M58 were submitted. The metabolite M58 for which new information was provided is included in Table 1.

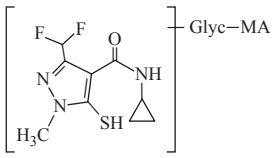
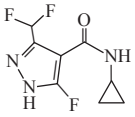
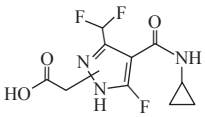
In these new studies, the isoflucypram and/or its major metabolite in rats, M58, were referred to in the interpretation of data on the in silico genotoxicity of metabolites. Therefore, isoflucypram and M58 were added to the table. Since M58 is a major metabolite in rats its toxicological properties are considered to be covered by the parent compound and is considered unlikely to be genotoxic.

***Table 1. List of metabolites with new information on in silico genotoxicity analysis***

| Number, name, identity | Structure/Markush structure* | Most likely structures or SMILES codes for metabolites that were expressed as Markush formats only |
|---|---|---|
| **Isoflucypram (parent)**<br>*N*-(5-chloro-2-isopropylbenzyl)-*N*-cyclopropyl-3-(difluoromethyl)-5-fluoro-1-methyl-*1H*-pyrazole-4-carboxamide [IUPAC] |  | |
| **M02**<br>Isoflucypram-2-propanol<br>BCS-DC20298<br>*N*-[5-chloro-2-(2-hydroxypropan-2-yl)-benzyl]-*N*-cyclopropyl-3-(difluoromethyl)-5-fluoro-1-methyl-*1H*-pyrazole-4-carboxamide [IUPAC] |  | |
| **M44**<br>Isoflucypram-desfluoro-homoGSH<br>γ-glutamyl-*S*-{4-[(5-chloro-2-isopropylbenzyl)(cyclopropyl)carbamoyl]-3-(difluoromethyl)-1-methyl-*1H*-pyrazol-5-yl} cysteinyl-beta-alanine [IUPAC] |  | |

| Number, name, identity | Structure/Markush structure* | Most likely structures or SMILES codes for metabolites that were expressed as Markush formats only |
|---|---|---|
| **M45**<br>Isoflucypram-desfluoro-Cys-MA<br>*N*-(carboxyacetyl)-*S*-{4-[(5-chloro-2-isopropylbenzyl)(cyclopropyl)carbamoyl]-3-(difluoromethyl)-1-methyl-*1H*-pyrazol-5-yl} cysteine [IUPAC] |  | |
| **M46**<br>Isoflucypram-desfluoro-mercapto-lactic acid-OH |  | <br>* shows locations of –OH group in four possible isomers of the Markush structure |
| **M47**<br>Isoflucypram-desfluoro-mercapto-lactic acid-Glyc<br>3-({4-[(5-chloro-2-isopropylbenzyl)(cyclopropyl)carbamoyl]-3-(difluoromethyl)-1-methyl-*1H*-pyrazol-5-yl}sulfanyl)-2-(hexopyranosyloxy)propanoic acid [IUPAC] |  | |
| **M48**<br>Isoflucypram-desfluoro-mercapto-lactic acid-propyl-OH-Glyc |  | * Substituent pair may be transposed to give isomers **a** and **b** |
| **M52**<br>Isoflucypram-desfluoro-*N*-methyl-cyclopropyl-pyrazole-carboxamide-OH-Cys | <br># Hydroxylation was not added | a<br><br>b<br> |

| Number, name, identity | Structure/Markush structure* | Most likely structures or SMILES codes for metabolites that were expressed as Markush formats only |
|---|---|---|
| **M54**<br>Isoflucypram-desfluoro-*N*-methyl-cyclopropyl-pyrazole-carboxamide-OH-GSH |  | a<br><br>b<br> |
| **M55**<br>Isoflucypram-desfluoro-*N*-methyl-cyclopropyl-pyrazole-carboxamide-desamino-Cys |  | a<br><br>b<br> |
| **M56**<br>Isoflucypram-desfluoro-*N*-methyl-cyclopropyl-pyrazole-carboxamide-mercapto-Glyc |  | Three possible structures; details were not provided as figures, but SMILES codes:<br>Cn1nc(C(F)F)c(C(=O)N(C2CC2)C3OC(CO)C(O)C(O)C3O)c1S<br>or<br>Cn1nc(C(F)F)c(C(=O)NC2CC2)c1SC3OC(CO)C(O)C(O)C3O<br>or<br>Cn1nc(C(F)F)c(\C(=N/C2CC2)\OC3OC(CO)C(O)C(O)C3O)c1S |

| Number, name, identity | Structure/Markush structure* | Most likely structures or SMILES codes for metabolites that were expressed as Markush formats only |
|---|---|---|
| **M57**<br>Isoflucypram-desfluoro-*N*-methyl-cyclopropyl-pyrazole-carboxamide-mercapto-Glyc-MA |  | Cn1nc(C(F)F)c(C(=O)N(C2CC2)C3OC(COC(=O)CC(=O)O)C(O)C(O)C3O)c1S<br>or<br>Cn1nc(C(F)F)c(C(=O)NC2CC2)c1SC3OC(COC(=O)CC(=O)O)C(O)C(O)C3O<br>or<br>Cn1nc(C(F)F)c(\C(=N/C2CC2)\OC3OC(COC(=O)CC(=O)O)C(O)C(O)C3O)c1S |
| **M58**<br>Isoflucypram-cyclopropyl-pyrazole-carboxamide<br>BCS-CX99798<br>*N*-cyclopropyl-3-(difluoromethyl)-5-fluoro-*1H*-pyrazole-4-carboxamide [IUPAC] |  | |
| **M68**<br>Isoflucypram-cyclopropyl-pyrazole-carboxamide-acetic acid |  | |

SMILES: Simplified molecular input line entry system; Based on Pellizzaro & Hynes, 2022

* Structures copied from the JMPR 2022 monograph;

\# The LC-MS/MS data contained $-H_2O$, which was not added when the data transferred to Markush format. Therefore hydroxylation (+OH) was not present in the actual structure; the Meeting added the information based on the rat ADME study of Bongartz & Bartelsen (2017), and the plant metabolism studies of Lamshoeft (2017) and Traub, (2017).

The Meeting noted a new finding, subsequent to JMPR 2022, concerning metabolites M52 and M54, arising from the raw liquid chromatography with tandem mass spectrometry (LC-MS/MS) data in a rat absorption, distribution, metabolism and excretion (ADME) study (Bongartz & Bartelsen, 2017) and in two plant metabolism studies (Lamshoeft, 2017; Traub, 2017). In these studies, submitted to JMPR 2022, not only were hydroxylation (+OH) and defluorination (–F) identified, but also dehydroxylation ($-H_2O$) in the metabolic processes leading to the formation of M52 and M54. Studies indicated no loss of the hydroxy group in their final formation. However, when the data were transcribed into the Markush format for the metabolite list provided in the studies, only hydroxylation (+OH) had been transferred to the format. The current Meeting concluded that the hydroxy group did not exist in the final Markush formats of M52 and M54.

***First in silico analysis for genotoxicity; metabolites M02, M44, M45, M46, M47, M48, M52, M54, M55 and M68***

An in silico analysis was conducted to predict the genotoxic potential of isoflucypram. Five of 10 metabolites had known structures, whilst five others have not had their structures fully characterized. For those metabolites with incompletely known structures, all possible structures have been taken in account in the analysis. Table 1 includes the characterized, or most likely predicted, structures of these metabolites.

All metabolites were assessed in this study using mutagenicity and chromosome damage models in DEREK Nexus (v. 6.2.1) and bacterial mutation and in vivo micronucleus mouse models in Leadscope (v. 2022.0.0-31). A read-across assessment was carried out by comparing the metabolite structures to isoflucypram using profilers associated with genotoxicity in the OECD QSAR Toolbox (v. 4.5). The parent compound, isoflucypram, is not genotoxic based on experimental data, so it can form a basis for the read-across assessment.

Data source used in the analysis to identify genotoxicity were as follows:

- ADME and genotoxicity studies on isoflucypram;
- the EFSA Public MetaPath database v. 2 (EFSA, 2021);
- databases available in OECD QSAR Toolbox v. 4.5;
- harmonized classification – Annex VI to Regulation (EC) No. 1272/2008 (CLP Regulation);
- JMPR monographs and EFSA conclusions for structurally related active substances.

From in silico predictions of genotoxicity using the mutagenicity and chromosome damage models in Derek NEXUS and the bacterial mutation and in vivo micronucleus mouse models in Leadscope, all metabolites were predicted to present no greater concern for genotoxicity than does isoflucypram.

As the first step in a read-across analysis, metabolites were compared to isoflucypram using profilers associated with genotoxicity end-points in the OECD QSAR Toolbox. Following this, active substances with a similar chemical structure were assessed to see whether they have any similar metabolic transformations. Finally the OECD QSAR Toolbox was searched for substances that contain the functional groups that are not present in parent isoflucypram to demonstrate that these metabolic transformations are of no concern.

Analysis indicated that isoflucypram activated alerts for DNA binding (OECD), protein binding (OECD), and in vivo mutagenicity (micronucleus) alerts by ISS profilers. All metabolites activated the same alerts with respect to protein binding (OECD) and in vivo mutagenicity (Micronucleus) by ISS profilers. Many of the metabolites activated the alert for DNA binding (OECD profilers), but some did not activate this alert. No metabolites activated any alert that was not activated by isoflucypram. Based on the analysis by OECD QSAR Toolbox profilers, none of the assessed metabolites showed any concern of genotoxicity additional to that for isoflucypram.

It has been proposed that the formation of the crop/livestock metabolites from isoflucypram is achieved by nine transformations (see Fig. 1)which include addition of alcohols, phenols, aryl/alkyl sulfide, and carboxylic acid, and dealkylation of the amide (Pellizzaro & Hynes, 2022). The genotoxic potential of each of these transformations was investigated by assessing the genotoxicity of analogues found in genotoxicity databases available in the OECD QSAR Toolbox, and from metabolites of active substances that are similar to isoflucypram. All metabolic transformations were determined to be of no greater genotoxic concern than isoflucypram.

In conclusion, all metabolites of isoflucypram investigated for genotoxicity predictions and read-across were considered to show no greater concern for genotoxicity than did isoflucypram (Pellizzaro & Hynes, 2022).

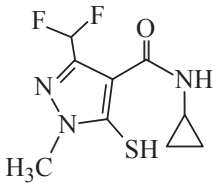
The Meeting noted that the chemical structure of M02 was similar to the parent. Metabolite M02 was found in rat plasma but its concentration was lower than that of other major rat metabolites such as M11 and M58 (JMPR, 2023). The meeting also noted that M68 was M58-acetic acid. Although M68 was not found in rats, M58 is a major metabolite in toxicity studies in mice, rats, rabbits and dogs. Also M58 is found at greater than 10% of total recovered radioactivity in plasma, liver and kidney during ADME study in rats (JMPR 2023).

***Second in silico analysis for genotoxicity; M56 and M57***

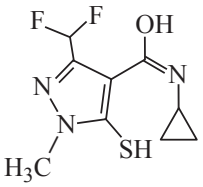
A second in silico analysis was conducted to predict the genotoxic potential of metabolites M56 and M57 (Table 1). As M56 and M57 are glycoside conjugates the two possible aglycons (aglycon 1 and aglycon 2) were assessed for genotoxic potential. The assessment was conducted using a weight of

evidence approach, based on the in silico predictions, structural similarity, read-across to isoflucypram (the parent), to M58 (the major rat metabolite), and additional specific read-across to assess any effect of the thiol group. Either aglycon can result from cleavage of any of the six possible conjugates. As the aglycons cannot be assigned to one of the metabolites they are treated as aglycon 1 and aglycon 2. Their structures are shown in Fig. 1. Aglycon 1 and aglycon 2 were evaluated for potential genotoxicity using a panel of in silico tools.

***Figure 1. Structures of the possible aglycons of M56 and M57 which were used for the in silico genotoxicity assessment.***




**Aglycon 1**
*N*-cyclopropyl-3-(difluoromethyl)-1-methyl-5-sulfanyl-*1H*-pyrazole




**Aglycon 2**
*N*-cyclopropyl-3-(difluoromethyl)-1-methyl-5-sulfanyl-*1H*-pyrazole-4-carboximidic acid

(Redrawn from Dronka & Tinwell, 2022)

In this second study QSAR analysis was carried out to predict the genotoxic potential (gene mutation and chromosomal aberrations, Chrom ab) of the possible aglycons of metabolites M56 and M57. The following tools were used:

- DEREK Nexus (Nexus v. 2.5.2, DEREK Nexus v. 6.2.1, and Knowledge Base 2022 1.0);
- Leadscope (Model Applier 3.1.0-40) using the Genetox Suite (statistical models);
- Toxtree (v. 3.1.0).

The end-points for each tool are parameters related to genotoxicity or Cramer class. In addition to the in silico predictions, a read-across analysis for the assessment of structural similarities of the substances was done using OECD QSAR Toolbox (version 4.5) profilers for genotoxicity and functional groups.

In the results of the predictions for genotoxicity no alerts for Ames mutagenicity, genotoxic carcinogenicity or in vivo micronucleus clastogenicity were found using Toxtree, with the exception of the structural alert SA34. This alert, in addition to its low predictivity (34% true positives against the training set) as stated by the developers (Benigni, Bossa & Worth, 2010), can be disregarded due to the negative results of the in vivo micronucleus test carried out on the parent compound.

Using Leadscope, expert alert bacterial mutation v. 8, all compounds were negative. For gene mutation in bacteria, the three models (Leadscope, Bacterial Mut v. 2, E. coli – Sal A-T Mut v 2 and Salmonella Mut v 4) gave consistently negative predictions for isoflucypram and M58, and only one indeterminate prediction for each aglycon (Leadscope, Bacterial Mut v. 2). It was therefore concluded that there was no concern over gene mutation potential in bacteria.

For gene mutation in mammalian cells, two models (Leadscope, HPRT Mut v. 1 and Mouse lymphoma Act v. 2 ) gave consistently negative predictions for the parent and its metabolites. The additional positive features revealed by the third model (Mouse lymphoma Unact v. 2) for aglycon 1 are also part of M58, which was assessed from the toxicity data of the parent and therefore was assumed to be non-genotoxic. On this basis, the predictions can be considered of low concern for this end-point.

For chromosome damage, the different Leadscope models for in vitro and in vivo chromosome aberration gave consistently negative predictions for isoflucypram. For the metabolites, four models gave negative predictions and two models positive predictions. All predictions were reported “out of domain”. However, due to the available negative experimental data for the parent, and the fact that M58 as a major rat metabolite was considered to be covered by the toxicity of the parent and has a high structural similarity to both aglycons, the majority negative predictions were considered reliable. Thus for the aglycons there remained some concern over this end-point, but only at a low level.

In the results in read-across analysis aglycon 1 and aglycon 2 trigger three additional profilers (DNA binding by OASIS, protein binding by OASIS and protein binding by OECD) compared to the parent and M58. All alerts were based on the additional thiol group of the aglycons. Aglycon 1 triggers two more alerts (alerts 3 and 7) which are also triggered by isoflucypram and M58. Profiling alert 6 is only triggered by M58. Profiling alert 7 was considered to correspond to SA34 of Toxtree; as already described this alert comes with a low predictivity.

In the analysis of functional group profilers, no additional groups were identified in M58 or aglycon 1 compared to the parent isoflucypram. An imidic acid functional group was reported for aglycon 2, however this functional group does not trigger any structural alert in DEREK or Toxtree, and only part of it (the cyano group) was detected as an additional positive feature in one model for chromosomal aberration in Leadscope, which however gave a negative "out of domain" prediction for aglycon 2.

A close structural similarity was observed between the two aglycons and the parent isoflucypram (Tanimoto distances 78.49%, 77.98% and 73.95% for aglycon 1, aglycon 2 and M58, respectively), and similarly towards M58 (73.95%, 84.77% and 81.21% for isoflucypram, aglycon 1 and aglycon 2, respectively), Based on this close structural similarity, indications of no structural alerts for the aglycons using the rule-based tools and from most of the negative predictions in Leadscope were considered plausible. However, a specific read-across was performed to address the limited uncertainty related to the three additional alerts triggered by the thiol group in the OECD Toolbox.

Specific read-across approaches were taken to assess the potential relevance of the thiol group present in the aglycons from M56 and M57. The thiol group was only involved in the prediction of two Ames models in Leadscope and did not trigger any structural alerts from the rule-based tools. A difference from the parent isoflucypram and M58 emerged from the mechanistic profilers in the OECD Toolbox. Three additional profilers (general mechanistic, end-point specific and structural) were triggered, and this is the basis for the search for analogues for which there is genotoxicity data. All approaches aimed to find analogues that triggered the same profilers based on the thiol subgroup, to assess if thiol could have a negative impact on the genotoxic potential of the assessed aglycons. Approaches 1 and 2 aimed to cover the Ames and in vivo micronucleus test end-points, but when it came to subcategorization for structural similarity only compounds with data for the Ames end-point remained, or at least almost so. Therefore a third approach was adopted to filter only for compounds with in vivo data for genotoxicity. Together the different approaches allow the conclusion, that the presence of the thiol group does not increase the risk of increased genotoxic potential with respect to the Ames end-point or for mammalian genotoxicity.

Based on the in silico prediction results, structural similarity and read-across to the parent compound isoflucypram and its metabolite M58, and on the additional specific read-across to assess thiol group effects, the overall weight of evidence indicated no concern regarding the genotoxic potential of the aglycons, and thus also of the conjugated metabolites M56 and M57. Structural similarities of both aglycons to the parent compound and M58 were demonstrated by the OECD Toolbox profilers used and the Tanimoto distances derived (Dronka & Tinwell, 2022).

The Meeting noted that for M56 and M57 measures of structural similarity other than the Tanimoto index had not been obtained.

## 3. Microbiological data

No new data available.

## 4. Observations in humans

No new data available

# Comments

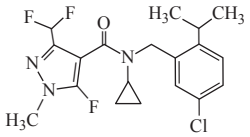
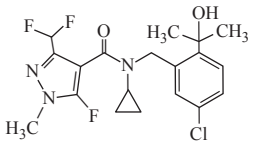
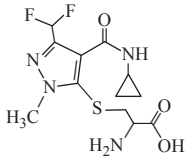
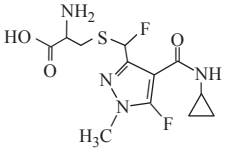
## Toxicological data on metabolites and/or degradates

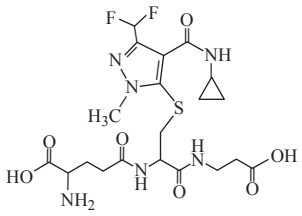
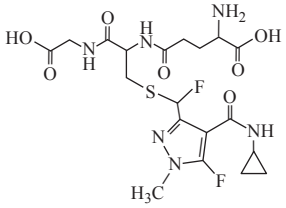
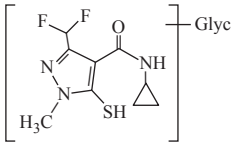
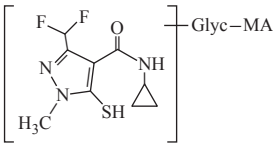
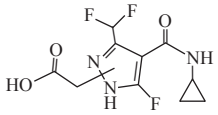
In silico analysis for genotoxic potential was conducted on 12 metabolites of isoflucypram: M02, M44, M45, M46, M47, M48, M52, M54, M55, M56, M57 and M68.

The parent and the major rat metabolite M58 were also analyzed for comparison with the other metabolites. The analysis included metabolites with known or partially characterized structures, and conjugated metabolites. Metabolites that were not fully structurally characterized were assessed for all their possible structures. Conjugated metabolites were assessed using their respective deconjugated forms. No concern with regard to genotoxic potential was raised for any of the metabolites assessed.

A summary of the toxicological evaluation of metabolites requested for residue definition is shown below in Table 2.

***Table 2. Summary of toxicological characterization of plant/livestock metabolites***

| **Compound, codes and structure** | **Major rat metabolite** (> 10% AD) | **Genotoxicity assessment** (data, QSAR, RA) | **General toxicity** | **Toxicological reference values** |
|---|---|---|---|---|
| **Isoflucypram**<br>(BCS-CN88460)<br> | Parent | Not genotoxic (data) | Full dataset | ADI:<br>0–0.06 mg/kg bw<br>ARfD: Unnecessary |
| **M02**<br>Isoflucypram-2-propanol<br> | No | Not genotoxic in (QSAR and RA) | No data but covered by the parent (Structural similarity) | Parent ADI |
| **M52***<br>Isoflucypram-desfluoro-*N*-methyl-cyclopropyl-pyrazole-carboxamide-OH-Cys<br>**a**<br><br>**b**<br> | No < 2% in rat bile | Not genotoxic (QSAR and RA) | No information | TTC<br>Cramer class III value:<br>1.5 µg/kg bw per day |

| Compound, codes and structure | Major rat metabolite (>10% AD) | Genotoxicity assessment (data, QSAR, RA) | General toxicity | Toxicological reference values |
|---|---|---|---|---|
| **M54*** Isoflucypram-desfluoro-*N*-methyl-cyclopropyl-pyrazole-carboxamide-OH-GSH **a**  **b**  | No <2% in rat bile | Not genotoxic (QSAR and RA) | No information | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **M56**** Isoflucypram-desfluoro-*N*-methyl-cyclopropyl-pyrazole-carboxamide-mercapto-Glyc  | Not found in rats | Not genotoxic (QSAR and RA)[a] | No information | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **M57**** Isoflucypram-desfluoro-*N*-methyl-cyclopropyl-pyrazole-carboxamide-mercapto-Glyc-MA  | Not found in rats | Not genotoxic (QSAR and RA) | No information | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **M68** Isoflucypram-cyclopropyl-pyrazole-carboxamide-acetic acid  | Not found in rats Structural similarity to M58; a major metabolite in mice, rats, rabbits and dogs | Not genotoxic (QSAR and RA) | No data but covered by the parent (Structural similarity to M58) | Parent ADI |

QSAR: Quantitative structure–activity relationship; RA: Read-across; TTC: Threshold of toxicological concern; ADI: Acceptable daily intake; ARfD: Acute reference dose;

[#] Data from JMPR evaluation in 2022;

* Showing the most likely structures of M52 and M54;

** As for M56 and M57, three possible chemical structures were not provided, therefore their Markush formats are shown in this table. SMILES codes of their most likely structures were as follow:

**For M56**: Cn1nc(C(F)F)c(C(=O)N(C2CC2)C3OC(CO)C(O)C(O)C3O)c1S,
Cn1nc(C(F)F)c(C(=O)NC2CC2)c1SC3OC(CO)C(O)C(O)C3O or
Cn1nc(C(F)F)c(\C(=N/C2CC2)\OC3OC(CO)C(O)C(O)C3O)c1S

**For M57:** Cn1nc(C(F)F)c(C(=O)N(C2CC2)C3OC(COC(=O)CC(=O)O)C(O)C(O)C3O)c1S,
Cn1nc(C(F)F)c(C(=O)NC2CC2)c1SC3OC(COC(=O)CC(=O)O)C(O)C(O)C3O or
Cn1nc(C(F)F)c(\C=N/C2CC2)\OC3OC(COC(=O)CC(=O)O)C(O)C(O)C3O)c1S

In silico analysis on genotoxicity of the two possible aglycons was conducted; both aglycons can be the cleavage product of these six possible conjugates.

## Toxicological evaluation (addendum)

The 2022 Meeting established an ADI of 0–0.06 mg/kg bw for isoflucypram based on a NOAEL of 6.27 mg/kg bw per day in the long-term study in rats and applying a safety factor of 100. The current Meeting concluded that the parent ADI applies to M01, M02, M11, M12, M62, M66, M67, M68 and M69.

The 2022 Meeting concluded that it was not necessary to establish an ARfD for isoflucypram and this conclusion applies to all the metabolites assessed at the present Meeting.

*Acceptable daily intake (ADI), applies to isoflucypram and metabolites M01, M02, M11, M12, M62, M66, M67, M68 and M69, expressed as isoflucypram*

0–0.06 mg/kg bw

*Acute reference dose (ARfD)*

Unnecessary

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# Isotianil

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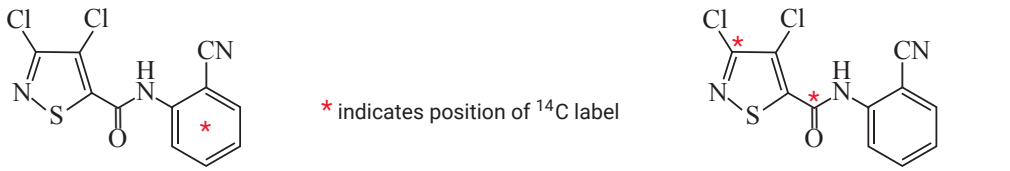
# Explanation

Isotianil is the International Organization for Standardization (ISO)-approved name for 3,4-dichloro-*N*-(2-cyanophenyl)-1,2-thiazole-5-carboxamide, for which the Chemical Abstracts Service number is 224049-04-1. Isotianil is a monocarboxylic acid amide fungicide of the thiodiazole-carboxamide chemical class (Fungicide Resistance Action Committee Group class P03, salicylic acid-like). Isotianil-induced fungal control is exerted via the induction in the plant of pathogenesis-related proteins, leading to increased systemic acquired resistance in uninfected plant parts. No comparable mechanism occurs in humans, although pathogenesis-related proteins are known human allergens (Arora et al., 2020).

Isotianil has not previously been evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed by the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR). All critical studies, except where stated, contained statements of compliance with good laboratory practice (GLP) and generally complied with currently accepted test methods. The purity of isotianil in all test articles was greater than 95%. All studies were validated by the appropriate use of controls and, when isotianil was administered via the diet, its stability and homogeneity were confirmed. A search of published scientific literature the US National Centre for Biotechnology Information, National Library of Medicine using the search term "isotianil" was performed. No relevant peer-reviewed, published scientific papers were identified.

Overall, the Meeting considered that the database was adequate for the risk assessment.

***Figure 1. Chemical structure of isotianil, and position of radiolabels in ADME studies***




[*Phenyl-U*-$^{14}$C]isotianil

[*Isothiazole-3*-$^{14}$C, *carboxamide*-$^{14}$C]isotianil

# Evaluation for acceptable daily intake

## 1. Biochemical aspects

### 1.1 Absorption, distribution and excretion

#### (a) Oral route

***Rat***

The absorption, distribution and excretion of isotianil following a single oral (gavage) dose of [*isothiazole-3*-$^{14}$C, *carboxamide*-$^{14}$C]isotianil or [*phenyl-U*-$^{14}$C]isotianil were evaluated in the following studies in male and female Wistar rats:

(a) a pilot pharmacokinetic experiment (4 or 200 mg/kg bw; 1 rat/sex/dose);

(b) a pivotal pharmacokinetic experiment (4 or 200 mg/kg bw; 4 rats/sex/dose);

(c) a pilot single dose excretion study (4 or 200 mg/kg bw; 2 rats/sex/dose); and

(d) a pivotal single dose excretion study consisting of two study arms;
  (i) single dose excretion, and
  (ii) distribution (4 or 200 mg/kg bw; 4 rats/sex/dose at the maximum termination times),

(e) and also a tissue distribution time-course arm
  (4 or 200 mg/kg bw; 3 rats/sex/dose/time point at the intermediate termination times).

Samples collected during the course of these experiments are summarized in Table 1.

***Table 1. Sampling schemes in rat oral absorption, distribution and excretion studies with isotianil***

| | Sampling scheme | | | | | | |
|---|---|---|---|---|---|---|---|
| | List A[a] | Tissue | | | Carcass | | |
| **Experiment/arm** | 10 times; 0.25–120h | 168 h | 0.25, 12, and 48h | 0.5, 24 and 72 h | 48 h | 72 h | 168 h |
| (a) Pilot pharmacokinetic | X | - | - | - | - | - | - |
| (b) Pivotal pharmacokinetic | X | - | - | - | - | - | - |
| (c) Pilot single dose excretion | - | X | - | - | - | - | X |
| (d) Pivotal single dose excretion: | - | - | - | - | - | - | - |
| single dose | - | - | - | - | - | - | - |
| distribution study | - | - | - | - | - | - | - |
| (e) Tissue distribution time course: | - | - | - | - | - | - | - |
| 4mg/kg group | - | - | X | - | X | - | - |
| 200mg/kg group | - | - | - | X | - | X | - |

h: Hours after dosing;

[a] List A samplings: volatiles and $^{14}C$-labelled carbon dioxide trapping, urine, faeces, cage wash, blood

Major metabolites were isolated and identified in urine and faeces samples (see section 1.2b) by two or more of the following approaches: spectral analysis, such as one- and two-dimensional nuclear magnetic resonance spectroscopy (1D- and 2D-NMR) and liquid chromatography with tandem mass spectrometry (LC-MS/MS), chemical modification, and comparison with reference standards using high-performance liquid chromatography (HPLC), two-dimensional thin layer chromatography (2-D TLC) and/or liquid chromatography–mass spectrometry (LC/MS).

*Pharmacokinetic parameters; isothiazole label*

Radioactivity derived from isotianil was predominantly found in the plasma fraction of whole blood. The plasma pharmacokinetic parameters of the radioactivity derived from [*isothiazole-3-*$^{14}C$, *carboxamide-*$^{14}C$]isotianil following oral dosing are shown in Table 2.

***Table 2. Plasma pharmacokinetic parameters of the radioactivity derived from [isothiazole-3-$^{14}C$, carboxamide-$^{14}C$]isotianil following oral dosing.***

| **Dosage group and sex** | **$T_{max}$ (h)** | **$C_{max}$ (µg equiv./g)** | **$C_{max}$; dose-normalized (µg equiv./g per mg/kg bw)** | **AUC (µg equiv. × h/g)** | **AUC; dose-normalized (µg equiv. × h/g per mg/kg bw)** | **Half-life (hours)** |
|---|---|---|---|---|---|---|
| Dosage 4 mg/kg bw | | | | | | |
| Males | 0.6 | 0.28 | 0.07 | 3.87 | 0.97 | 13.9 |
| Females | 0.3 | 0.36 | 0.09 | 4.84 | 1.21 | 14.5 |
| Male : female ratio | 2 | 0.77 | 0.78 | 0.80 | 0.80 | 0.96 |
| Dosage 200 mg/kg bw | | | | | | |
| Males | 3.3 | 2.57 | 0.01 | 65.19 | 0.32 | 17.9 |
| Females | 0.9 | 4.17 | 0.02 | 106.65 | 0.53 | 18.6 |
| Male : female ratio | 3.67 | 0.62 | 0.50 | 0.61 | 0.60 | 0.96 |

$C_{max}$: Maximum concentration; $T_{max}$: Time to $C_{max}$; h: Hours Source: Gupta, Shah & Savides, 2006

AUC: Area under the concentration–time curve

Oral exposure to [*isothiazole-3*-$^{14}$C, *carboxamide*-$^{14}$C]isotianil over the 4–200 mg/kg bw dose range was associated with higher plasma exposure in females compared to males, based on the total area under the concentration–time curve ($AUC_{0\rightarrow\infty}$) and concentration maximum ($C_{max}$) values. Plasma $T_{max}$ was lower in males compared with females. There were no biologically meaningful sex differences in the plasma half-life ($t_{½}$). The $AUC_{0\rightarrow\infty}$ and $C_{max}$ increased with dose, however, dose-normalized $AUC_{0\rightarrow\infty}$, and dose-normalized $C_{max}$ were sublinear over the 4–200 mg/kg bw range. The degree of sub-linearity, which was up to about a 3.7-fold change for dose-normalized $C_{max}$ and about a 2.1-fold difference for dose-normalized AUC between 4 and 200 mg/kg bw, was considered biologically significant.

*Pharmacokinetic parameters; phenyl label*

Within blood the radioactivity derived from isotianil was predominantly found in the plasma fraction. The plasma pharmacokinetic parameters for radioactivity derived from [*phenyl-U*-$^{14}$C]isotianil following oral dosing are shown in Table 3.

***Table 3. Plasma pharmacokinetic parameters of the radioactivity derived from [phenyl-$^{14}$C]isotianil following oral dosing***

| Dosage group and sex | $T_{max}$ (h) | $C_{max}$ (µg equiv./g) | $C_{max}$; dose-normalized (µg equiv./g per mg/kg bw) | AUC (µg equiv. × h/g) | AUC; dose-normalized (µg equiv. × h/g per mg/kg bw) | Half-life (hours) |
|---|---|---|---|---|---|---|
| Dosage 4 mg/kg bw | | | | | | |
| Males | 0.4 | 0.20 | 0.05 | 4.35 | 1.01 | 15.8 |
| Females | 1.7 | 0.26 | 0.07 | 4.71 | 1.18 | 17.8 |
| Male : female ratio | 4.25 | 0.77 | 0.71 | 0.92 | 0.86 | 0.89 |
| Dosage 200 mg/kg bw | | | | | | |
| Males | 3.3 | 3.50 | 0.018 | 81.43 | 0.41 | 18.2 |
| Females | 10.6 | 3.72 | 0.019 | 111.82 | 0.56 | 20.5 |
| Male : female ratio | 3.2 | 0.94 | 0.95 | 0.73 | 0.73 | 0.89 |

$C_{max}$: Maximum concentration; $T_{max}$: Time to $C_{max}$; h: Hours Source: Gupta, Shah & Savides, 2006

AUC: Area under the concentration–time curve

Oral exposure to [*phenyl-U*-$^{14}$C]isotianil over the dose range 4–200 mg/kg bw was associated higher plasma exposure (based on $AUC_{\infty}$ and $C_{max}$ values) in females compared with males. Plasma $T_{max}$ was lower in males than in females. There were no biologically meaningful sex differences in the plasma $t_{½}$. Plasma $AUC_{0\rightarrow\infty}$ and $C_{max}$ increased with dose, however, based on dose-normalized $AUC_{0\rightarrow\infty}$, and dose-normalized $C_{max}$ values the plasma exposure was sublinear over the range 4–200 mg/kg bw. The degree of the dose sub-linearity was considered biologically significant.

*Single dose excretion and distribution; isothiazole label*

The overall recoveries of administered isothiazole radiolabel were greater than 93.1% for all dose groups. At the end of the 168-hour exposure period total residues in tissues were less than 0.2% of the administered dose (AD) and total residues in the residual carcass were less than 0.4% of AD. The predominant route of excretion of radioactivity over the 4–200 mg/kg bw dose range was via the faeces, with urine being a secondary route. The ratio between radioactivity excreted in faeces and urine varied with dose, with a higher fraction of the administered radioactivity being excreted in the faeces at the higher dose level (Table 4).

***Table 4. Faecal and urinary excretion of radioactivity derived from [isothiazole-3-$^{14}$C, carboxamide-$^{14}$C]isotianil following oral dosing***

| | Percentage recovery of administered dose | | | |
|---|---|---|---|---|
| | 4 mg/kg bw dose level | | 200 mg/kg bw dose level | |
| **Matrix** | **Male** | **Female** | **Male** | **Female** |
| Urine | 31.64 | 30.12 | 12.21 | 11.38 |
| Faeces | 57.20 | 58.90 | 82.17 | 80.63 |
| Urine:faeces ratio | 0.55 | 0.51 | 0.15 | 0.14 |

Source: Gupta, Shah & Savides, 2006

These data, when combined with the results of the tissue distribution arm of the study in which the highest tissue levels were detected in the stomach following dosing at 200 mg/kg bw, imply that sub-dose-linear oral absorption occurred at the 200 mg/kg bw dose level. This interpretation is consistent with the sub-linearity, with respect to dose, seen in plasma $AUC_{0\rightarrow\infty}$ and $C_{max}$ observed following oral dosing with the isothiazole label.

*Single dose excretion and distribution; phenyl label*

The overall recoveries of administered phenyl radiolabel were greater than 95.5% for all dose groups. After 168 hours total residues in tissues were below 0.2% of AD, and total residues in the carcass were 0.12% of AD or less. The predominant route of excretion of radioactivity over the 4–200 mg/kg bw dose range was via the faeces, with urine being a secondary route. The ratio between radioactivity excreted in faeces and urine varied with dose, with a higher fraction of the administered radioactivity being excreted in the faeces at the higher dose level (Table 4). These data, when combined with the results of the tissue distribution arm of the study in which the highest tissue levels were detected in the stomach following dosing at 200 mg/kg bw, imply that sub-dose-linear oral absorption occurred at the 200 mg/kg bw dose level. This interpretation is consistent with the sub-linearity, with respect to dose, seen in plasma $AUC_{0\rightarrow\infty}$ and $C_{max}$ observed following oral dosing with the phenyl label.

***Table 5. Faecal and urinary excretion of radioactivity derived from [phenyl-$^{14}$C]isotianil following oral dosing***

| **Matrix** | Percentage recovery of administered dose | | | |
|---|---|---|---|---|
| | 4 mg/kg bw dose level | | 200 mg/kg bw dose level | |
| | **Male** | **Female** | **Male** | **Female** |
| Urine | 27.23 | 25.69 | 9.04 | 8.94 |
| Faeces | 63.76 | 66.47 | 86.75 | 88.17 |
| Urine:faeces ratio | 0.43 | 0.39 | 0.10 | 0.10 |

Source: Gupta, Shah & Savides, 2006

*Single dose tissue distribution; isothiazole label*

Within the 4 mg/kg bw dose group, the highest residue levels were found in liver and kidney. Residue levels at the 4 mg/kg bw dose level for all other tissues were less than 0.03 mg/kg or were not detectable. No sex-related differences in tissue residue levels were observed. Among the 200 mg/kg bw dose group, the highest residue levels in male rats were found in stomach, intestine, kidney and liver. Residue levels at the 200 mg/kg bw dose level for all other tissues were less than 0.6 mg/kg or not detectable.

*Single dose tissue distribution; phenyl label*

Within the 4 mg/kg bw dose group, the highest residue levels were found in liver and kidney. Residue levels at the 4 mg/kg bw dose level for all other tissues were less than 0.02 mg/kg or were not detectable. No sex-related differences in tissue residue levels were observed. Among the 200 mg/kg bw dose group, the highest residue levels in male rats were found in stomach, kidney, liver and packed cells. Residue levels at the 200 mg/kg bw dose level for all other tissues were less than 0.6 mg/kg or not detectable. No sex-related differences in tissue residue levels were observed.

At both the 4 mg/kg bw and 200 mg/kg bw dose levels, concentrations in most of the tissues were maximal after 0.25 hours for the low-dose group and at 0.5 hours for the high-dose group for both radiolabels, after which their concentration continually decreased with time. There were no biologically important differences according to label or sex. Based on the available data, isotianil has no potential for accumulation in the body (Gupta, Shah & Savides, 2006).

*Biliary excretion*

The biliary excretion of isotianil was investigated in bile duct-cannulated male and female Wistar rats (four of each sex per radiolabel) using [*isothiazole-3*-$^{14}$C, *carboxamide*-$^{14}$C]isotianil and [*phenyl-U*-$^{14}$C]isotianil. The rats were orally (gavage) administered either labelled isotianil at 4 mg/kg bw. Excreta (urine, faeces and bile) were collected once a day for two days following dosing. Metabolites in bile, urine and faeces were identified by HPLC co-chromatography (see section 1.2b).

Male rats excreted 24.6%, 46.2% and 13.1% (total 83.9%) of administered [*isothiazole-3*-$^{14}$C, *carboxamide*-$^{14}$C]isotianil into urine, bile and faeces, respectively in the two days after dosing: for female rats the corresponding values were 14.7%, 56.1% and 12.8% (total 83.6%) in urine, bile, and faeces, respectively. On day 2 following dosing 1.7% and 1.5% of dosed $^{14}$C was detected in the carcass and gastrointestinal (GI) contents, respectively, of male rats: for female rats the corresponding values were 2.6% and 2.4% of dosed $^{14}$C detected in the carcass and GI contents, respectively. Derived from these results, the total recoveries of dosed $^{14}$C were 87.1% and 88.6% for males and females, respectively. The oral absorption rates of $^{14}$C, estimated from the sum of $^{14}$C excreted into urine and bile and that remaining in the carcass, were 72.5% and 73.4% for males and females, respectively. The administered radioactivity was excreted rapidly.

Male rats excreted 18.8%, 59.4% and 5.2% (total 83.4%) of administered [*phenyl*-$^{14}$C]isotianil into urine, bile, and faeces respectively in the two days after dosing; for female rats corresponding values were 19.1%, 63.8% and 4.6% (total 87.5%) in urine, bile and faeces, respectively. On day 2 following dosing 1.9% and 11.8% of dosed $^{14}$C were detected in the carcass and GI contents, respectively, of male rats: for female rats the corresponding values were 3.0% and 4.8% of dosed $^{14}$C detected in the carcass and GI contents, respectively. Derived from these results, the total recoveries of dosed $^{14}$C were 97.2% and 95.3% for males and females, respectively. The oral absorption rates of $^{14}$C estimated from the sum of $^{14}$C excreted into urine and bile and that remaining in the carcass, were 80.2% and 85.9% for males and females, respectively.

Irrespective of the radiolabel position, these data demonstrate that radioactivity derived from orally dosed isotianil at 4 mg/kg bw is rapidly absorbed with an estimated oral absorption at or above 72.5%. The major route of excretion of isotianil-derived radioactivity was via the faeco-biliary pathway. Elimination in urine represented a minor excretory pathway (Mikata, 2009).

An incomplete progress report (GLP certificate not supplied) on a biokinetic study in rats was evaluated but provided no additional useful information. It has not been summarized (Justus, 2002).

**(b) Dermal route**

No data was submitted.

## 1.2 Biotransformation

**(a) In vitro**

In an in vitro comparative metabolism study, [*isothiazole-3*-$^{14}$C, *carboxamide*-$^{14}$C]isotianil metabolism was compared across species using human, rat, mouse, dog and rabbit liver microsomes. Pooled, mixed-sex liver microsomes from humans (28 male and seven female donors), rat (126 males and 14 females) and mouse (404 males and 61 females), and pooled mixed liver microsomes from three male dogs, and similar pooled microsomes from four female rabbit, were incubated for 0, 60 or 120 minutes with either 1 µM or 5 µM [*isothiazole-3*-$^{14}$C, *carboxamide*-$^{14}$C]isotianil. Reactions were started by adding a NADPH-generating system, and stopped after incubation by adding cooled acetonitrile and by cooling the samples in an ice bath. Control incubations with both concentrations of labelled isotianil were conducted without the use of a NADPH regenerating system to demonstrate the stability

of the test item in the incubation system. The metabolic systems were validated by their capacity to metabolize a testosterone positive control. The detected putative metabolites were characterized based on their retention times in radio-HPLC. Determination of the metabolites' chemical structures was not undertaken, and a metabolic pathway was not derived. The chemical identity of the parent compound was confirmed using normal phase TLC.

After a 60 minute incubation the mean radioactivity recoveries were between 90.2% and 108.9% and after 120 minutes, between 90.6% and 109.7%. The metabolic transformation rate after incubation for 120 minutes with human mixed liver microsomes for [*isothiazole-3*-$^{14}$C, *carboxamide*-$^{14}$C]isotianil was up to 100.0% for the low concentration incubations and up to 97.1% for incubations at the high concentration of isotianil. The rate after incubation for 120 minutes with rat mixed liver microsomes was up to 98.1% for the low isotianil concentration incubations, and up to 93.6% for incubations at the high concentration. The rate after incubation for 120 minutes with male dog liver microsomes was up to 100.0% for low concentration incubations, and up to 92.6% for incubations at the high concentration. The rate after incubation for 120 minutes with mouse mixed liver microsomes was up to 100.0% for low concentration incubations, and up to 100.0% for incubations at the high concentration. The rates after incubation for 120 minutes with rabbit female liver microsomes was up to 86.4% for low concentration incubations, and up to 100.0% for incubations at the high concentration. Incubations at low and high concentrations of [*isothiazole-3*-$^{14}$C, *carboxamide*-$^{14}$C]isotianil with human mixed, rat mixed, male dog, mouse mixed and female rabbit liver microsomes generated 20 putative metabolites as described in Tables 6 and 7.

With human, rat, dog and mouse microsomes putative metabolite 18 was the most prevalent at both concentrations. Putative metabolite 18 was detected in human mixed liver microsome incubations with the highest abundance, reaching 76.5% after 60 minutes of incubation at the isotianil high concentration. The major putative metabolites (numbered 12, 15, 16 and 18; present at 5% or above of the applied radioactive dose) were detected after an incubation time of 120 minutes in human mixed liver microsomes and also occurred in the other species. No uniquely human, major putative metabolite was detected. Putative metabolite 6 (2.0% of the applied radioactive dose) was transiently detected in the human preparations after 60 minutes of incubation at a low dose concentration of [*isothiazole-3*-$^{14}$C, *carboxamide*-$^{14}$C]isotianil only (Tobias, 2021a).

***Table 6. In vitro comparative metabolism study – metabolic distribution after incubation of 1 µM [isothiazole-3-$^{14}$C, carboxamide-$^{14}$C]isotianil with liver microsomes from five species (mean values[a])***

| | Percentage of the radioactivity (sum of all peaks) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Incubation time** | **0 minutes** | | | | | **60 minutes** | | | | | **120 minutes** | | | | |
| **Putative metabolite** | **Human** | **Rat** | **Dog** | **Mouse** | **Rabbit** | **Human** | **Rat** | **Dog** | **Mouse** | **Rabbit** | **Human** | **Rat** | **Dog** | **Mouse** | **Rabbit** |
| 1 | - | - | - | - | - | - | - | - | - | - | - | - | - | 4.7 | - |
| 2 | - | - | - | - | - | - | - | - | 1.6 | - | - | - | - | - | - |
| 3 | - | - | - | - | - | - | 2.6 | - | - | - | - | - | - | - | - |
| 4 | - | - | - | - | - | 0.5 | - | - | 1.8 | - | - | 2.7 | - | 6.6 | - |
| 5 | - | - | - | - | - | - | 0.9 | - | - | - | - | 1.3 | - | - | - |
| 6 | - | - | - | - | - | 2.0 | - | - | - | - | - | - | - | - | - |
| 7 | - | - | - | - | - | - | - | - | - | - | - | 1.4 | 3.0 | - | - |
| 8 | - | 0.5 | - | - | - | - | 2.9 | - | - | - | - | 2.6 | - | - | - |
| 9 | - | - | - | - | - | - | - | - | - | 0.7 | - | - | - | - | 0.8 |
| 10 | - | - | - | - | - | - | 1.4 | - | - | 1.7 | - | 1.5 | - | - | 0.8 |
| 11 | - | - | - | - | 5.1 | - | - | - | - | 58.1 | - | - | - | - | 65.2 |
| 13 | - | - | - | - | - | - | 0.9 | - | - | - | - | 0.8 | - | - | - |
| 14 | - | - | - | - | - | - | 0.5 | - | - | - | - | 1.4 | - | - | - |
| 15 | - | - | - | - | - | 24.5 | 5.1 | 5.9 | 17.6 | 24.2 | 33.1 | 7.8 | 8.6 | 11.9 | 12.9 |

| Percentage of the radioactivity (sum of all peaks) | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Incubation time** | **0 minutes** | | | | | **60 minutes** | | | | | **120 minutes** | | | | |
| **Putative metabolite** | **Human** | **Rat** | **Dog** | **Mouse** | **Rabbit** | **Human** | **Rat** | **Dog** | **Mouse** | **Rabbit** | **Human** | **Rat** | **Dog** | **Mouse** | **Rabbit** |
| 16 | - | - | - | - | - | 5.6 | 13.2 | 5.3 | 18.0 | 3.0 | 5.2 | 14.3 | 7.6 | - | 0.9 |
| 17 | - | - | - | - | - | 1.2 | 1.2 | - | 8.8 | - | 3.1 | 2.9 | 4.1 | 15.3 | - |
| 18 | - | - | - | - | - | 66.2 | 68.8 | 88.8 | 50.8 | 5.6 | 58.6 | 59.8 | 76.7 | 55.4 | 2.2 |
| 19 | - | - | - | - | - | - | - | - | - | 0.4 | - | - | - | - | - |
| 20 | - | - | - | - | - | - | 0.4 | - | 1.5 | 4.5 | - | 1.6 | - | 6.1 | 4.3 |
| Isotianil | 100.0 | 99.5 | 100.0 | 100.0 | 94.9 | - | 2.1 | - | - | 1.9 | - | 1.9 | - | - | 12.8 |
| Applied radioactivity: 100% | | | | | | | | | | | | | | | |

[a] All values rounded to one decimal place; -: Not detected; Source: Tobias, 2021a

***Table 7. In vitro comparative metabolism study – metabolic distribution after incubation of 1 µM [isothiazole-3-$^{14}$C, carboxamide-$^{14}$C]isotianil with liver microsomes from five species (mean values[a])***

| Percentage of the radioactivity (sum of all peaks) | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Incubation time** | **0 minutes** | | | | | **60 minutes** | | | | | **120 minutes** | | | | |
| **Putative metabolite** | **Human** | **Rat** | **Dog** | **Mouse** | **Rabbit** | **Human** | **Rat** | **Dog** | **Mouse** | **Rabbit** | **Human** | **Rat** | **Dog** | **Mouse** | **Rabbit** |
| 7 | - | - | - | - | - | - | - | 2.0 | - | - | - | - | 1.7 | - | - |
| 10 | - | - | - | - | - | - | 1.8 | - | 3.2 | 2.2 | - | 1.6 | 1.5 | 2.3 | - |
| 12 | - | - | - | 0.7 | 15.9 | 4.8 | 7.9 | 4.3 | 29.4 | 62.2 | 7.0 | 19.7 | 8.0 | 44.0 | 69.8 |
| 15 | - | - | - | - | - | 9.2 | 1.0 | 2.7 | 7.4 | 16.6 | 17.4 | - | 1.5 | 5.0 | 15.9 |
| 16 | - | - | - | - | - | 4.9 | 5.7 | 4.0 | 12.7 | 6.3 | 8.9 | - | 3.0 | 13.6 | 8.7 |
| 17 | - | - | - | - | - | - | - | - | - | - | - | - | 1.4 | - | - |
| 18 | - | - | - | - | - | 76.5 | 69.0 | 76.4 | 47.4 | 12.7 | 63.9 | 72.6 | 75.6 | 35.1 | 5.6 |
| Isotianil | 100.0 | 100.0 | 100.0 | 99.3 | 84.1 | 4.7 | 14.7 | 10.6 | - | - | 2.9 | 6.2 | 7.4 | - | - |
| Accountability/Applied radioactivity: 100% | | | | | | | | | | | | | | | |

[a] All values rounded to one decimal place; -: Not detected; Source: Tobias, 2021a

In a second in vitro comparative metabolism study [*phenyl-U*-$^{14}$C]isotianil at approximately 1 µM and 5 µM was incubated (for 0, 60 or 120 minutes) with pooled, mixed-sex liver microsomes from five species. The microsomes were sourced as follows: human (28 males and seven females), rat (154 males and 14 females), mouse (399 males, 61 females), dog (32 males) and rabbit (four females). The reactions were started by the addition of a NADPH-generating system, and were stopped after 120 minutes of incubation by adding cooled acetonitrile and by cooling the samples in an ice bath. Control incubations with both concentrations of [*phenyl-U*-$^{14}$C]isotianil were conducted without the use of a NADPH-regenerating system to show the stability of the test item in the incubation system. The metabolic systems were validated by their capacity to metabolize a testosterone positive control. The detected putative metabolites were characterized based on their retention times in radio-HPLC. Determination of the metabolites' chemical structures was not undertaken, and a metabolic pathway was not derived. The chemical identity of the parent compound was confirmed using normal phase TLC.

After incubation for 60 minutes the mean radioactivity recoveries were between 88.7% and 103.9% for the test item. After a 120 minute incubation the mean recoveries were between 85.7% and 107.3%. The metabolic transformation rates determined after incubation for 120 minutes with human mixed liver microsomes was up to 97.8% for low concentration incubations, and up to 83.2% for incubations at the high concentration of isotianil. The rates after incubation with rat mixed liver microsomes for 120 minutes was up to 96.9% for low concentration incubations, and up to 69.2% for the

high concentration. The rates after incubation with dog male liver microsomes for 120 minutes was up to 100.0% for incubations with both low and high isotianil concentrations. The rates after incubation with mouse mixed liver microsomes for 120 minutes was up to 100.0% for low concentration incubations, and up to 88.3% for incubations at the high concentration. The rates after incubation with rabbit female liver microsomes for 120 minutes was up to 100.0% for incubations with both low and high isotianil concentrations. Incubations of low and high dose [*phenyl-U-*$^{14}$C]isotianil with human mixed, rat mixed, male dog, mouse mixed and female rabbit liver microsomes generated 20 putative metabolites as described in Tables 8 and 9.

With male dog microsomes, putative metabolite 10 detected after an incubation time of 60 minutes was the most abundant metabolite in both the low and high concentration incubations (90.6% and 96.5%, respectively). Putative metabolite 10 was also the most abundant metabolite in human preparations. No uniquely human major putative metabolite was detected. The major metabolites (5% or more of the applied radioactivity; metabolites 2, 7, 8, 9, 10 and 11) were detected after an incubation time of 120 minutes with human mixed liver microsomes, and these occurred also in the other species (Tobias, 2021b).

Considering the results of both in vitro comparative metabolism studies there were no major (5% or more of the administered radioactivity) unique human putative metabolites and the available animal species used were adequate models of the potential in vivo human hepatic microsomal metabolism of isotianil. The enzymes involved in metabolism were not identified.

***Table 8. In vitro comparative metabolism study – Metabolic distribution after incubation of 1 µM [phenyl-UL-$^{14}$C]isotianil with liver microsomes from five species (mean values[a])***

| | Percentage of the radioactivity (sum of all peaks) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Incubation time** | **0 minutes** | | | | | **60 minutes** | | | | | **120 minutes** | | | | |
| **Putative metabolite** | **Human** | **Rat** | **Dog** | **Mouse** | **Rabbit** | **Human** | **Rat** | **Dog** | **Mouse** | **Rabbit** | **Human** | **Rat** | **Dog** | **Mouse** | **Rabbit** |
| 1 | - | - | - | - | - | - | - | - | 0.7 | - | - | - | - | - | - |
| 2 | - | - | - | - | - | 5.5 | 2.5 | - | 27.1 | - | 5.7 | 3.8 | - | 29.6 | - |
| 3 | - | - | - | - | - | - | - | 1.7 | 1.5 | 46.1 | - | - | 4.5 | - | 63.3 |
| 4 | - | - | - | - | - | - | - | - | 1.1 | - | - | - | - | - | - |
| 5 | - | - | - | - | - | - | - | - | - | 1.4 | - | - | - | - | - |
| 6 | - | - | - | - | 27.8 | - | 1.7 | 3.0 | - | - | - | 2.4 | 4.5 | - | - |
| 7 | - | - | - | - | - | - | 2.6 | - | 6.0 | - | 1.4 | 3.4 | - | - | - |
| 8 | - | - | - | - | - | 21.4 | 4.7 | 4.7 | 10.0 | 34.8 | 29.6 | 7.1 | 5.1 | 10.4 | 36.7 |
| 9 | - | - | - | - | - | 7.9 | 10.3 | - | 11.1 | 5.7 | 14.0 | 17.0 | - | - | - |
| 10 | - | - | - | - | - | 59.5 | 74.5 | 90.6 | 32.8 | 9.7 | 39.5 | 62.3 | 85.9 | 49.7 | - |
| 11 | - | - | - | - | - | - | - | - | - | - | 5.6 | - | - | 6.8 | - |
| 12 | - | - | - | - | - | 1.4 | - | - | 5.4 | 2.3 | 2.1 | 1.0 | - | 3.5 | - |
| Isotianil | 100.0 | 100.0 | 100.0 | 100.0 | 72.2 | 4.3 | 3.7 | - | 4.2 | - | 2.2 | 3.1 | - | - | - |
| Applied radioactivity: 100% | | | | | | | | | | | | | | | |

[a] All values rounded to one decimal place; -: Not detected; Source: Tobias, 2021b

***Table 9. In vitro comparative metabolism study – Metabolic distribution after incubation of 5 µM [phenyl-UL-$^{14}$C]isotianil with liver microsomes from five species (mean values[a])***

| Percentage of the radioactivity (sum of all peaks) | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Incubation time** | **0 minutes** | | | | | **60 minutes** | | | | | **120 minutes** | | | | |
| | **Human** | **Rat** | **Dog** | **Mouse** | **Rabbit** | **Human** | **Rat** | **Dog** | **Mouse** | **Rabbit** | **Human** | **Rat** | **Dog** | **Mouse** | **Rabbit** |
| 3 | - | - | - | - | - | - | - | - | 12.1 | 25.9 | - | - | - | 43.7 | 75.1 |
| 5 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1.7 |
| 7 | - | - | 1.0 | - | 11.4 | 5.0 | 5.7 | - | 7.2 | 62.6 | 6.7 | 10.9 | - | 3.7 | 1.8 |
| 8 | - | - | - | - | - | 1.9 | - | 6.0 | - | - | 4.9 | - | 3.5 | - | 14.7 |
| 9 | - | - | - | - | - | - | - | 1.9 | - | - | - | 1.0 | - | 2.0 | - |
| 10 | - | - | - | - | - | 58.8 | 4.1 | 92.2 | 31.5 | 11.6 | 71.7 | 57.4 | 96.5 | 39.0 | 6.7 |
| Isotianil | 100.0 | 100.0 | 99.0 | 100.0 | 88.6 | 34.3 | 90.2 | - | 49.2 | - | 16.8 | 30.8 | - | 11.7 | - |
| Accountability/Applied radioactivity: 100% | | | | | | | | | | | | | | | |

[a] All values rounded to one decimal place; -: Not detected; Source: Tobias, 2021b

**(b) In vivo**

Excreta and tissue metabolites were evaluated in samples collected for the study of Gupta, Shah & Savides (2006); for details see section 1.1, metabolite profiles summarized in Tables 10 and 11. The following major systemic metabolites were identified, using the criterion 10% or more of AD or 10% or more of the area under the HPLC curve:

- 4′-hydroxy-isotianil (kidney, plasma),
- glucuronide of 4'-hydroxy-isotianil (kidney),
- 4′,5′-dihydroxy-isotianil plus 3′,4′-dihydroxy-isotianil (kidney and plasma),
- 3,4-dichloroisothiazole-5-carboxylic acid; DCIT-acid (liver, kidney and plasma),
- sulfate of 2-amino-5-hydroxybenzonitrile (liver, kidney, plasma)

(Gupta, Shah & Savides, 2006)

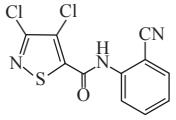
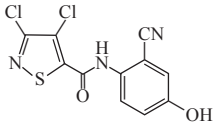
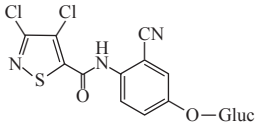
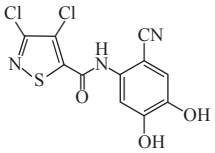
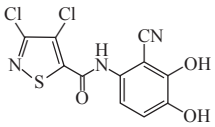
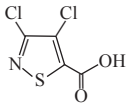
Based on samples collected from the rat biliary excretion study (Mikata, 2009; summarized in section 1.1 and metabolite profile in Table 11) four major metabolites were identified in the excreta:

- 3,4-dichloroisothiazole-5-carboxylic acid; DCIT-acid (12.8% of the administered radioactivity in males, 5.5% in females),
- 4′-hydroxy-isotianil,
- glucuronide of 4′-OH-isotianil
- trihydroxy-isotianil.

Considering the results of both Gupta, Shah & Savides (2006) and Mikata (2009), DCIT-acid was present in excreta at up to 7.95% plus12.8% in urine and bile, a total of 20.75% of AD in males and at up to 7.03 plus 5.5%, totalling 12.53% in females, following oral dosing with 4 mg/kg bw of [*isothiazole-3*-$^{14}$C, *carboxamide*-$^{14}$C]isotianil. This supports the conclusion of Gupta, Shah & Savides, that DCIT-acid is a major rat metabolite of isotianil.

A proposed metabolic pathway for isotianil in rats, based on Gupta, Shah & Savides (2006) is shown in Fig. 2. The major route of metabolism is hydroxylation followed, in some cases, by glucuronidation. The major enzymes involved in metabolism were not identified.

***Table 10. Identified metabolites in excreta (urine, faeces, and cage wash), plasma and tissues after administration of $^{14}C$ isotianil to male and female rats.***

| Compound/metabolite, codes and structure | Matrix | Percentage of administered dose | |
|---|---|---|---|
| | | 4 mg/kg bw (male/female) | 200 mg/kg bw (male/female) |
| **Isothiazole label** | | | |
| Isotianil (S-2310)  | Faeces | 20.52 / 23.89 | 70.09 / 71.47 |
| 4′-Hydroxy-isotianil (4′-OH-S-2310)  | Faeces | 5.00 / 5.12 | 2.65 / 3.84 |
| | Urine | 6.48 / 6.57 | 2.71 / 3.30 |
| Glucuronide of 4′-hydroxy-isotianil (4′-OH-S-2310 glucuronide)  | Urine | 5.26 / 5.91 | 1.64 / 0.49 |
| 4′,5′-Dihydroxy-isotianil (4′,5′-di-OH-S-2310)  | Urine | 3.68 / 2.97 | 1.53 / 1.38 |
| | Faeces | 8.63 / 8.50 | 3.43 / 0.74 |
| 3′,4′-Dihydroxy-isotianil (3′,4′-di-OH-S-2310)  | Faeces | 5.28 / 7.09 | 0.43 / 0.50 |
| 3,4-Dichloroisothiazole-5-carboxylic acid (DCIT-acid)  | Urine | 7.95 / 7.03 | 2.80 / 3.12 |
| **Phenyl label** | | | |
| Isotianil (S-2310) | Faeces | 29.69 / 32.32 | 76.88 / 80.21 |
| 4′-Hydroxy-isotianil (4′-OH-S-2310) | Urine | 2.88 / 2.72 | 1.57 / 1.80 |
| | Faeces | 5.67 / 6.88 | 1.42 / 1.57 |
| Glucuronide of 4′-hydroxy-isotianil (4′-OH-S-2310 glucuronide) | Urine | 5.05 / 4.41 | 1.42 / 1.09 |
| 4′,5′-Dihydroxy-isotianil (4′,5′-di-OH-S-2310) | Urine | 0.71 / 1.43 | 0.32 / 0.53 |
| | Faeces | 8.09 / 8.09 | 1.34 / 0.45 |
| 3′,4′-Dihydroxy-isotianil (3′,4′-di-OH-S-2310) | Faeces | 4.10 / 3.17 | 1.21 / 0.46 |

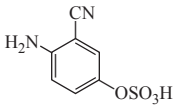
| Compound/metabolite, codes and structure | Matrix | Percentage of administered dose | |
|---|---|---|---|
| | | 4 mg/kg bw (male/female) | 200 mg/kg bw (male/female) |
| Sulfate of 2-amino-5-hydroxybenzonitrile | Urine | 5.11 / 4.96 | 2.32 / 1.46 |
|  | Faeces | 1.25 / 1.01 | 0 / 1.00 |



***Table 11. Characterized metabolites in plasma and tissues following administration of $^{14}C$ isotianil in the rat; quantitative percentage from HPLC curve shown (% male / %female)***

| Metabolite | Matrix and dose | | | | | |
|---|---|---|---|---|---|---|
| | Liver[a] | | Kidney[a] | | Plasma[b] | |
| | 4 mg/kg bw | 200 mg/kg bw | 4 mg/kg bw | 200 mg/kg bw | 4 mg/kg bw | 200 mg/kg bw |
| **Isothiazole label** | | | | | | |
| Isotianil (S-2310) | 0/0 | 0/0 | 0/0 | 2.37/4.66 | 8.65/4.30 | 11.01/14.57 |
| Sulfate of 2-amino-5-hydroxybenzonitrile | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | |
| 4′-Hydroxy-isotianil | 0/0 | 0/0 | 0.87/8.31 | 8.68/8.93 | 28.02/15.97 | 27.41/27.72 |
| Glucuronide of 4'-hydroxy-isotianil | 0/0 | 0/0 | 19.86/5.47 | 4.15/3.92 | 7.31/14.15 | 2.17/10.98 |
| 4′,5′-Dihydroxy-isotianil plus 3′,4′-Dihydroxy-isotianil | 0/0 | 0/0 | 11.98/38.23 | 41.88/37.79 | 17.50/15.33 | 38.64/17.17 |
| DCIT-acid | 11.89/16.27 | 15.74/17.46 | 14.23/14.77 | 0/0 | 13.96/20.01 | 10.65/16.04 |
| **Phenyl label** | | | | | | |
| Isotianil (S-2310) | 11.73/24.80 | 21.35/13.62 | 10.50/0 | 3.69/5.59 | 3.29/7.23 | 2.60/3.99[c] |
| Sulfate of 2-amino-5-hydroxybenzonitrile | 32.37/35.44 | 28.77/39.12 | 31.92/42.04 | 11.10/14.72 | 18.17/12.14 | 5.86/7.76[c] |
| 4′-Hydroxy-isotianil | 2.00/4.37 | 5.02/9.20 | 17.39/15.31 | 10.72/0 | 11.21/10.79 | 9.28/10.24[c] |
| Glucuronide of 4′-Hydroxy-isotianil | 0/0 | 0/0 | 2.17/0 | 8.42/10.06 | 20.66/25.39 | 11.67/12.43[c] |
| 4′,5′-Dihydroxy-isotianil plus 3′,4′-Dihydroxy-isotianil | 0/0 | 0/0 | 3.10/4.61 | 8.14/4.61 | 16.28/28.22 | 24.71/24.04[c] |

[a] 0.25 hours following dosing;

[b] 12 hours following dosing;

[c] 0.5 hours following dosing;

Source: Gupta, Shah & Savides, 2006

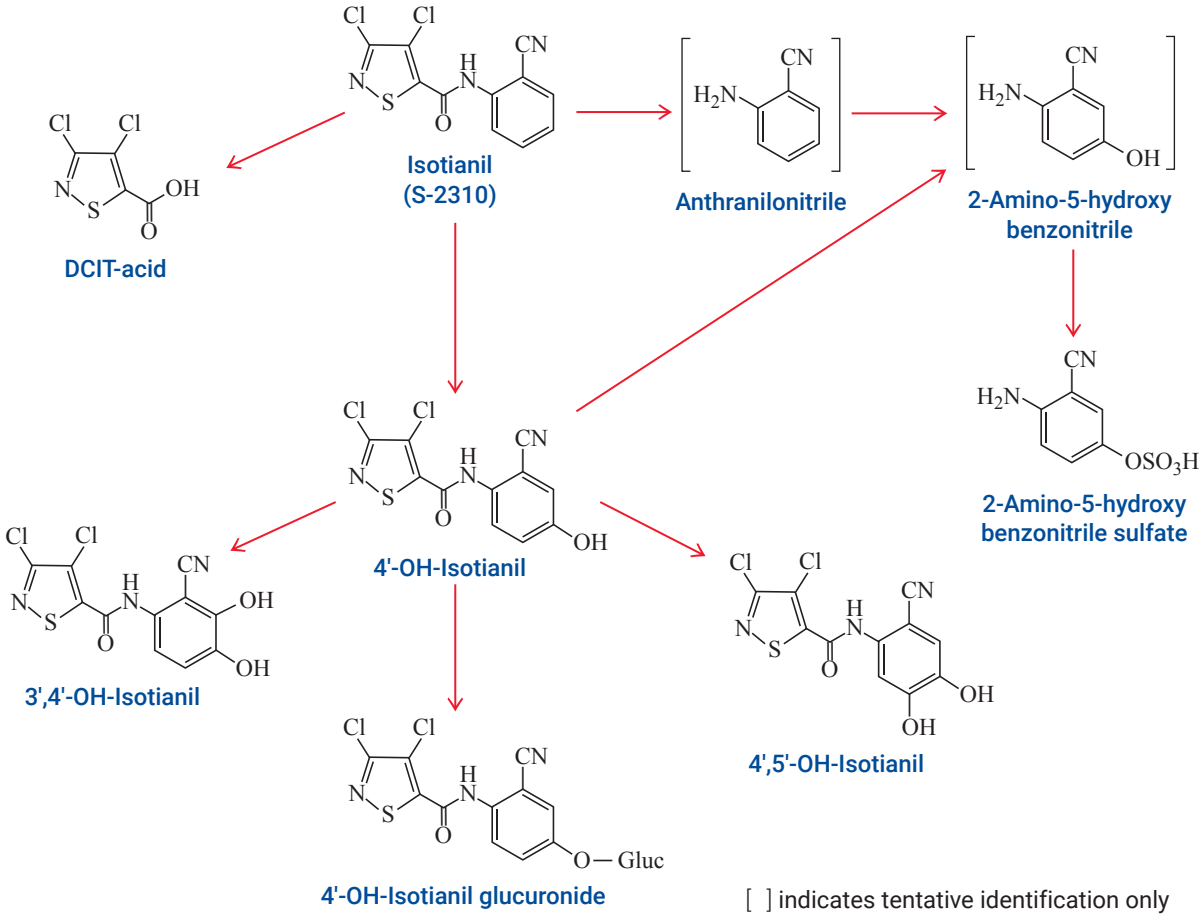
***Table 12. Rat biliary excretion study – metabolites identified in urine, bile and faeces after administration of isothiazole- or phenyl-labelled isotianil to rats at a dose of 4 mg/kg bw; (percentages of administered dose shown)***

| Compound/metabolite | Dosage group; all at 4 mg/kg bw[a] | | | |
|---|---|---|---|---|
| | Isothiazole-labelled isotianil | | Phenyl-labelled isotianil | |
| | Males | Females | Males | Females |
| Isotianil (S-2310) | 11.7 | 11.9 | 4.7 | 3.9 |
| Sulfate of 2-amino-5-hydroxybenzonitrile | - | - | 7.7 | 5.4 |
| DCIT-acid | 12.8 | 5.5 | - | - |
| 4′-Hydroxy-isotianil | 13.5 | 8.7 | 12.6 | 12.6 |
| Glucuronide of 4′-hydroxy-isotianil | 15.3 | 27.7 | 18.0 | 29.5 |

| | Dosage group; all at 4 mg/kg bw[a] | | | |
|---|---|---|---|---|
| | Isothiazole-labelled isotianil | | Phenyl-labelled isotianil | |
| **Compound/metabolite** | **Males** | **Females** | **Males** | **Females** |
| Glucuronide of 3′,4′-dihydroxy-isotianil | 0.8 | 0.5 | 1.7 | 3.0 |
| Glucuronide of 4′,5′-dihydroxy-isotianil | 4.9 | 5.9 | 7.0 | 4.0 |
| Trihdroxy-isotianil | 9.7 | 12.7 | 7.1 | 7.8 |

[a] The sum of urine, bile and faeces from days 0–2, expressed as percentage of administered dose; Source: Justus, 2002

***Figure 2. Summary of proposed metabolic pathway for isotianil in the rat.***




## 1.3 Effects on enzymes and other biochemical parameters

Limited information on isotianil-induced hepatic metabolic enzyme induction is provided in the summaries of the repeat dose toxicology studies in rats.

# 2. Toxicological studies

## 2.1 Acute toxicity

All studies complied with relevant test guidelines.

### (a) Lethal doses

*Table 13. Summary of acute oral, dermal and inhalation toxicity studies with isotianil*

| Route | Species | Sex | Result | | References |
|---|---|---|---|---|---|
| | Rat | F | $LD_{50}$ | > 2000 mg/kg bw | Schuengel, 2005 |
| Oral | Rat | M | $LD_{50}$ | > 2000 mg/kg bw | Andrews & Brendler-Schwaab, 2001 |
| | Rat | F | $LD_{50}$ | > 2000 mg/kg bw | Andrews & Brendler-Schwaab, 2001 |
| Dermal (occlusive) | Rat | M, F | $LD_{50:}$ | > 2000 mg/kg bw | Ose, 2009 |
| Inhalation (dust aerosol, nose only 4 hour exposure)[a] | Rat | M, F | $LC_{50}$: | > 5 mg/L | Deguchi, 2009 |

M: Male; F: Female; $LD_{50}$: Median lethal dose; $LC_{50}$: Median lethal concentration;

[a] Mass median aerodynamic diameter 5.26 µm, geometric standard deviation 2.16 (MMAD higher than OECD TG 403 recommended maximum value)

### (b) Dermal irritation

Semi-occlusive dermal exposures of rabbits (strain not stated) to 0.5 g of isotianil moistened with water for four hours was not irritant and nontoxic (Leuschner, 2002a; Nakamura, 2009a).

### (c) Ocular irritation

Administration of 100 mg of isotianil into the conjunctival fornix of three male Himalayan rabbits was not irritant and did not induce any systemic toxicity (Leuschner, 2002b). In a second study administration of 0.1 mL of isotianil into the conjunctival cul de sacs of three male New Zealand White rabbits were also not irritant and did not induce any systemic toxicity (Nakamura, 2009b).

### (d) Dermal sensitization

Isotianil was tested using an intradermal induction at 5%, a topical induction at 50%, and a challenge of 50% isotianil (all w/v in a polyethylene glycol 400 vehicle). The test article was nontoxic, but a skin sensitizer in a Magnusson & Kligman Guinea pig maximization test (Vohr, 2005).

## 2.2 Short-term studies of toxicity

### (a) Oral administration

*Mouse*

In a 28-day mouse oral toxicity study, isotianil was administered ad libitum in the diet to groups of Crl:CD-l(ICR)BR mice (five of each sex per group) at concentrations of 0, 100, 1000 or 10 000 ppm (equal to 0, 21, 196 and 1874 mg/kg bw per day for males, 0, 25, 223 and 2631 mg/kg bw per day for females). There were no adverse test article-associated effects on clinical signs, survival, body weight or food consumption. Small (ca 8% or less reductions compared to controls at $p < 0.05$), nonadverse, reductions in haematocrit (Ht), that were not dose-related, occurred in females treated with isotianil at 100 ppm and above. Compared to controls there were significant increases ( $p < 0.05$) in absolute mean liver (by 32%) and spleen weights (by 27%) and an increase by 22% or more in the relative weights of both these organs in the high dietary concentration cohorts. However, these changes were not accompanied by any adverse anatomic or clinical pathology changes. The study NOAEL was 10 000 ppm (equal to 1874 mg/kg bw per day), the highest dose tested (Lesser & Ruehl-Fehlert, 2003).

In a 92-day mouse oral toxicity study, isotianil was administered ad libitum in the diet to groups of Crl:CD-l(ICR)BR mice (10 of each sex per group) at concentrations of 0, 150, 1000 or 7000 ppm

(equal to 0, 33.1, 204.1 and 1309.5 mg/kg bw per day for males, 0, 54.8, 401.1 and 2474.6 mg/kg bw per day for females). There were no test article-associated adverse effects on clinical signs, mortality, body weight, haematology or clinical chemistry parameters. Food intake was reduced (by about 17% compared with controls) in the 7000 ppm male cohort. Relative liver weight was significantly increased ( $p < 0.05$) in high-dose males (by ca 21%), and in high-dose females (by ca 18%), both compared to controls. This change did not correlated with any adverse anatomical pathology findings. The study NOAEL was 7000 ppm (equal to 1309.5 mg/kg bw per day), the highest dose tested (Leser & Hartmann, 2004).

***Rat***

In a 28-day rat oral toxicity study isotianil was administered ad libitum in the diet to groups of HsdCpb: WU Wistar rats (five of each sex per group) at concentrations of 0, 1000, 3000 or 10 000 ppm (equal to 0, 82.68, 286.19 and 948.51 mg/kg bw per day for males, 0, 111.04, 278.97 and 1040.70 mg/kg bw per day for females). There were no test article-associated adverse effects on clinical signs, body weight, food or water intake, haematology, coagulation parameters, urinalysis, immunotoxicity, functional observation battery findings, motor activity, locomotor activity measurements or anatomical pathology. Significant ( $p < 0.05$), dose-related increases (by 27% or more compared to controls) in serum cholesterol occurred in males at 3000 ppm and above. Increases in serum cholesterol (not dose-related; significant at $p < 0.05$ only at 3000 ppm) occurred in females at 3000 ppm and above. These increases, whilst test article-related, did not exceed the historical (normal) range, and were not accompanied by any other evidence of dyslipidaemia or hepatotoxicity. Accordingly, these isotianil-induced changes were not regarded as adverse. Physiologically adaptive changes in liver metabolic tissue enzyme activities were also noted (see Table 14). Significant dose-related increases ( $p < 0.05$) in relative liver weight occurred in males at 1000 ppm and above, and in females at 10 000 ppm, but in the absence of any correlates of hepatotoxicity. As such these changes were regarded as adaptive and nonadverse. The study NOAEL was 10 000 ppm (equal to 948 mg/kg bw per day) the highest dose tested (Kroetlinger & Hastings, 2003).

***Table 14. Rat 28-day oral toxicity study with isotianil; selected clinical chemistry results and enzyme activities in the liver***

| | Serum chemistry measurements mean, [% change compared with control] | | | |
|---|---|---|---|---|
| | Cholesterol (mmol/L) | | Bilirubin (µmol/L) | |
| Dose (ppm) | Males | Females | Males | Females |
| 0 | 2.08 | 1.97 | 1.1 | 1.3 |
| 1000 | 2.28 | 2.19 | 1.1 | 1.0 |
| 3000 | 2.65* [27%] | 2.68* [36%] | 1.2 | 1.0 |
| 10 000 | 3.19* [53%] | 2.48 [26%] | 1.4* [27%] | 1.1 |
| Historical control (normal) range | 0.85 – 3.83[a] | 0.44 – 3.70[a] | 0.86 – 2.57[b] | 0.86 – 3.08[ab] |

| | Mean liver enzyme activities | | | | | |
|---|---|---|---|---|---|---|
| Dose (ppm) | ECOD nmol/g.min | EROD nmol/g.min | ALD nmol/g.min | EH nmol/g.min | GST µmol/g.min | UGP-GT nmol/g.min |
| | | | Males | | | |
| 0 | 10.1 | 0.53 | 298.1 | 654 | 107 | 1259 |
| 1000 | 10.1 | 0.48 | 232.6* | 950* | 119 | 1870* |
| 3000 | 9.2 | 0.40 | 240.4 | 1028* | 141* | 1993* |
| 10 000 | 10.0 | 0.67 | 197.1* | 1521* | 149* | 2642* |
| | | | Females | | | |
| 0 | 5.7 | 0.39 | 30.4 | 428 | 83 | 880 |
| 1000 | 4.0* | 0.34 | 17.2* | 940* | 136* | 1880* |
| 3000 | 5.2 | 0.43 | 25.4 | 529 | 111* | 1173* |
| 10 000 | 5.0 | 0.42 | 23.9* | 844* | 168* | 1820* |

| | Mean relative liver weight (mg/100 g bw) | |
|---|---|---|
| **Dose (ppm)** | **Males** | **Females** |
| 0 | 4452 | 4308 |
| 1000 | 4870* | 4400 |
| 3000 | 4986* | 4595 |
| 10 000 | 5593* | 5029* |

[a] Based on 25 studies; [b] based on Giknis & Clifford 2008; Source: Kroetlinger & Hastings, 2003

ECOD: 7-Ethoxycoumarin deethylase (major catalysts in rats are cytochrome P450 (CYP450) 1A1, 1B, 2D6 and 3A4);

EROD: 7-Ethoxyresorufin-*O*-deethylase (major catalyst in rats is CYP450 1A1);

ALD: Aldrin epoxidase (major catalyst in rats is CYP450);

EH: Epoxide hydrolase; GST: Glutathione-*S*-transferase; UDP-GT: Uridine diphosphate glucuronyltransferase;

* Statistically significant at $p \leq 0.05$

In a 90-day rat oral toxicity study isotianil was administered ad libitum in the diet to groups of Wistar HsdCpb:WU rats (10 of each sex per group) at concentrations of 0, 100, 500, 2500 or 20 000 ppm (equal to 0, 6.71, 34.18, 166.02 and 1441.42 mg/kg bw per day for males, 0, 7.71, 39.04, 187.14 and 1559.57 mg/kg bw per day for females). Additional groups of 10 rats per sex were treated with 0 or 20 000 ppm for 13 weeks, and thereafter they were observed for four weeks for recovery.

There were no test article-associated adverse effects on clinical signs, mortality, food and water intake, body weight, haematology, coagulation parameters, liver tissue cytochrome p450 levels, ophthalmological findings, or microscopic anatomical pathology findings. Significantly increased serum cholesterol ($p < 0.05$) occurred consistently in males at 2500 ppm and above. The same effect was seen at $p < 0.05$ at all dose levels in females (Table 15). These increases, whilst probably test article-related, did not exceed the historical (normal) range, and were not accompanied by any other evidence of dyslipidaemia or hepatotoxicity. Accordingly, these changes were not regarded as adverse. Induction of phase I and phase II hepatic metabolic enzymes occurred at 2500 ppm and above. The induction pattern was similar in both sexes, but the effects were more pronounced in females. The most marked induction was seen in 7-ethoxyresorufin deethylase (EROD; CYP 1A1). Where enzyme induction was observed it was seen to be an effect that was reversible by the end of the four-week recovery period. Significant increases (by 13% or more relative to control) in relative liver weight ($p < 0.05$) occurred at 2500 ppm and above, but did not correlate with any adverse anatomical pathology findings. Accordingly, whilst the effects on hepatic metabolic enzyme induction and liver weight were considered test article-related, they were not regarded as adverse. The NOAEL was 20 000 ppm (equal to 1441 mg/kg bw per day), the highest dose tested (Kroetlinger & Hartmann, 2004).

***Table 15. Ninety-day rat oral toxicity study with isotianil: serum cholesterol***

| | Mean serum cholesterol (mmol/L) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Males | | | | | Females | | | | |
| **Dietary dose (ppm)** | **0** | **100** | **500** | **2500** | **20 000** | **0** | **100** | **500** | **2500** | **20 000** |
| Day 29/30 | 1.77 | 2.15* | 2.02 | 2.69* | 2.98 | 1.64 | 1.77 | 1.75 | 2.17* | 2.43* |
| Day 92/93 | 2.19 | 2.77 | 2.44 | 2.95* | 3.34* | 1.55 | 1.81* | 1.96* | 2.22* | 2.75* |
| Day 119/120 | 2.59 | NA | NA | NA | 2.61 | 1.90 | NA | NA | NA | 2.18* |
| Historical control (normal) range[a] | 0.85 – 3.83[a] | | | | | 0.44 – 3.70[a] | | | | |

[a] Based on 25 rat studies conducted between 2004 and 2012; Source: Kroetlinger & Hartmann, 2004

* Statistically significant at $p \leq 0.05$ ; NA: Not applicable;

*Table 16. Ninety-day rat oral toxicity study with isotianil: liver enzyme activities*

| | Mean liver enzyme activities | | | | | | |
|---|---|---|---|---|---|---|---|
| **Dose (ppm)** | **P 450 (nmol/g.min)** | **ECOD nmol/g.min** | **EROD nmol/g.min** | **ALD nmol/g.min** | **EH nmol/g.min** | **GST µmol/g.min** | **UGP-GT nmol/g.min** |
| **Males (day 93)** | | | | | | | |
| 0 | 40.2 | 5.1 | 0.31 | 159.0 | 465 | 110 | 925 |
| 100 | 38.3 | 4.3 | 0.32 | 132.8 | 526 | 128 | 860 |
| 500 | 39.6 | 4.8 | 0.42 | 150.0 | 594 | 135 | 1145 |
| 2500 | 41.3 | 4.7 | 0.65* | 134.6 | 625 | 168* | 1206 |
| 20000 | 41.2 | 7.8* | 1.12* | 148.7 | 933* | 176* | 2053* |
| **Males (day 122)** | | | | | | | |
| 0 | 41.7 | 6.8 | 0.32 | 229.9 | 608 | 153 | 1217 |
| 20000 | 36.0* | 4.2* | 0.30 | 155.5* | 493* | 137 | 835* |
| **Females (day 94)** | | | | | | | |
| 0 | 38.2 | 4.2 | 0.28 | 28.4 | 274 | 85 | 813 |
| 100 | 34.9 | 3.4 | 0.25 | 24.9 | 229 | 117* | 686 |
| 500 | 34.8 | 4.8 | 0.39 | 27.7 | 427* | 156* | 1291* |
| 2500 | 36.2 | 4.4 | 0.66* | 21.8* | 600* | 189* | 1508* |
| 20000 | 38.1 | 6.6* | 1.30* | 20.2* | 1089* | 229* | 2375* |
| **Females (day 122)** | | | | | | | |
| 0 | 37.3 | 3.4 | 0.30 | 24.2 | 254 | 101 | 788 |
| 20000 | 33.4* | 3.5 | 0.37 | 25.9 | 300 | 105 | 752 |

Source: Kroetlinger & Hartmann, 2004

ECOD: 7-Ethoxycoumarin deethylase (major catalysts in rats are cytochrome P450 (CYP450) 1A1, 1B, 2D6 and 3A4);

EROD: 7-Ethoxyresorufin-*O*-deethylase (major catalyst in rats is CYP450 1A1);

ALD: Aldrin epoxidase (major catalyst in rats is CYP450);

EH: Epoxide hydrolase; GST: Glutathione-*S*-transferase; UDP-GT: Uridine diphosphate glucuronyltransferase

* Statistically significant at $p \leq 0.05$

In a second 90-day rat oral toxicity study, isotianil was administered ad libitum in the diet to groups of SPF Wistar Hannover (BrlHan:Wist@Jcl(GALAS)) rats (10 of each sex per group) at concentrations of 0, 20, 500, 2500 or 20000 ppm (equal to 0, 1.18, 29.7, 148 and 1238 mg/kg bw per day for males, 0, 1.39, 35.1, 178 and 1398 mg/kg bw per day for females). There were no test article-associated adverse effects on clinical signs, mortality, body weight, food consumption, functional observational battery (FOB) findings, ophthalmology findings, haematology, or clinical pathology. A two-fold increase compared with controls (significant at $p < 0.05$) in serum γ-glutamyl transpeptidase (GGTP) occurred in males at 20000 ppm (Table 17). However, this was not accompanied by any clinical chemistry or anatomic pathology evidence of cholestasis or biliary injury and is consistent with hepatic enzyme induction (Ennulat et al., 2010). Accordingly, whilst this effect was regarded as test article-related, it was not considered adverse. Dose-related, increases in serum cholesterol (by up to 28% compared with controls; significant at $p < 0.05$) occurred in dose groups at 2500 ppm and above. These increases, whilst article related, did not exceed the historical (normal) range, and were not accompanied by any other evidence of dyslipidaemia or hepatotoxicity. Accordingly, these changes were not regarded as adverse. Compared with controls, relative liver weights were increased at 2500 ppm and above, by 11% or more (significant at $p < 0.05$) . Relative kidney weight was increased in the high-dose female group when compared with controls, but there were no anatomical pathology or clinical chemistry correlates. Mucosal epithelial hyperplasia in the limiting ridge of the forestomach was observed in 7/10 males and 5/10 females in the high-dose groups. This gastric finding at 20000 ppm was considered to be of

limited toxicological significance to humans because: (a) humans lack of forestomach; (b) there were no comparable isotianil-associated effects in the canine oesophagus, which is histologically similar to the rat forestomach; (c) this is a high concentration effect with no correlates at lower doses; (d) there were no other microscopic anatomical pathology findings. The NOAEL was 20 000 ppm (equal to 1238 mg/kg bw per day), the highest dose tested (Kuwahara, 2009).

The combined NOAEL for the two 90-day dietary studies in rats was 1441 mg/kg bw per day, the highest dose tested.

***Table 17. Findings in the 90-day rat oral toxicity study with isotianil***

| | Sex and dose (ppm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Males | | | | | Females | | | | |
| **Parameter** | **0** | **20** | **500** | **2500** | **20 000** | **0** | **20** | **500** | **2500** | **20 000** |
| | **Clinical pathology** | | | | | | | | | |
| γ-Glutamyl transpeptidase, GGTP (U/L) | 1 | 0 | 1 | 1 | 2** | 0 | 0 | 1 | 1 | 1** |
| Total cholesterol (mg/dL) | 54 | 64 | 63 | 69** | 84** | 52 | 57 | 57 | 67* | 81** |
| Historical control range[a] | | | 33–103[a] | | | | | 17–88[a] | | |
| | **Organ weights** | | | | | | | | | |
| Relative liver weight (percentage of control) | 100 | 103 | 101 | 111** | 129** | 100 | 102 | 104 | 113** | 126** |
| | **Microscopic anatomical pathology** | | | | | | | | | |
| Gastric limiting ridge epithelial hyperplasia | 0/10 | 0/10 | 0/10 | 0/10 | 7/10** | 0/10 | 0/10 | 0/10 | 0/10 | 5/10* |

[a] Range based on HCD from 25 rat studies conducted between 2004 and 2012; Source: Kuwahara, 2009

Statistically significant at: * $p < 0.05$; ** $p < 0.01$;

In a one-year toxicity study in rats, isotianil was administered ad libitum for 52 weeks in the diet to groups of SPF Wistar Hannover (BrlHan:Wist@Jcl(GALAS)) rats (21 of each sex per group) at concentrations of 0, 60, 600, 6000 or 20 000 ppm (equal to 0, 2.83, 27.9, 291 and 979 mg/kg bw per day for males, 0, 3.70, 37.3, 381 and 1254 mg/kg bw per day for females). There were no test article-associated adverse effects on body weight, food consumption, ophthalmology findings or FOB findings. Significant ($p < 0.05$), but non-adverse, reductions, by 2%–3% in the circulating erythron mass compared with controls, accompanied by significantly increased ( $p < 0.05$ for males) reticulocyte count in the high-dose groups occurred consistently over the course of the study. Significantly increased ($p < 0.05$) serum GGTP activity occurred at 6000 ppm and above in males; above control values by two-fold at 6000 ppm and four-fold at 20 000 ppm. The same increase was seen in females at 20 000 ppm, by two-fold. In both sexes these changes occurred in the absence of any other correlates of cholestasis or hepatotoxicity (Table 18). Significant increases ($p < 0.05$) in serum cholesterol occurred at 600 ppm and above, but in the absence of any other correlates of dyslipidaemia or hepatotoxicity. Acicular urinary crystals occurred at 6000 ppm and above. At 6000 ppm the presence of acicular urinary crystals was not associated with correlates of adverse changes in renal function or structure. However, at 20 000 ppm acicular urinary crystals did correlate with adverse micro-anatomical changes in the kidneys.

Significant increases ($p < 0.05$) in relative liver weight occurred at 600 ppm and above. This correlated with a significantly increased ($p < 0.05$) incidence of diffuse hepatocyte hyperplasia in the male high-dose group. Relative kidney and thyroid weights were also significantly increased ($p < 0.05$) in the male high-dose group. The changes in relative kidney weight correlated with a significantly increased incidence ($p < 0.05$) of renal proximal tubular changes (dilation of the pars recta lumen and renal proximal tubular epithelial basophilic change) in the male high-dose rats. There were no microscopic anatomical abnormalities in the thyroid glands. A significantly increased incidence ($p < 0.05$) of hyperplasia of the gastric limiting ridge epithelia occurred at 20 000 ppm in both sexes and at 6000 ppm in males. This finding is of limited human relevance because humans and dogs lack a forestomach, and because there were no correlating isotianil-associated effects in the canine oesophagus, which is histologically similar to the rat forestomach. A significantly increased incidence ($p < 0.05$) of lung alveolar wall bronchiolization occurred in the male high-dose group. The NOAEL for this study was 6000 ppm (equal to 291 mg/kg bw per day) due to increased incidences of acicular urinary crystals correlating with renal proximal tubule changes, alveolar wall bronchiolization, and diffuse hepatocyte hyperplasia, all at 20 000 ppm (equal to 979 mg/kg bw per day) (Kuwahara, 2007a).

***Table 18. Key findings in the one-year rat oral toxicity study with isotianil***

| | | Sex and dose (ppm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | | Females | | | | |
| **Parameter** | **Week/s** | **0** | **60** | **600** | **6000** | **20 000** | **0** | **60** | **600** | **6000** | **20 000** |
| **Clinical chemistry** | | | | | | | | | | | |
| GGTP (U/L) | 52 | 1 | 1 | 1 | 2* | 4* | 1 | 1 | 1 | 1 | 2* |
| Cholesterol (mg/dL) | 52 | 67 | 82 | 90* | 108* | 103* | 65 | 79 | 98* | 83 | 101* |
| Historical control (normal) range | 47–148 | 39–143 | | | | | | | | | |
| **Urinalysis** | | | | | | | | | | | |
| Acicular urinary crystals[a] | 51 (M) 52 (F) | 0/10 | 0/10 | 0/10 | 2/10 | 7/10 | 0/10 | 0/10 | 0/10 | 1/10 | 3/10 |
| **Organ weights** | | | | | | | | | | | |
| Relative liver weight (%) | 2.15 | 2.17 | 2.38* | 2.35* | 2.68* | 2.03 | 2.13 | 2.31* | 2.41* | 2.56* | |
| Relative kidney weight (%) | 0.50 | 0.51 | 0.53 | 0.54 | 0.56* | 0.57 | 0.58 | 0.59 | 0.60 | 0.63 | |
| Relative thyroid weight % (× 100) | 0.50 | 0.54 | 0.58 | 0.50 | 0.57* | 0.79 | 0.73 | 0.86 | 0.78 | 0.79 | |
| **Microscopic anatomical pathology[a]** | | | | | | | | | | | |
| Lung alveolar wall bronchiolization | 0/21 | 0/21 | 0/21 | 1/21 | 13/21* | 0/21 | 0/21 | 0/21 | 0/21 | 1/21 | |
| Gastric limiting edge mucosal epithelial hyperplasia | 0/21 | 0/21 | 0/21 | 9/21* | 16/21* | 0/21 | 0/21 | 0/21 | 4/21 | 15/21* | |
| Diffuse hepatocyte hyperplasia | 0/21 | 0/21 | 0/21 | 0/21 | 10/21* | 0/21 | – | 0/1 | – | 0/21 | |
| Renal proximal tubule pars recta luminal dilation | 0/21 | 0/21 | 0/21 | 1/21 | 7/21* | 0/21 | – | 0/1 | – | 0/21 | |
| Renal proximal tubular epithelial basophilic change | 2/21 | 3/21 | 7/21 | 6/21 | 12/21* | 4/21 | – | 0/1 | – | 6/21 | |

[a] Incidence, expressed as number affected/number examined; Source: Kuwahara, 2007a

* Statistically significant at $p \leq 0.05$

***Dog***

In a 28-day toxicity study in dogs, isotianil was administered daily in the diet to groups of beagle dogs (two of each sex per group) at concentrations of 0, 1600, 8000 or 40 000/20 000 ppm (equal to 0, 59.2, 204 and 311.5 mg/kg bw per day for males, 0, 47.8, 236.5 and 657.5 mg/kg bw per day for females). The high dose was reduced from 40 000 ppm to 20 000 ppm after study day 15 due to vomiting of food and marked decreases in body weight and food consumption in the high-dose groups. No adverse effects on mortality or ophthalmologic parameters occurred during the study. In the 40 000/20 000 ppm group, vomiting was observed in all dogs and loose or watery stools occurred in one of the two males and in both females. Reduced body weights compared to controls (by 10% or more) occurred in the high-dose group and this correlated with substantial reductions in food consumption in males and females. Transient, mild haematuria occurred at 1600 ppm and above. When compared to controls, increases in serum alkaline phosphatase (ALP) up to about 2.4-fold, GGPT (up to about 2.1-fold) and cholesterol (up to about 1.9-fold), correlated with increased liver weights, liver swelling and hepatocyte centrilobular hypertrophy, all of which were seen at 8000 ppm and above. Non-adverse increases in serum triglycerides (up to about 1.5-fold compared with controls) occurred at 1600 ppm and above. The NOAEL was 1600 ppm (equal to 47.8 mg/kg bw per day) based on serum chemical evidence of bile duct injury at 8000 ppm (equal to 204 mg/kg bw per day) (Enomoto, 2006).

In a 91- to 94-day toxicity study in dogs, isotianil was administered daily via the diet to groups of beagle dogs (four of each sex per group) at concentrations of 0, 500, 2000 or 8000 ppm (equal to 0, 12.2, 51.1 and 200 mg/kg bw per day for males, 0, 13.4, 54.4 and 211 mg/kg bw per day for females). There were no test article-associated adverse effects on clinical signs, body weight, food consumption, ophthalmological findings, haematology, coagulation parameters or urinalysis. Significant increases compared to the controls ($p < 0.05$) in serum alanine transaminase (ALT; six-fold or more), GGTP (three-fold), aspartate transaminase (AST; 1.5-fold or more), and ALP (2.7-fold) occurred in males at 8000 ppm (see Table 19). Females also displayed significantly ($p < 0.05$) increased serum ALP (3.4-fold) at 8000 ppm. This correlated with a greater than 2.3-fold increase in serum GGTP. Changes in serum liver enzymes correlated with significant increases ($p < 0.05$) in relative liver weight and hepatic bile duct proliferation at 8000 ppm. In females at 8000 ppm serum cholesterol was significantly elevated (1.6-fold or more) compared to controls. This correlated with significantly increased ($p < 0.05$; 1.6-fold compared to controls) serum triglyceride at 2000 ppm and above. The changes in serum lipids remained within the historical control (normal) range and so were not considered adverse. The NOAEL was 2000 ppm (equal to 51.1 mg/kg bw per day) based on the presence of hepatotoxicity, manifesting as increased serum hepatic enzymes (ALP, GGTP) indicative of cholestasis, increased serum hepatic enzymes (ALT, AST) indicative of hepatocyte cell membrane leakage, and an increased incidence of hepatic bile duct proliferation in both sexes at 8000 ppm (equal to 200 mg/kg bw per day) (Enomoto, 2009).

***Table 19. Key findings in the 90-day dog dietary toxicity study with isotianil***

| | Dose level (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Parameters** | **0** | **500** | **2000** | **8000** | **0** | **500** | **2000** | **8000** |
| | **Serum chemistry; week 13** | | | | | | | |
| Alanine transaminase (U/L) | 30 | 31 | 40 | 182* | 28 | 29 | 58 | 171 |
| Aspartate transaminase (U/L) | 31 | 33 | 33 | 49* | 29 | 31 | 33 | 38 |
| Alkaline phosphatase (U/L) | 293 | 334 | 351 | 790* | 241 | 369 | 420 | 836* |
| γ-Glutamyl transpeptidase (U/L) | 4 | 4 | 5 | 12* | 3 | 4 | 4 | 7* |
| Total cholesterol (mg/dL) | 166 | 136 | 190 | 195 | 128 | 149 | 154 | 226* |
| Historical control (normal) range | 82–246 | | | | 67–247 | | | |
| Triglycerides (mg/dL) | 22 | 24 | 33* | 30 | 20 | 27 | 32* | 32* |
| Historical control (normal) range | 9–54 | | | | 6–65 | | | |
| | **Gross anatomic pathology** | | | | | | | |
| Liver: relative weight (% relative to control) | 100 | 99 | 120 | 132* | 100 | 94 | 109 | 123* |

| | Dose level (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Parameters** | **0** | **500** | **2000** | **8000** | **0** | **500** | **2000** | **8000** |
| Liver: coarse surface (% affected) | 0 | 0 | 0 | 100* | 0 | 0 | 0 | 75 |
| | **Microscopic anatomical pathology** | | | | | | | |
| Liver centrilobular hepatocyte hypertrophy (% affected) | 0 | 0 | 0 | 100* | 0 | 0 | 0 | 100* |
| Liver bile duct proliferation (% affected) | 0 | 0 | 0 | 100* | 0 | 0 | 0 | 100* |

* Statistically significant at $p \leq 0.05$ Source: Enomoto, 2009

In a one-year dog toxicity study, isotianil was administered in the diet to groups of beagle dogs (four of each sex per group) at concentrations of 0, 200, 1000 or 5000/3000 ppm (equal to 0, 5.22, 27.2 and 107 mg/kg bw per day for males, 0, 5.33 26.9 and 110 mg/kg bw per day for females). The high dose was reduced to 3000 ppm after week 31 (males) or week 30 (females) due to serum chemical evidence of hepatotoxicity and decreased food consumption at week 26. There were no test article-associated adverse effects on mortality, urinalysis or ophthalmological findings. Emaciation (1/4 animals), decreased spontaneous motor activity (2/4 animals), and vomiting (2/4 animals) occurred in the female high-dose group. Skin lesions (interdigital swelling, skin swelling on the head, thorax and hind limbs, periocular swelling, subcutaneous lymphangiectasis with regional lymphadenitis), conjunctivitis and excessive lacrimation occurred in the high-dose animals. Interdigital skin swelling also occurred in 1/4 females at 1000 ppm. Reduced mean body weight gain (by more than10% below control values) occurred in males at all dose levels (Table 20). However, mean body weights in males at 1000 ppm and above were approximately 90% of those of the control group value by week 52 ($p < 0.05$). Mild (nonadverse) macrocytic, hypochromic anaemia occurred in the high-dose groups. This correlated with increases (by 61% compared with controls) in spleen weight, increased incidence of splenomegaly and microscopic anatomic changes in the spleen in these animals. Hepatotoxicity manifesting as adverse and significant increases ($p < 0.05$) in serum ALT, AST, ALP, GGTP and total bilirubin, increased liver weight in males and liver surface changes, hepatitis and hepatic bile duct proliferation occurred at 5000/3000 ppm. Dose-related increases in serum cholesterol occurred in males, but remained within the historical (normal) control range. Accordingly, these changes, whilst test article-related, were not regarded as adverse. A significantly increased in incidence ($p < 0.05$) of renal proximal tubular epithelial cell lipofuscinosis occurred in the high-dose female cohort. The NOAEL in this study was 200 ppm (equal to 5.22 mg/kg bw per day) based on the increased incidence of hepatic bile duct proliferation in males at 1000 ppm (equal to 29.6 mg/kg bw per day). This correlated with anaemia, hepatotoxicity, microscopic anatomic changes in the spleen and renal proximal tubular lipofuscinosis, at the highest dose o5000/3000 ppm (equal to 107 mg/kg bw per day) (Yoshida, 2007). Although the increased incidence of hepatic bile duct proliferation in males at 1000 ppm was not statistically significant, reactions of this type in the hepatic duct are uncommon, spontaneous lesions in experimental beagle dogs (historical control incidence 0%) and its detection in toxicology studies using the dog is most commonly an induced change (Woicke et al., 2021).

***Table 20. Key findings in the one-year dog dietary toxicity study with isotianil***

| **Parameters** | **Dietary dose (ppm)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Males** | | | | **Females** | | | |
| | **0** | **200** | **1000** | **5000/3000** | **0** | **200** | **1000** | **5000/3000** |
| **Body weight** | | | | | | | | |
| Body weight; week 52 (kg) | 12.0 | 11.1 | 10.5 | 10.9 | 10.3 | 11.4 | 11.2 | 10.1 |
| Body weight gain; weeks 0–52 (kg) | 2.5 | 1.6 | 1.0 | 1.4 | 1.6 | 2.7 | 2.5 | 1.2 |
| **Serum chemistry (week 52)** | | | | | | | | |

| Parameters | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | 0 | 200 | 1000 | 5000/3000 | 0 | 200 | 1000 | 5000/3000 |
| ALT (U/L) | 41 | 40 | 79 | 842* | 37 | 31 | 41 | 333 |
| AST (U/L) | 31 | 34 | 34 | 187* | 33 | 28 | 27 | 112 |
| ALP (U/L) | 189 | 260 | 547 | 7419* | 231 | 221 | 283 | 4038 |
| GGTP (U/L) | 4 | 4 | 6 | 21* | 3 | 4 | 4 | 15* |
| Total bilirubin (mg/dL) | 0.10 | 0.10 | 0.12 | 0.17* | 0.09 | 0.09 | 0.10 | 0.17 |
| Cholesterol (mg/dL) | 111 | 132 | 149* | 161 | 126 | 167 | 169 | 156 |
| Historical control (normal) range | 82–184 | | | | 67–247 | | | |
| Triglycerides (mg/dL) | 17 | 21 | 23 | 14 | 35 | 40 | 28 | 27 |
| Historical control (normal) range | 9–54 | | | | 6 – 65 | | | |
| **Anatomic pathology** | | | | | | | | |
| Liver: relative weight (% of control value) | 2.30 | 2.65 | 3.05** | 3.07** | 2.53 | 2.63 | 2.64 | 3.32 |
| Liver: coarse surface[a] | 0/4 | 0/4 | 1/4 | 4/4* | 0/4 | 0/4 | 0/4 | 4/4* |
| Kidney: relative weight (% of control value) | 100 | 110 | 103 | 156 | 100 | 86 | 93 | 141 |
| Coloured kidneys[a] | 0/4 | 0/4 | 0/4 | 1/4 | 0/4 | 0/4 | 0/4 | 1/4 |
| **Microscopic anatomical pathology[a]** | | | | | | | | |
| Hepatic centrilobular hepatocyte hypertrophs | 0/4 | 0/4 | 2/4 | 1/4 | 0/4 | 0/4 | 0/4 | 1/4 |
| Hepatitis | 0/4 | 0/4 | 0/4 | 2/4 | 0/4 | 0/4 | 0/4 | 1/4 |
| Hepatic bile duct proliferation | 0/4 | 0/4 | 2/4 | 4/4* | 0/4 | 0/4 | 0/4 | 4/4* |
| Historical control incidence[b] | 0% | 0% | | | | | | |
| Renal tubular dilation | 0/4 | 0/4 | 0/4 | 1/4 | 0/4 | 0/4 | 0/4 | 2/4 |
| Renal proximal tubular lipofuscinosis | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 1/4 | 4/4* |
| Splenic red pulp reticular cell hyperplasia | 0/4 | 0/4 | 0/4 | 4/4 | 0/4 | 0/4 | 0/4 | 3/4 |
| Splenic red pulp lymphoid cell infiltration | 0/4 | 0/4 | 0/4 | 1/4 | 0/4 | 0/4 | 0/4 | 2/4 |
| Splenic capsular/subcapsular fibrosis | 0/4 | 0/4 | 0/4 | 4/4* | 0/4 | 0/4 | 0/4 | 2/2 |

[a] Incidence, expressed as number affected/number examined; Source: Yoshida, 2007

[b] Based on 12 studies conducted over the previous five years utilising 48 dogs;

* Statistically significant at $p \leq 0.05$

*(b) Dermal application*

In a 28-day repeated dermal exposure toxicity study isotianil was applied topically (semi-occlusive) to Wistar Hanover IGS [CRL:WI(Han)] rats (ten of each sex per dose) for six hours per day, for five consecutive days each week over four weeks, plus the day prior to necropsy during the fifth week; a total of 21 doses. Dose levels were 0, 100, 300 or 1000 mg/kg bw per day. No isotianil-induced adverse effects on clinical signs, body weight, food consumption, ophthalmological observations, haematological parameters, clinical chemistry parameters, organ weights, gross anatomic pathology or microscopic anatomical pathology were noted. The only statistical significant difference from controls was an increase in the absolute (28%) and relative (31%) ovary weights at the high dose, but this in

the absence of any microscopic anatomical correlates (including no effects on estrus cyclicity). The largest ovary weight in the present study (0.197 g for one female in the 1000 mg/kg bw dose group) was within the historical control range. The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Eigenberg, 2009).

*(c) Exposure by inhalation*

No data on this aspect was submitted.

## 2.3 Long-term studies of toxicity and carcinogenicity

*Mouse*

In a combined chronic/carcinogenicity study, isotianil was administered ad libitum for 78 weeks in the diet to groups of SPF ICR mice (Crj:CD-l) at concentrations of 0, 70, 700 or 7000 ppm (equal to 0, 6.89, 71.5 and 706 mg/kg bw per day for males, 0, 6.66, 67.2 and 667 mg/kg bw per day for females). Each dose group consisted of sixty-eight males and sixty-eight females, comprising 56 per sex for the main groups and 12 per sex for the satellite groups (sacrificed after week 52 of exposure). There were no test article-associated adverse effects on mortality, general condition, body weight, food consumption, haematology, or anatomical pathology. Significantly increased (by 30% compared to controls; $p < 0.05$) relative liver weight was detected after 52 weeks of treatment in the male high-dose group. At necropsy, the 7000 ppm group of males from the 78-week exposure cohort had a significantly increased ($p < 0.05$) overall incidence of macroscopic liver masses (mostly due to an increased incidence of this lesion in animals found dead or killed in extremis). A significantly increased incidence ($p < 0.05$) of liver masses in animals found dead or killed in extremis was also observed amongst the 700 ppm group males from the 78-week exposure cohort, although there was no significant increase ($p \geq 0.05$) in the overall incidence for this group. Based on the microscopic anatomical pathology evaluation there were no significant differences ($p < 0.05$) between the control and 7000 ppm animals in the incidence of hepatocellular adenoma and carcinoma or the combined incidences of these neoplastic lesions (the number of animals with hepatocellular adenoma and/or carcinoma). Additionally, there were no significant differences ($p \geq 0.05$) between the control and 7000 ppm group males in incidences of non-neoplastic, proliferative lesions such as eosinophilic, basophilic or clear cell hepatocellular foci. There were no other test article-associated adverse microscopic anatomical pathology findings. The NOAEL for toxicity and carcinogenicity was 7000 ppm (equal to 667 mg/kg bw per day), the highest dose tested (Nakashima, 2007).

***Table 21. Key findings in the mouse carcinogenicity study***

| | | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | Females | | | |
| **Site and lesion** | **Group** | **0** | **70** | **700** | **7000** | **0** | **70** | **700** | **7000** |
| | | **Clinical signs** | | | | | | | |
| Tactile hair loss | 78-week exposure cohort[a] [%] | 0/56 - | 3/56 [5%] | 0/56 - | 1/56 [2%] | 12/56 [21%] | 7/56 [12.5%] | 19/56 [34%] | 22/56* [39%] |
| | Historical control range | 0%–12.5% | | | | 11%–48% | | | |
| | | **Anatomical pathology** | | | | | | | |
| Liver: relative weight (% of control) | 52-week exposure | 100 | 110 | 105 | 130* | 100 | 90 | 98 | 100 |
| | 78-week exposure | 100 | 110 | 109 | 110 | 100 | 99 | 105 | 131 |
| Lung adenoma[a] [%] | All cohorts combined | 6/56 [11%] | 10/35 [29%] | 9/29 [31%] | 10/56 [18%] | 10/56 [18%] | 5/25 [20%] | 10/21 [48%] | 6/56 [11%] |
| Liver hepatocellular adenoma[a] [%] | All cohorts combined | 16/56 [29%] | 13/38 [34%] | 18/37 [49%] | 24/56 [43%] | 1/56 [2%] | 2/25 [8%] | 3/18 [17%] | 4/56 [7%] |

| | | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | Females | | | |
| Site and lesion | Group | 0 | 70 | 700 | 7000 | 0 | 70 | 700 | 7000 |
| Liver hepatocellular carcinoma[a] [%] | All cohorts combined | 1/56 [2%] | 2/38 [5%] | 5/37 [14%] | 1/56 [2%] | 0/56 [0%] | 1/25 [4%] | 0/18 [0%] | 1/56 [2%] |
| Liver hepato-cellular adenoma plus carcinoma[a] [%] | All cohorts combined | 16/56 [29%] | 13/38 [34%] | 19/37 [51%] | 24/56 [43%] | 1/56 [2%] | 3/25 [12%] | 3/18 [17%] | 5/56 [9%] |

[a] Incidence, expressed as number affected/number examined; Source: Nakashima, 2007

* Statistically significant at $p \leq 0.05$

***Rat***

In a carcinogenicity study in rats, isotianil was administered ad libitum for 104 weeks in the diet to groups of SPF Wistar Hannover (BrlHan:Wist@Jcl(GALAS)) rats (51 of each sex per group) at concentrations of 0, 2000, 6000 or 20 000 ppm (equal to 0, 79.2, 242 and 823 mg/kg bw per day for males, 0, 105, 311 and 1052 mg/kg bw per day for females). There were no test article-associated adverse effects on mortality, body weight, food consumption parameters or neoplasia. A significantly increased incidence ($p \geq 0.05$) of soiled fur and opacity of the eye occurred in the male high-dose cohort (Table 22). A significantly decreased incidence ($p \geq 0.05$) of incisor elongation occurred in all isotianil-exposed males, although a dose–response relationship was lacking. Dose-related and significantly decreased ($p < 0.05$) incidence of tail masses occurred in males at 6000 ppm and above, whilst dose-related and significantly decreased incidence ($p < 0.05$) of calluses on the extremities occurred in both sexes at 6000 ppm and above. Significantly decreased ($p < 0.05$) total leukocyte, neutrophil and large unstained cell counts occurred in males, and were likely test article-related, however the extent of change was not regarded as adverse when the absolute cell counts were considered in relation to their relevant normal biological ranges.

By the end of the study the following significant ($p < 0.05$) test article-related adverse effects were noted: an increased incidence of eye opacity in males at 20 000 ppm, an increased incidence of kidneys with coarse surfaces in males at all dose levels (consistent with chronic progressive nephropathy), an increased incidence of pulmonary alveolar wall bronchiolization in males at 6000 ppm and above and in females at 20 000 ppm, an increased incidence of luminal dilation of the pars recta of the renal proximal tubules (consistent with chronic progressive nephropathy) in males at 20 000 ppm, an increased incidence of chronic nephropathy at 2000 ppm and above (a significant increase in severity in females occurred at 2000 ppm and above), an increased incidence of foci of hepatocellular eosinophilic change (potentially preneoplastic change and reversible, but lacked any neoplastic correlates) at 2000 ppm and above in males, and an increased incidence of gastric limiting ridge mucosal epithelial hyperplasia (limited toxicological relevance to humans) at 2000 ppm and above. Chronic progressive nephropathy is a common spontaneous lesion in aged rats and exposure to isotianil probably exacerbated the severity of this disease process. However, chemically induced exacerbation of chronic progressive nephropathy in rats is of limited relevance to humans given there is no equivalent entity in humans that presents with the same combination or pattern of histological features seen with the rat disease (Hard, Johnson & Cohen, 2009; Hard et al. 2013).

The NOAEL for carcinogenesis was 20 000 ppm (equal to 823 mg/kg bw per day), the highest dose tested. The NOAEL for systemic toxicity was 2000 ppm (equal to 79.2 mg/kg bw per day) due to a significantly increased incidence (exceeding concurrent and historical control incidences) of lung alveolar wall bronchiolization in males at 6000 ppm (equal to 242 mg/kg bw per day) (Blanck & Nepelska, 2021; Kuwahara, 2007b, 2014).

In a follow up immunohistochemical study, immunohistochemical identification of hepatic foci positive for glutathione-*S*-transferase placental form (GST-P) was undertaken in all surviving male rats. The expression of GST-P-positive foci with a diameter of at least 0.1 mm was significantly reduced in a dose-related manner at 6000 ppm and above, when compared to controls, and a dose-related decrease ($p < 0.05$) occurred in the 2000 ppm group. This supported the evaluation conclusions

of Blanck & Nepelska, 2021, Kuwahara, 2007b and Kuwahara, 2014, that the increased incidence of foci of hepatocellular eosinophilic change observed in males from the rat carcinogenesis study was not a dose-related, preneoplastic change (Kushida, 2007).

***Table 22. Key findings in the rat carcinogenicity study with isotianil***

| Parameters | Sex and dose group (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | 0 | 2000 | 6000 | 20 000 | 0 | 2000 | 6000 | 20 000 |
| | **Clinical signs; incidence**[a] | | | | | | | |
| Soiled fur | 5/51 | 6/51 | 5/51 | 14/51* | 8/51 | 10/51 | 10/51 | 8/51 |
| Eye opacity | 3/51 | 4/51 | 1/51 | 10/51* | 2/51 | 2/51 | 1/51 | 0/51 |
| Elongation of incisors | 11/51 | 3/51* | 4/51* | 2/51* | 11/51 | 6/51 | 10/51 | 7/51 |
| Callus on extremities | 33/51 | 30/51 | 22/51* | 17/51* | 12/51 | 5/51 | 4/51* | 4/51* |
| Tail masses | 8/51 | 4/51 | 1/51* | 0/51* | 0/51 | 0/51 | 0/51 | 0/51 |
| | **Anatomical pathology; incidence**[a] | | | | | | | |
| Hair loss[b] | 12/43 | 7/45 | 4/44* | 4/42* | 5/42 | 11/42 | 6/38 | 9/42 |
| Callus on extremities[b] | 30/43 | 27/45 | 19/44* | 15/42* | 11/42 | 5/42 | 4/38 | 4/42* |
| Mammary gland hypertrophy[b] | 0/43 | 0/45 | 0/44 | 0/42 | 22/42 | 10/42* | 9/38* | 8/42* |
| Kidneys with coarse surface[b] | 2/51 | 8/51* | 9/51* | 8/51* | 0/51 | 2/51 | 1/51 | 2/51 |
| Soft epididymis[b] | 6/43 | 1/45* | 2/44 | 2/42 | - | - | - | - |
| Liver: relative weight (% control) | 100 | 105 | 123 | 132* | 100 | 111 | 125* | 127* |
| Lung alveolar wall bronchiolization [as %] | 0/51 [0] | 3/51 [5.9] | 12/51* [23.5] | 39/51* [78] | 0/51 [0] | 0/51 [0] | 1/51 [2.0] | 16/51* [31.4] |
| Historical control range | 1%–10% | | | | 2%–3% | | | |
| Gastric mucosal limiting ridge epithelial hyperplasia | 4/51 | 38/51* | 47/51* | 45/50* | 3/51 | 25/51* | 33/51* | 44/51* |
| Diffuse hepatocellular hypertrophy | 1/51 | 2/51 | 8/51* | 10/50* | 0/51 | 0/51 | 0/51 | 9/51* |
| Hepatic bile duct hyperplasia | 25/51 | 13/51* | 4/51* | 4/50* | 33/51 | 10/51* | 5/51* | 2/51* |
| Focal hepatocyte alteration: | | | | | | | | |
| eosinophilic | 3/51 | 18/51* | 17/51* | 13/50* | 4/51 | 6/51 | 5/51 | 8/51 |
| basophilic | 29/51 | 7/51* | 5/81* | 9/50* | 26/51 | 9/51* | 4/51* | 8/51* |
| Luminal dilation of the pars rectal of the renal proximal tubule | 0/51 | 0/51 | 0/51 | 8/50* | 0/51 | 0/51 | 0/51 | 0/51 |
| Chronic nephropathy: all | 36/51 | 37/51 | 46/51* | 43/50 | 14/51 | 26/51* | 37/51* | 42/51* |
| severity + | 21/51 | 22/51 | 22/51 | 16/50 | NA | NA | NA | NA |
| severity ++ | 14/51 | 7/51 | 17/51 | 18/50 | NA | NA | NA | NA |
| severity +++ | 1/51 | 8/51 | 7/51 | 9/50 | NA | NA | NA | NA |
| Renal calculus | 9/51 | 2/51* | 1/51* | 0/50* | 25/51 | 18/51 | 14/51* | 9/51* |
| Renal pyelonephritis | 7/51 | 1/51* | 0/51* | 0/50* | 2/51 | 1/51 | 0/51 | 0/51 |

NA: Severity not assessed; Source: Blanck & Nepelska, 2021; Kuwahara, 2007b, 2014

[a] Incidence, expressed as number affected/number examined;

[b] Terminal kill animals;

* Statistically significant at $p \leq 0.05$

## 2.4 Genotoxicity

Overall, isotianil was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found.

### (a) In vitro studies

Isotianil was tested in an adequate battery of in vitro genotoxicity and mutagenicity tests (see Table 23). There was no indication of any genotoxic response in the presence or absence of metabolic activation. Based on the available data isotianil showed no genotoxic potential in vitro.

### (b) In vivo studies

Isotianil was tested in three in vivo micronucleus studies (see Table 23) all of which were negative. Exposure of the bone marrow to the test article appears likely to have occurred. Based on the available data isotianil showed no genotoxic potential in vivo.

***Table 23. Summary of genotoxicity studies***

| Test type | Test system | Concentration/dose range tested | Result | References |
|---|---|---|---|---|
| | | **In vitro** | | |
| Reverse mutation | *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 Plate incorporation assay and pre-incubation assay | 16–5000 µg/plate in DMSO (± S9) | Negative | Wirnitzer, 2005; Herbold, 2001 |
| Mammalian forward mutation | Chinese hamster V79 cells | 2–128 µg/mL in DMSO (± S9) | Negative | Herbold, 2002a |
| Chromosome aberration | Chinese hamster V79 cells | 7, 14, 28 µg/mL in DMSO (± S9) | Negative | Thum, 2005 |
| Chromosome aberration | Chinese hamster V79 cells | 7, 14, 28 µg/mL in DMSO (± S9) | Negative | Herbold, 2002b |
| Unscheduled DNA synthesis | Male rat (Wistar) primary hepatocytes | 0.5–25 µg/ml in DMSO | Negative | Merker, 2011 |
| Micronucleus formation | Micronucleus test in human lymphocytes | 3.9–600 µg/mL; 2 experiments; (± S9) | Negative | Sokolowski, 2021a |
| | | **In vivo** | | |
| Micronucleus formation | Male NMRI mouse, bone marrow | 1000, 2000, 4000 mg/kg bw; 2 i.p. doses at interval of 24 hours | Negative | Herbold, 2002c |
| Micronucleus formation | Crlj:CDl(ICR) mouse: Toxicity test – male and female; Micronucleus test – male bone marrow | 2000 mg/kg bow: 2 oral doses at interval of 24 hours | Negative | Odawara, 2009 |
| Micronucleus formation | Female NMRI mouse, bone marrow | 2000 mg/kg bw; single oral dose | Negative | Dony, 2014 |

i.p.: Intraperitoneal; S9: Activation using at liver supernatant fraction obtained by centrifuging at 9000 *g*

## 2.5 Reproductive and developmental toxicity

### (a) Multigeneration studies

Isotianil was administered to groups of 24 Wistar Hannover BrlHan:Wist@Jcl(GALAS) rats of each sex (the F0 generation) at dietary concentrations of 0, 50, 1000 or 10 000 ppm (equal to mean isotianil intakes for the F0 males, F1 males, F0 females, and F1 females in that order of: 2.97, 3.42, 4.87 and 5.13 mg/kg bw per day for the 50 ppm group; 59.7, 68.2, 97.6 and 103 mg/kg bw per day for the 1000 ppm group; 590, 695, 976 and 1027 mg/kg bw per day for the 10 000 ppm group). This diet was provided for 10 weeks prior to mating, continuously through the mating (up to three weeks), gestation and lactation periods, for production of the next generation of animals. Animals from the F1 generation (24 of each sex per group) were randomly selected for production of the F2 generation; F1rats received the same dietary concentrations as their parents, starting at weaning, for 10 weeks prior to mating, then continuously through the mating, gestation and lactation periods.

#### *F0 generation*

There were no test article-associated adverse effects on clinical signs, body weight, food consumption, reproductive parameters or macroscopic anatomical pathology. Significant ($p < 0.05$) increases (by up to 123% of controls in males and up to 126% in females) in liver weight parameters occurred at 1000 ppm and above. These changes lacked microscopic anatomic pathology correlates and were not regarded as adverse. Significantly increased ($p < 0.05$) kidney weights occurred in the female high-dose group. These changes lacked microscopic anatomical pathology correlates, and were therefore not regarded as adverse.

#### *F1 generation*

There were no test article-associated adverse effects on clinical signs, food consumption, reproductive parameters, or macroscopic anatomical pathology. Both male and female pup weights were significantly ($p < 0.05$) and consistently reduced at 10 000 ppm compared to controls over lactational days (LDs) 7–21 (see Table 24). Reduced body weights ($p < 0.05$) occurred in males and females dosed at 1000 ppm on LD 21. Nonadverse (greater than 90% of control value), but significantly reduced ($p < 0.05$) body weight in males at 1000 ppm and above were seen during the premating growth and breeding periods (treatment weeks 1–18). Transiently reduced body weight ($p < 0.05$) occurred in the female high-dose group over treatment weeks 2–4. However, consistent, and sustained effects on body weight did not occur at any dose level in females, and the transient changes over treatment weeks 2–4 were not regarded as adverse. Significantly increased ($p < 0.05$) relative liver weights (to 111% of controls) occurred in males dosed at 1000 ppm. Significantly increased ($p < 0.05$) absolute and relative liver weights (up to 124% of controls) occurred in males and females dosed at 10 000 ppm. These changes lacked microscopic anatomical pathology correlates and were not therefore regarded as adverse. Increased relative kidney weight (to 107% of controls) occurred in the male high-dose group, whilst increased relative spleen weight (up to 112% of controls) occurred in the high-dose groups. As with liver weights, there were no microscopic anatomical pathology correlates for either of these effects and they were therefore not regarded as adverse.

#### *F2 generation*

Significant reductions ($p < 0.05$) in body weight occurred during the lactational period at 1000 ppm and above. Dose-related reductions ($p < 0.05$) in thymus weight occurred at 1000 ppm in females, and in both males and females at 10 000 ppm, but lacked microscopic anatomical pathology correlates. Accordingly, the effects were not regarded as being adverse.

No other test article-associated adverse effects were noted.

The parental systemic NOAEL was 10 000 ppm (equal to 590 mg/kg bw per day), the highest dose tested. The reproductive NOAEL was 10 000 ppm (equal to 590 mg/kg bw per day), the highest dose tested. The developmental (offspring) NOAEL was 1000 ppm (equal to 68.2 mg/kg bw per day), based on decreased pup body weight (more than a 5% reduction compared to controls) in both the F1 and F2 generations at 10 000 ppm (equal to 695 mg/kg bw per day) (Aoyama, 2007).

*Table 24. Group mean body weights of F1 and F2 rat pups in two generation isotianil study*

| | | Pup weight (g) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Males; lactation day | | | | | Females; lactation day | | | | |
| Litter | Dietary dose level (ppm) | 0 | 4 | 7 | 14 | 21 | 0 | 4 | 7 | 14 | 21 |
| F1 | 0 | 6.1 | 10.9 | 17.6 | 36.1 | 57.3 | 5.8 | 10.6 | 17.1 | 35.0 | 54.7 |
| | 50 | 6.2 | 11.1 | 18.0 | 36.6 | 57.7 | 5.9 | 10.7 | 17.5 | 35.7 | 55.7 |
| | 1000 | 5.9 | 10.8 | 17.6 | 35.3 | 54.9* | 5.6 | 10.3 | 16.8 | 34.0 | 52.1* |
| | Change compared to controls | | | | | −4.2% | | | | | −4.8% |
| | 10 000 | 5.6* | 10.0* | 16.7 | 33.7* | 52.2* | 5.3* | 9.7* | 16.2 | 32.9* | 50.1* |
| | Change compared to controls | −8.2% | −8.3% | | −6.6% | −8.9% | −8.6% | −8.5% | | −6.0% | −8.4% |
| F2 | 0 | 6.0 | 10.6 | 17.3 | 35.8 | 56.5 | 5.6 | 10.3 | 16.9 | 34.9 | 54.1 |
| | 50 | 5.8 | 10.5 | 17.1 | 35.9 | 56.8 | 5.5 | 10.3 | 16.7 | 35.2 | 54.8 |
| | 1000 | 5.7 | 10.2 | 16.6 | 34.5 | 53.8* | 5.4 | 9.8 | 16.2 | 33.5 | 51.6 |
| | Change compared to controls | | | | | −4.8% | | | | | |
| | 10 000 | 5.6* | | | 34.1* | 52.2* | 5.3* | | | | 49.8* |
| | Change compared to controls | −6.6% | 10.2 | 16.8 | −4.7% | −7.6% | –5.3% | 9.9 | 16.2 | 33.0 | −7.9% |

* Statistically significant at $p \leq 0.05$ Source: Aoyama, 2007

## (b) Developmental toxicity

### *Rat*

In a limit dose prenatal developmental toxicity study, 11 inseminated Wistar rats received 1000 mg/kg bw per day of isotianil by oral gavage from gestation day (GD) 6 to GD 19. Fetuses were delivered by laparohysterectomy on GD 20. There was no control group; rather, all observed findings were compared to historical control data (HCD). One-third of the fetuses were evaluated for skeletal anomalies; the remainder underwent evaluation for visceral malformations. Further, tabulated data were not presented in the study summary. No signs of systemic toxicity were reported in any of the dams, although only seven of eleven dams showed evidence of implantations. Treatment with isotianil did not adversely affect any reproductive parameters, including gestation rate, number of viable fetuses per litter, the male : female ratio of fetuses, and placental weight and appearance. There was, however, a slight reduction in mean fetal body weights compared to the HCD mean fetal weights, however the weights were within the HCD range and so the finding was not considered adverse. There was also a slight increase in post-implantation loss that could not be excluded as possibly treatment-related, however, this result may have been influenced by the small number of litters available for evaluation. Although a few external and visceral anomalies were noted, these were not considered related to treatment. In addition, no treatment-related adverse skeletal findings were observed (Klaus, 2001).

In the pivotal rat prenatal developmental toxicity study, isotianil was administered by oral gavage to Wistar (Crl:WI(HAN)) rats (30 of each sex per dose) on GDs 6–19 at 0, 99.5, 300 or 1180 mg/kg bw per day. The homogeneity and stability of the dosing suspensions were confirmed. There were no test article-associated adverse effects on maternal body weight, food consumption, organ weights, reproductive parameters, number or proportion of live fetuses per litter, median percentage of male fetuses, mean weights of fetuses and placentas, malformations, or variations. The NOAEL for both maternal and developmental toxicity was 1180 mg/kg bw per day, the highest dose tested (Young, 2002).

***Rabbit***

In a limit-dose, prenatal rabbit developmental toxicity study, three mated female Himalayan rabbits each were treated orally by gavage with isotianil from GD 6 to postcoitum day (PCD) 28 at doses of 0, 50, 250 or 1000 mg/kg bw per day. The fetuses were delivered by caesarean section on PCD 29. Marginally increased post-implantation loss occurred in the 1000 mg/kg bw per day group (Langewische, 2002a).

In a prenatal rabbit developmental toxicity study, isotianil was administered orally (gavage) to 25 artificially inseminated Japanese White rabbits (Kbl:JW) per group, at doses of 0, 100, 300 or 1000 mg/kg bw per day from GD 6 to GD 27. No test article-associated adverse effects on mean gravid weights, mean numbers of corpora lutea and implants, pre-implantation losses, numbers of live fetuses, resorptions, fetal deaths, sex ratio and the incidences of malformations and variations were observed. An significant increase ($p < 0.05$) in the incidence of supernumerary ribs (12.8%–29.3% of fetuses) was noted as a skeletal abnormality in all treatment groups. However, the incidence was within the HCD range (maximum 34.8% of fetuses) and there was no significant difference ($p < 0.05$) in the number of litters affected. A significant reduction ($p < 0.05$) in food consumption occurred in the second half of gestation in the high-dose group (Table 25). This correlated with reduced body weights on GD 21, significantly reduced body weight gain ($p < 0.05$) over GDs 6–21, and significant reductions ($p < 0.05$) in fetal and placental weights and hepatomegaly, with an accentuated lobular pattern occurring in these animals. No treatment-related adverse effects on maternal animals were noted in the 100 and 300 mg/kg bw per day groups. The maternal NOAEL was 300 mg/kg bw per day due to reduced maternal body weight gain over gestational days 6 to 21 at 1000 mg/kg bw per day, the highest dose tested. The developmental NOAEL was 300 mg/kg bw per day due to reduced fetal and placental weights at 1000 mg/kg bw per day (Takahashi, 2009).

***Table 25. Key findings in the rabbit prenatal developmental study with isotianil***

| Dose level (mg/kg bw per day) | Maternal food consumption (mean; g per day) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Gestation days | | | | | | | | | |
| | 0–3 | 3–6 | 6–9 | 9–12 | 12–15 | 15–18 | 18–21 | 21–24 | 24–27 | 27–28 |
| 0 | 174 | 180 | 170 | 166 | 151 | 162 | 162 | 140 | 122 | 121 |
| 100 | 176 | 180 | 170 | 163 | 155 | 158 | 160 | 147 | 115 | 117 |
| 300 | 180 | 179 | 172 | 165 | 150 | 155 | 155 | 143 | 124 | 126 |
| 1000 | 167 | 175 | 161 | 145 | 116 | 110* | 116* | 101* | 85* | 74* |

| Dose level (mg/kg bw per day) | Maternal body weight gain (mean; g) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Gestation days | | | | | | | |
| | 6–15 | 6–12 | 6–15 | 6–18 | 6–21 | 6–24 | 6–27 | 6–28 |
| 0 | 0 | 30 | 102 | 123 | 170 | 212 | 250 | 265 |
| 100 | 10 | 48 | 111 | 125 | 166 | 212 | 234 | 242 |
| 300 | −3 | 32 | 90 | 104 | 131 | 181 | 222 | 246 |
| 1000 | 1 | 21 | 65 | 38 | 18* | 135 | 140 | 144 |

| Dose level (mg/kg bw per day) | Fetal parameters (mean) | | | | |
|---|---|---|---|---|---|
| | Number of females with live fetuses | Fetal weight (g) | | Placental weight (mg) | Sex ratio |
| | | Males | Females | | |
| 0 | 21 | 39.3 | 39.2 | 5360 | 0.538 |
| 100 | 23 | 36.0 | 35.8 | 5096 | 0.489 |
| 300 | 25 | 37.3 | 36.1 | 5246 | 0.484 |
| 1000 | 19 | 32.0* | 31.3* | 4727* | 0.560 |

* Statistically significant at $p \leq 0.05$; Source: Takahashi, 2009

## 2.6 Special studies

### (a) Neurotoxicity

No specific data were submitted. Based on the data submitted for other toxicity studies, isotianil is unlikely to be neurotoxic.

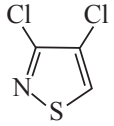
### (b) Immunotoxicity

No specific data were submitted. Based on the data submitted for other toxicity studies, isotianil is unlikely to be immunotoxic.

### (c) Studies on metabolites

***3,4-Dichloroisothiazole (3,4-dichloro-1,2-thiazole, DCIT)***

***Figure 3. Chemical structure of 3,4-dichloroisothiazole (DCIT)***



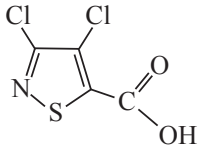

3,4-Dichloroisothiazole (DCIT)

The metabolite DCIT, is a high-temperature hydrolysis product associated with baking, brewing, boiling and sterilization. The formation of DCIT, based on data provided by the sponsor, is primarily of concern in processed citrus juices. Based on the sponsor's submission, the processing of citrus juice is performed by pasteurization which does not create any DCIT residues at all. This approach therefore needs to be seen in the light of a worst-case scenario. Tested in a bacterial reverse mutation assay DCIT was not mutagenic and did not induce micronuclei in an in vitro human lymphocyte micronucleus test (Chang, 2022; Naumann, 2022a). Overall, DCIT is unlikely to be genotoxic.

Based on the limited available data, exposure to DCIT via the diet should be limited to less than, or equal to, the Cramer Class III threshold of toxicological concern (TTC) value of 1.5 µg/kg bw per day.

***3,4-Dichloroisothiazole-5-carboxylic acid (DCIT-acid)***

***Figure 4. Chemical structure of 3,4-dichloroisothiazole-5-carboxylic acid (DCIT-acid)***




3,4-Dichloroisothiazole-5-carboxylic acid (DCIT-acid)

DCIT-acid is a plant, soil, and major rat metabolite found in liver, kidney and plasma. Based on an acute toxic class oral toxicity test in female Crl:CD(SD) rats the acute oral $LD_{50}$ of DCIT-acid is more than 300 mg/kg bw but less than 2000 mg/kg bw (Deguchi, 2006a). DCIT-acid was classified as a skin corrosive based on the results of a validated, in vitro reconstructed human epidermis, skin corrosivity test (Kovács, 2016). It was classified as a severe eye irritant based on the results of a validated in vitro isolated chicken eye corrosivity test (Váliczkó, 2014). DCIT-acid is also a skin sensitizer, with an EC3 of 3%, based on the findings of a local lymph node assay (LLNA) in female CBA/JN mice (Odawara, 2017).

In a 28-day rat oral toxicity study, DCIT-acid was administered ad libitum in the diet to groups of Wistar Rj:WI (IOPS HAN) rats (10 of each sex per group) at concentrations of 0, 600, 1500, 3000 or 6000 ppm (equal to 0, 41.1, 106, 210 and 417 mg/kg bw per day for males, 0, 47, 120, 235 and 486 mg/kg bw per day for females). There were no test article-associated effects on mortality, clinical signs, food consumption, haematology, clinical chemistry, or anatomical pathology. Mean body weight gain was slightly reduced at 3000 and 6000 ppm in males and at 1500 ppm and above in females. This effect was observed with no dose–response relationship and showed large variability between individuals. At the end of the study the mean body weight compared with controls was reduced in males

by 8% and 4% and in females by 6% and 3% at 3000 ppm and 6000 ppm respectively. The NOAEL was 6000 ppm (equal to 417 mg/kg bw per day), the highest dose tested (Blanck, 2017a).

In a 90-day rat oral toxicity study, DCIT-acid was administered ad libitum in the diet to groups of Wistar Rj:WI (IOPS HAN) rats (10 of each sex per group) at concentrations of 0, 600, 2000 or 6500 ppm (equal to 0, 31.6, 105 and 349 mg/kg bw per day for males, 0, 42.0, 133 and 478 mg/kg bw per day for females). There were no test article-associated effects on mortality, clinical signs, food consumption, neurotoxicity evaluations (exploratory locomotor activity, open-field observations, sensory reactivity, and grip strength), ophthalmology, haematology, clinical chemistry, urinalysis, or anatomical pathology findings. Females treated at 6500 ppm showed a reduction in mean body weight gain at the beginning of the treatment period until day 43. Thereafter, the mean body weight gain was comparable to the controls. Overall, at the end of the study, the mean body weight compared with controls was reduced by 5% and the mean body weight gain between days 1 and 92 was reduced by 14% ($p < 0.05$). The NOAEL was 2000 ppm (equal to 133 mg/kg bw per day) based on the reduced body weight gain in females at 6500 ppm (equal to 478 mg/kg bw per day) (Blanck, 2017b).

An appropriately validated bacterial reverse mutation study consisted of a dose range-finding assay (78.1–5000 µg/plate) and a main assay (156–5000 µg/plate), all conducted with and without S9, with triplicate plating, and using *Salmonella typhimurium* (TA100, TA98, TA1535, TA1537) and *Escherichia coli* (WP2 uvrA). Cytotoxicity occurred in strains TA100, TA98, TA1535 and TA1537 at 5000 µg/plate with and without S9 activation. No precipitation of the test article occurred at any concentration. In both the dose range-finding and main assays no genotoxicity was detected (Kitamoto, 2006). DCIT-acid was not genotoxic in an in vitro human lymphocyte micronucleus test (Naumann, 2022b).

In a non-GLP, dose range-finding, prenatal developmental toxicity study, inseminated female Wistar rats (seven per group) received daily doses of 0, 200 or 500 mg/kg bw of DCIT-acid by oral gavage over GDs 6 to 19. There were signs of maternal toxicity from administration of DCIT-acid, including one death and one premature termination of a moribund female in the 500 mg/kg bw per day group. Clinical signs of toxicity were also evident, including discoloured fur at 200 mg/kg bw per day and above, and salivation, reduced water intake, piloerection, light coloured faeces, and reddish vaginal discharge at 500 mg/kg bw per day. Other significant effects observed in the high-dose group included reduced maternal food consumption, body weight gain and final body weight. All surviving dams in the 500 mg/kg bw per day group showed total litter resorption. Necropsy of the high-dose females revealed four incidences of enlarged spleen and single incidences of gaseous intestinal contents, reddish brown spots in the gastric mucosa, tightly filled urinary bladder and light-coloured kidney. At 200 mg/kg bw per day, slightly reduced body weight gain was apparent during treatment, which resulted in a slightly reduced final maternal mean body weight and body weight gain when this latter was corrected. Gestation rate, post-implantation loss, the number of live fetuses per litter, sex ratio, and placental appearance were not significantly affected in the 200 mg/kg bw per day group. However, placental weights and fetal weights were slightly below the HCD range. There were increased incidences of visceral malformations in fetuses at 200 mg/kg bw per day. This included five cases of microphthalmia within two litters, three cases of interatrial septal defects within three litters, and a vascular malformation (aortic spur; not seen in HCD) in another litter. Visceral variations included slight dilation of the renal pelvis and possibly altered position of blood vessels on the aortic arch. Based on the results of this pilot study, it was decided that a second pilot study at 250 mg/kg bw per day was required (Klaus, 2000).

In a second, non-pivotal, non-GLP, dose range-finding prenatal developmental toxicity study, inseminated female Wistar rats (eight per group) received daily doses of 0 or 250 mg/kg bw DCIT-acid by oral gavage over GDs 6–19. No maternal mortalities resulted from the treatment with DCIT-acid. During the last days of treatment clinical signs of maternal toxicity, including discoloured fur, piloerection, salivation, reduced faeces and decreased water consumption, were observed. One female was transiently hypoactive. Maternal body weight and body weight gain were reduced in the treated group. Necropsy revealed only yellowish discoloured contents in the small intestine of one female. The only reproductive effects reported were reduced weights of placenta and reduced fetal body weights. There were no effects observed on gestation rate, appearance of placentas or the fetal sex ratio. External examination of the fetuses revealed no treatment-related findings. Given the effects on maternal toxicity and intrauterine development, a dose of 250 mg/kg bw per day was considered to be suitable for use in the main developmental toxicity study as the high dose (Langewische, 2002b).

In the rat prenatal developmental toxicity study DCIT-acid was administered daily by oral gavage to the inseminated Wistar (Hsd Cpb:WU) rats (25 per group) over GDs 6 to19 at doses of 0, 10, 50 or 250 mg/kg bw per day. Following interim results of incidentally low fertility rates at the high dose, five animals were added at 250 mg/kg bw per day, making a total of 30 dams. There were no test article-associated adverse effects on reproductive parameters (gestation rate, litter size, post-implantation loss, percentage of male fetuses, placental appearance), fetal external or visceral malformations or variations, incidence of total malformations or variations or any gender-related differences. At 250 mg/kg bw per day, two mortalities occurred on GD 19. Both females exhibited significant body weight losses, discoloured fur and piloerection prior to death; one of the females additionally displayed respiratory sounds, reduced faeces, and decreased water consumption. Other dams at 250 mg/kg bw per day exhibited the same clinical signs. The mean maternal body weights of the 50 and 250 mg/kg bw per day dams over GDs 0–20 were 86.2% and 78.4% of the control mean, respectively (both significant at $p < 0.05$; see Table 26). The corrected body weight gains of dams in the 50 and 250 mg/kg bw per day dose groups were 91.6% and 69.9% respectively that of the controls (both significant at $p < 0.05$). Other test article-associated effects at 50 and 250 mg/kg bw per day included altered water intake throughout gestation, and reduced food consumption. Placental weights and fetal body weights were significantly reduced ($p < 0.05$) at 250 mg/kg bw per day. A significantly increased ($p < 0.05$) fetal incidence of retarded ossification (distal phalanges of toes, metacarpals, sternebrae, as well as cervical, sacral, and caudal vertebrae) occurred at 250 mg/kg bw per day. Based on the litter incidence, a significantly increased ($p < 0.05$) incidence compared with controls of incomplete ossification of the fourth sacral vertebral arch occurred at 250 mg/kg bw per day. The maternal NOAEL was 10 mg/kg bw per day based on a 20% or greater depression compared with controls in maternal body weight gain at 50 mg/kg bw per day. The developmental NOAEL was 50 mg/kg bw per day based on decreased fetal body weight at 250 mg/kg bw per day. These effects were accompanied by an increased incidence of delayed ossification which is regarded as a reversible developmental variation that is most likely secondary to the effects on maternal body weight (Langewische, 2004).

***Table 26. Key findings in the rat prenatal developmental study with DCIT-acid***

| | Dose level (mg/kg bw per day) | | | |
|---|---|---|---|---|
| **Gestation day** | **0** | **10** | **50** | **250** |
| | **Maternal mean body weight (g)** | | | |
| 6–19 | 82.9 | 73.7 | 66.2* | 58.5* |
| Percentage of control | 100% | 89% | 80% | 71% |
| 0–20 | 114.7 | 107.6 | 98.9* | 89.9* |
| | **Corrected maternal mean body weight gain (g)** | | | |
| 0–20 | 43.9 | 41.6 | 40.2 | 30.7* |
| | **Maternal mean food consumption (g/rat per day)** | | | |
| 0–3 | 20.02 | 20.11 | 20.75 | 19.33 |
| 3–6 | 21.08 | 21.10 | 20.83 | 21.93 |
| 6–9 | 20.77 | 20.63 | 19.94 | 17.13* |
| 9–12 | 21.77 | 21.13 | 20.68 | 18.28* |
| 12–15 | 22.00 | 21.25 | 20.24* | 19.08* |
| 15–18 | 24.85 | 24.29 | 23.21 | 21.23* |
| 18–20 | 25.25 | 24.75 | 23.60 | 23.23* |
| | **Fetal parameters (mean)** | | | |
| Body weight (g) | 3.59 | 3.58 | 3.57 | 3.26* |
| Percentage of control | 100% | 100% | 99% | 91% |
| | **Placental parameters (mean)** | | | |
| Placental weight (g) | 0.64 | 0.60 | 0.62 | 0.56* |

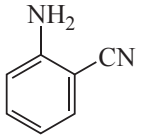
* Statistically significant at $p \leq 0.05$ Source: Langewische, 2004

Overall, DCIT-acid has moderate acute oral toxicity, is a skin and eye corrosive/severe irritant, is a sensitizer but is not genotoxic based on bacterial reverse mutation studies. Based on the available acute oral toxicity data, repeat oral dietary toxicity study data and prenatal developmental toxicity data, that DCIT-acid is more toxic than its parent molecule (isotianil). This is because DCIT-acid is corrosive to the skin, a severe eye-irritant and a skin sensitizer whereas isotianil is not. likely secondary to irritancy and therefore not relevant to dietary risk assessment. Furthermore, DCIT-acid was maternotoxic (NOAEL 10 mg/kg bw per day; manifesting as reduced mean body weight, reduced corrected mean body weight gain, and reduced placental weight) and developmentally toxic (NOAEL 50 mg/kg bw per day) manifesting as decreased fetal body weight and delayed ossification) in rats whereas isotianil was not at doses up to 1180 mg/kg bw per day.

Overall, the key effects in the rodent oral toxicity studies are on body weight parameters. These effects are likely secondary to irritancy and thus not relevant to dietary risk assessment. Given this and the fact that DCIT-acid is a major rat metabolite the health-based guidance values for the parent molecule are adequately protective.

***2-Aminobenzonitrile (anthranilonitrile)***

***Figure 5. Chemical structure of 2-aminobenzonitrile***




2-Aminobenzonitrile (anthranilonitrile)

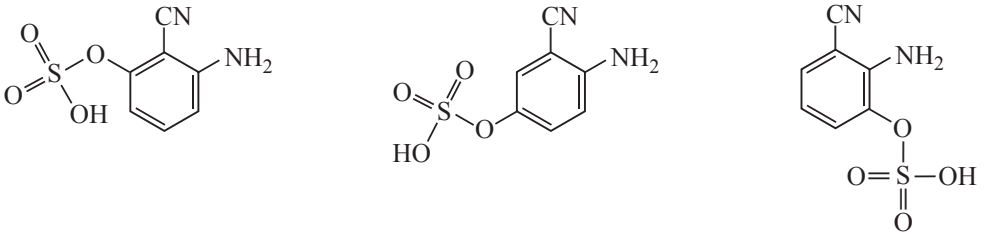
The compound 2-aminobenzonitrile is a plant metabolite of isotianil. An appropriately validated bacterial reverse mutation study was conducted consisting of a dose range-finding assay (78.1–5000 µg/plate) and a main assay (156–5000 µg/plate), both conducted with and without S9 activation and plated in triplicate, using *Salmonella typhimurium* (TA 100, TA98, TA1535, TA1537) and *Escherichia coli* (WP2 uvrA). Cytotoxicity was observed at 5000 µg/plate. Precipitation of the test article did not occur at any concentration tested. In both the dose range-finding and main assays no genotoxicity was detected (Kitamoto, 2006).

Based on an acute toxic class oral toxicity test in female Crl:CD(SD) rats, the acute oral $LD_{50}$ of 2-aminobenzonitrile was more than 300 mg/kg bw but less than 2000 mg/kg bw (Deguchi, 2006b). Tremors, ataxic gait, and prone position were observed at 300 and 2000 mg/kg bw. Lateral position, hypothermia, lacrimation, red tears, ptosis, and a decrease in spontaneous activity were observed in the group receiving 2000 mg/kg bw.

No other data was available on 2-aminobenzonitrile. Based on the limited data available it was concluded that 2-aminobenzonitrile is more acutely toxic than the parent isotianil, but does not induce reverse mutations in bacteria. It was concluded that human exposure to 2-aminobenzonitrile via the diet should be limited to the Cramer Class III TTC value of 1.5 µg/kg bw per day.

***Sulfonic acid conjugates of 2-aminobenzonitrile***

***Figure 6. Sulfonic acid conjugates of 2-aminobenzonitrile***




No data is available for these compounds. Based on quantitative structure–activity relationship (QSAR) analysis, genotoxic potential cannot be categorically excluded. Accordingly human exposure via the diet should be limited to the TTC for genotoxicity of 0.0025 µg/kg bw per day.

### (d) Rat forestomach cell proliferation assays

Studies on cell proliferation in the mucosal epithelium of the rat forestomach were conducted because mucosal epithelial hyperplasia in the limiting ridge of the forestomach was noted in males and females in the rat 90-day toxicity study, the rat one-year study and the rat carcinogenicity study.

*One-week proliferation assay (non-GLP)*

Isotianil was administered in the diet for one week to male Wistar Hannover (BrlHan:WIST@Jcl(GALAS)) rats (20 per dose) at concentrations of 0, 60 or 20 000 ppm (equal to 0, 6.72 and 2358 mg/kg bw per day), and the proliferation stimulating potential in the forestomach was examined using the bromodeoxyuridine (BrdU) labelling index on study day 7. There were no deaths during the study. Reduced body weight gain occurred at 20 000 ppm. There was no increase in the BrdU labelling index in the gastric limiting ridge at 60 ppm. At 20 000 ppm the number of BrdU labelled cells was increased by about 1.3-fold (significant at $p < 0.05$) and the total limiting ridge cells increased about 1.2-fold compared with controls (significant at $p < 0.05$). This correlated with a slightly increased level of keratinization in the limiting ridge of the forestomach in the 20 000 ppm treatment group. The NOAEL for body weight effects and the increased BrdU labelling index on the gastric ridge was 60 ppm (equal to 6.72 mg/kg bw per day) based on the above changes seen at 20 000 ppm (Tamano 2007).

*Ninety-day proliferation assay (non-GLP)*

Microscopic anatomical pathology samples of the forestomach and limiting ridge collected as part of the rat 90-day dietary exposure toxicity study (Kuwahara, 2009) were immunohistochemically stained for Ki-67 (a nonhistone nuclear protein marker of cell proliferation) and proliferating cell nuclear antigen (PCNA). Because the duration of fixation of samples from the 20, 500 and 2500 ppm dose groups was more than ten times longer than for the control (0 ppm, eight samples) and high-dose (20 000 ppm, nine samples) groups and the effect this potentially had on the staining procedure, only samples from the control and high-dose groups were evaluated.

In the 0 ppm group samples, the Ki-67 labelling index for the limiting ridge was higher than in the forestomach, that is, the basal limiting ridge mucosal cell proliferation was relatively high compared with the basal mucosal cell proliferation activity in the forestomach. In samples take from the 20 000 ppm group, the limiting ridge mucosal cell Ki-67 labelling index was up to about 1.6-fold higher ($p < 0.05$) than in controls. In the female 20 000 ppm group the forestomach Ki-67 labelling index was about 1.3-fold higher ($p < 0.05$) than in controls. Histopathologically, the male and female rats in the 20 000 ppm group showed mild hyperplasia at the limiting ridge of the forestomach, however no abnormalities were found (Tamano, 2007).

Overall, the finding of an increased incidence of gastric limiting ridge mucosal epithelial hyperplasia following exposure of rats to high dietary concentrations of isotianil was considered to be of limited toxicological significance to humans because: (a) humans lack a forestomach and limiting ridge; (b) there were no correlating isotianil-associated effects in the canine oesophagus (histologically similar to the rat forestomach); (c) this was a high concentration effect that has no correlates at lower doses; (d) there were no other microscopic anatomical pathology findings.

### (e) Phototoxicity

In a phototoxicity study, isotianil (99.8% purity) was tested in BALB/c 3T3 cells. The treatment regime involved exposing cells treated with eight concentrations of isotianil to artificial sunlight for 50 minutes, with the cytotoxic effect measured in comparison to a solvent control. A duplicate plate of cells that were kept in the dark was used to assess the cytotoxic effect of isotianil in the absence of simulated sunlight exposure. The poor solubility of isotianil in the solvent, dimethyl sulfoxide (DMSO) limited the maximum concentration tested to 31.25 µg/mL. No cytotoxic effects were seen in cells exposed to isotianil, neither in the presence nor absence of artificial sunlight. Since the cell viability after treatment with the non-irradiated test item did not decrease below 50% in either experiment, a half-maximal inhibitory concentration ($IC_{50}$) value could not be determined, therefore neither could the photo-irritation factor (PIF) be calculated. The mean photo effect (MPE) values were determined as 0.030 and –0.037, in

the range finder and main experiment, indicating that the test item had no phototoxic potential. Isotianil is not phototoxic (Sokolowski, 2021b).

## 3. Microbiological aspects

There was no information available in the public domain and no experimental data were submitted that addressed the possible impact of isotianil residues on the human intestinal microbiome.

## 4. Observations in humans

Regular and routine occupational medical surveillance of workers involved in the production of isotianil has not demonstrated any adverse effects on clinical chemistry, lung function testing, electrocardiograms or audiometry. No cases of human poisoning have been reported or published in the peer-reviewed scientific literature.

# Comments

## Biochemical aspects

In rats the radioactivity derived from orally gavaged isotianil was rapidly absorbed, however the time to reach maximum concentration ($T_{max}$) was dose-dependent and increased from 0.3 hours in males and 0.6 hours in females following dosing at 4 mg/kg bw, to 3.3 hours in males and 0.9 hours in females following dosing at 200 mg/kg bw. Over the dose range 4–200 mg/kg bw, values for total plasma area under the concentration–time curve ($AUC_{0\rightarrow\infty}$) and maximum concentration ($C_{max}$) increased with dose, but these increases were less than dose proportional. In this dose range, higher plasma exposure, based on $AUC_{0\rightarrow\infty}$ and $C_{max}$, and urinary pathways in rodent mass balance studies at 4 mg/kg bw, represented 72.5% or more of administered radioactivity (AR). The highest levels of isotianil-derived radioactivity following oral dosing occurred in the stomach, intestine, kidney and liver, reflecting the routes of administration and excretion. Concentrations of isotianil-derived radioactivity in most tissues were maximal after 0.25–0.5 hours, depending on dose. After 168 hours total residues in tissues were below 0.2% of AR and total residues in the carcass were 0.12% of AR or less. The ratio of radioactivity excreted in the faeces compared to that in the urine varied with dose, a larger fraction of the AR being excreted in the faeces at higher dose levels (Gupta, Shah & Savides, 2006). Based on a bile duct cannulation study in rats, biliary excretion was extensive (greater than 46% of AR) with the biliary pathway being the major route of excretion and urinary excretion being a minor pathway after oral dosing at 4 mg/kg bw (Mikata, 2009).

Based on in vitro comparative metabolite studies using pooled, mixed sex, human, rat, dog, and rabbit liver microsomes there appeared to be no human-specific metabolites (Tobias, 2021b).

The following major (greater than 10% of AR and/or greater than 10% of the area under the HPLC curve) in vivo metabolites were identified in rats following oral dosing with 4 or 200 mg/kg bw of isotianil:

- 4′-hydroxy-isotianil (kidney, plasma),
- glucuronide of 4′-hydroxy-isotianil (kidney),
- 4′,5′-dihydroxy-isotianil, plus 3′,4′-dihydroxy-isotianil (kidney and plasma),
- DCIT-acid (liver, kidney and plasma), and
- sulfate of 2-amino-5-hydroxybenzonitrile (liver, kidney, plasma).

Based on samples collected from the rat biliary excretion study three major metabolites were identified in the excreta: DCIT-acid, 4′-hydroxy-isotianil, the glucuronide of 4′-hydroxy-isotianil and trihydroxy-isotianil. Metabolism of isotianil in rats predominantly involves hydroxylation followed by glucuronidation in the case of 4′-hydroxy-isotianil (Gupta, Shah & Savides, 2006; Mikata, 2009).

## Toxicological data

The acute oral median lethal dose ($LD_{50}$) of isotianil in rats was greater than 2000 mg/kg bw (Andrews & Brendler-Schwaab, 2001; Schuengel, 2005). The acute dermal (occlusive) $LD_{50}$ of isotianil in rats was greater than 2000 mg/kg bw (Ose, 2009), and the median lethal concentration for inhalation ($LC_{50}$) was greater than 5 mg/L (Deguchi, 2009). Isotianil was not irritant to the skin or eyes of rabbits (Leuschner, 2002a; Nakamura, 2009a, b). Isotianil was nontoxic but sensitizing in the Guinea pig maximization test (Magnusson & Kligman) (Vohr, 2005).

The repeat daily dose oral (dietary) toxicological properties of isotianil were evaluated in mice, rats and dogs. The key effects were on body weight parameters, the liver, the kidney and the lung.

In a 92-day oral toxicity study, isotianil was administered to groups of mice at concentrations of 0, 150, 1000 or 7000 ppm (equal to 0, 33.1, 204.1 and 1309.5 mg/kg bw per day for males, 0, 54.8, 401.1 and 2474.6 mg/kg bw per day for females). The study NOAEL was 7000 ppm (equal to 1309.5 mg/kg bw per day), the highest dose tested (Leser & Hartmann, 2004).

In a 90-day oral toxicity study isotianil was administered via the diet to groups of rats at concentrations of 0, 100, 500, 2500 or 20 000 ppm (equal to 0, 6.7, 34, 166 and 1441 mg/kg bw per day for males, 0, 7.7, 39, 187 and 1560 mg/kg bw per day for females). Additional groups of ten rats per sex were treated with 0 or 20 000 ppm for 13 weeks. The NOAEL was 20 000 ppm (equal to 1441 mg/kg bw per day), the highest dose tested (Kroetlinger & Hartmann, 2004).

In a second 90-day rat oral toxicity study, isotianil was administered via the diet to groups of rats at concentrations of 0, 20, 500, 2500 or 20 000 ppm (equal to 0, 1.18, 29.7, 148 and 1238 mg/kg bw per day for males, 0, 1.39, 35.1, 178 and 1398 mg/kg bw per day for females). The NOAEL was 20 000 ppm (equal to 1238 mg/kg bw per day), the highest dose tested (Kuwahara, 2009).

The combined NOAEL for the two 90-day dietary studies in rats was 1441 mg/kg bw per day, the highest dose tested.

In a 91 to 94-day toxicity study, isotianil was administered via the diet to groups of dogs at concentrations of 0, 500, 2000 or 8000 ppm (equal to 0, 12.2, 51.1 and 200 mg/kg bw per day for males, 0, 13.4, 54.4 and 211 mg/kg bw per day for females). The NOAEL was 2000 ppm (equal to 51.1 mg/kg bw per day) due to the presence of hepatotoxicity at 8000 ppm (equal to 200 mg/kg bw per day) (Enomoto, 2009).

In a one-year toxicity study, isotianil was administered via the diet to groups of dogs at concentrations of 0, 200, 1000 or 5000/3000 ppm (equal to 0, 5.22, 27.2 and 107 mg/kg bw per day for males, 0, 5.33, 26.9 and 110 mg/kg bw per day for females). The high dose was reduced to 3000 ppm after week 31 (males) and week 30 (females), due to hepatotoxicity and decreased food consumption. The NOAEL was 200 ppm (equal to 5.22 mg/kg bw per day) due to the presence of hepatotoxicity in males at 1000 ppm (equal to 2.72 mg/kg bw per day) (Yoshida, 2007).

In a combined chronic/carcinogenicity study, isotianil was administered for 78-weeks via the diet to groups mice at concentrations of 0, 70, 700 or 7000 ppm (equal to 0, 6.89, 71.5 and 706 mg/kg bw per day for males, 0, 6.66, 67.2 and 667 mg/kg bw per day for females). The NOAEL for systemic toxicity and carcinogenicity was 7000 ppm (equal to 667 mg/kg bw per day), the highest dose tested (Nakashima, 2007).

In a one-year chronic toxicity study isotianil was administered for 52 weeks via the diet to groups of rats at concentrations of 0, 60, 600, 6000 or 20 000 ppm (equal to 0, 2.83, 27.9, 291 and 979 mg/kg bw per day for males, 0, 3.70, 37.3, 381 and 1254 mg/kg bw per day for females). The NOAEL was 6000 ppm (equal to 291 mg/kg bw per day) due to increased incidences of acicular urinary crystals that correlated with renal proximal tubule changes, alveolar wall bronchiolization and diffuse hepatocyte hyperplasia at 20 000 ppm (equal to 979 mg/kg bw per day) (Kuwahara, 2007a).

In a carcinogenicity study isotianil was administered ad libitum for 104 weeks via the diet to groups of rats at concentrations of 0, 2000, 6000 or 20 000 ppm (equal to 0, 79.2, 242 and 823 mg/kg bw per day for males, 0, 105, 311 and 1052 mg/kg bw per day for females). The NOAEL for carcinogenesis was 20 000 ppm (equal to 823 mg/kg bw per day), the highest dose tested. The NOAEL for systemic toxicity was 2000 ppm (equal to 79.2 mg/kg bw per day) due to an increased incidence

of lung alveolar wall bronchiolization in males at 6000 ppm (equal to 242 mg/kg bw per day) (Blanck & Nepelska, 2021; Kuwahara, 2007b, 2014).

The Meeting concluded that isotianil is not carcinogenic in mice or rats.

Isotianil did not induce genotoxicity in an adequate range of in vitro and in vivo assays.

The Meeting concluded that isotianil is unlikely to be genotoxic.

In view of the lack of genotoxicity and the absence of carcinogenicity in mice and rats, the Meeting concluded that isotianil is unlikely to pose a carcinogenic risk to humans.

In a multigenerational study isotianil was administered to groups of rats (the F0 generation) at dietary concentrations of 0, 50, 1000 or 10 000 ppm (equal to mean isotianil intakes for the F0 males, F1 males, F0 females, and F1 females, in that order, of 0 mg/kg bw per day, then: 2.97, 3.42, 4.87 and 5.13 mg/kg bw per day for the 50 ppm group; 59.7, 68.2, 97.6 and 103 mg/kg bw per day for the 1000 ppm group; and 590, 695, 976 and 1027 mg/kg bw per day for the 10 000 ppm group) The parental systemic NOAEL was 10 000 ppm (equal to 590 mg/kg bw per day), the highest dose tested. The reproductive NOAEL was 10 000 ppm (equal to 590 mg/kg bw per day), the highest dose tested. The offspring NOAEL was 1000 ppm (equal to 68.2 mg/kg bw per day) based on decreased pup body weight in both the F1 and F2 generations at 10 000 ppm (equal to 695 mg/kg bw per day) (Aoyama, 2007).

In a prenatal developmental toxicity study, isotianil was administered via oral gavage to rats on gestation days (GDs) 6–19 at 0, 99.5, 300 or 1180 mg/kg bw per day. The NOAEL for both maternal and embryo/fetal toxicity was 1180 mg/kg bw per day, the highest dose tested (Young, 2002).

In a prenatal developmental toxicity study, isotianil was administered orally (gavage) to rabbits at doses of 0, 100, 300 or 1000 mg/kg bw per day from GD 6 to GD 27. The maternal NOAEL was 300 mg/kg bw per day due to reduced maternal body weight gain over GDs 6–21 and reduced placental weights, at 1000 mg/kg bw per day, the highest dose tested. The embryo/fetal NOAEL was 300 mg/kg bw per day due to reduced fetal weights at 1000 mg/kg bw per day (Takahashi, 2009).

The Meeting concluded that isotianil is not teratogenic.

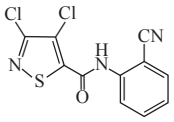
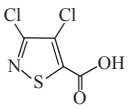
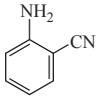
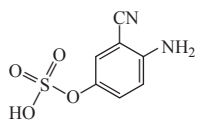
No neurotoxicity studies were submitted for evaluation. Based on the currently available database the meeting concluded that isotianil is unlikely to be neurotoxic.

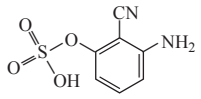
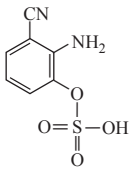
No immunotoxicity studies were submitted for evaluation. Based on the currently available database the meeting concluded that isotianil is unlikely to be immunotoxic.

## Toxicological data on metabolites and/or degradates

A summary of the results for plant and livestock metabolites of isotianil is shown in Table 27, below.

***Table 27. Summary of toxicological characterization of plant/livestock metabolites***

| Compound, codes and structure | Major rat metabolite (> 10% AD) | Genotoxicity assessment (data, QSAR, read across) | General toxicity | Toxicological reference values |
|---|---|---|---|---|
| **Isotianil**<br> | Parent | | | ADI:<br>0–0.05 mg/kg bw<br>ARfD:<br>Unnecessary |
| **DCIT**<br>(3,4-Dichloro-1,2-thiazole; 3,4-dichloro-isothiazole)<br> | No | Negative (bacterial reverse mutation assay) | No data | Cramer Class III<br>TTC:<br>1.5 µg/kg bw per day |
| **DCIT-acid**<br>(3,4-Dichloroisothiazole-5-carboxylic acid)<br> | Yes | Negative (bacterial reverse mutation assay) | Acute oral $LD_{50}$:<br>> 300 < 2000 mg/kg bw<br>Skin corrosive, severe eye irritant, skin sensitizer<br>28-day rat oral toxicity<br>NOAEL 417 mg/kg bw per day (highest dose tested)<br>90-day rat oral toxicity<br>NOAEL 133 mg/kg bw/ day<br>Rat prenatal developmental toxicity:<br>Maternal NOAEL<br>10 mg/kg bw per day;<br>Developmental NOAEL<br>50 mg/kg bw per day | More toxic than parent<br>Key effect in repeat oral studies (rodents) on body weight, likely secondary to irritancy and therefore not relevant to dietary risk assessment<br>Parent ADI is adequately protective |
| **2-Aminobenzonitrile**<br>(anthranilonitrile)<br> | No | Negative (bacterial reverse mutation assay) | Acute rat oral $LD_{50}$<br>> 300 < 2000 mg/kg bw<br>Neurotoxicity (tremor, ataxic gait, and prone position) at 300 mg/kg bw;<br>lateral position, hypothermia, lacrimation, red tears, ptosis, and decrease in spontaneous activity at 2000 mg/kg) | Cramer Class III<br>TTC:<br>1.5 µg/kg bw per day |
| **Sulfonic acid conjugates of 2-aminobenzonitrile** | | | | |
| Not specifically named<br> | No | Based on QSAR genotoxic potential cannot be categorically excluded | No data | TTC for genotoxicity:<br>0.0025 µg/kg bw per day |

| Compound, codes and structure | Major rat metabolite (> 10% AD) | Genotoxicity assessment (data, QSAR, read across) | General toxicity | Toxicological reference values |
|---|---|---|---|---|
| Not specifically named  | No | (As above) | No data | TTC for genotoxicity: 0.0025 µg/kg bw per day |
| Not specifically named  | No | (As above) | No data | TTC for genotoxicity: 0.0025 µg/kg bw per day |

QSAR: Quantitative structure–activity relationship; TTC: Threshold of toxicological concern; AD: Administered dose
ADI: Acceptable daily intake; ARfD: Acute reference dose

## Microbiological aspects

There was no information available in the public domain and no experimental data were submitted that addressed the possible impact of isotianil residues on the human intestinal microbiome.

## Human data

In reports on manufacturing plant personnel, no adverse health effects were noted.

The Meeting concluded that the existing database on isotianil was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

# Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) for isotianil of 0–0.05 mg/kg bw, based on a NOAEL of 5.22 mg/kg bw per day in the one-year oral (dietary exposure) toxicity study in dogs. A safety factor of 100 was used.

The Meeting concluded that it was not necessary to establish an ARfD for isotianil in view of its low acute oral toxicity and the absence of developmental toxicity or any other toxicological effects likely to be elicited by a single dose.

***Levels relevant to risk assessment of isotianil***

| Species | Study | Effect | NOAEL | LOAEL |
|---|---|---|---|---|
| Mouse | 92-day toxicity study [a] | None | 7000 ppm, equal to 1309.5 mg/kg bw per day [c] | - |
| | 78-week study of toxicity and carcinogenicity [a] | Toxicity | 7000 ppm, equal to 667 mg/kg bw per day [c] | - |
| | | Carcinogenicity | 7000 ppm, equal to 667 mg/kg bw per day [c] | - |

| Species | Study | Effect | NOAEL | LOAEL |
|---|---|---|---|---|
| Rat | 90-day toxicity studies [a, d] | No adverse toxic effects observed | 20 000 ppm, equal to 1441 mg/kg bw per day [c, d] | - |
| | One-year toxicity study [a] | Toxicity | 6000 ppm, equal to 291 mg/kg bw per day | 20 000 ppm, equal to 979 mg/kg bw per day |
| | 104-week carcinogenicity study [a] | Toxicity | 2000 ppm, equal to 79.2 mg/kg bw per day | 6000 ppm, equal to 242 mg/kg bw per day |
| | | Carcinogenicity | 20 000 ppm, equal to 823 mg/kg bw per day [c] | - |
| | Two-generation study of reproductive toxicity [a] | Reproductive toxicity | 10 000 ppm equal to 590 mg/kg bw per day [c] | - |
| | | Parental toxicity | 10 000 ppm, equal to 590 mg/kg bw per day [c] | - |
| | | Offspring toxicity | 1000 ppm, equal to 68.2 mg/kg bw per day | 10 000 ppm, equal to 695 mg/kg bw per day |
| | Developmental toxicity study [b] | Maternal toxicity | 1180 mg/kg bw per day [c] | - |
| | | Embryo/fetal toxicity | 1180 mg/kg bw per day [c] | - |
| Rabbit | Developmental toxicity study [b] | Maternal toxicity | 300 mg/kg bw per day | 1000 mg/kg bw per day |
| | | Embryo/fetal toxicity | 300 mg/kg bw per day | 1000 mg/kg bw per day |
| Dog | 91 to 94-day toxicity study [a] | Toxicity | 2000 ppm, equal to 51.1 mg/kg bw per day | 8000 ppm, equal to 200 mg/kg bw per day |
| | One-year toxicity study [a] | Toxicity | 200 ppm, equal to 5.22 mg/kg bw per day | 1000 ppm, equal to 29.6 mg/kg bw/day |
| | One-year toxicity study [a] | Toxicity | 200 ppm, equal to 5.22 mg/kg bw per day | 1000 ppm, equal to 29.6 mg/kg bw/day |
| **3,4-Dichloroisothiazole-5-carboxylic acid (DCIT-acid) [e]** | | | | |
| Rat | 28-day study of toxicity [a] | Toxicity | 6000 ppm, equal to 417 mg/kg bw per day [c] | - |
| | 90-day study of toxicity [a] | Toxicity | 2000 ppm, equal to 133 mg/kg bw per day | 6500 ppm, equal to 478 mg/kg bw per day |
| | Developmental toxicity study [b] | Maternal toxicity | 10 mg/kg bw per day | 50 mg/kg bw per day |
| | | Embryo/fetal toxicity | 50 mg/kg bw per day | 250 mg/kg bw per day |

[a] Dietary administration; [b] Gavage administration; [c] Highest dose tested; [d] Two or more studies combined
[e] Major metabolite in rats

*Acceptable daily intake (ADI) applies to isotianil and DCIT-acid, expressed as isotianil*

*0–0.05 mg/kg bw*

*An acute reference dose (ARfD)*

*Unnecessary*

## Information that would be useful for the continued evaluation of the compound

Investigation of the mode of action of hepatotoxicity in dogs; information on the genotoxicity of the sulfates of 2-aminobenzonitrile.

*Critical end-points for setting guidance values for exposure to isotianil*

| | |
|---|---|
| **Absorption, distribution, excretion and metabolism in mammals** | |
| Rate and extent of oral absorption | Rapid, subproportional to dose, 72.5%–73.4% (estimated) |
| Dermal absorption | No data |
| Distribution | Wide; highest concentrations in stomach, intestine, liver and kidney reflecting the routes of administration and excretion |
| Potential for accumulation | No evidence of accumulation |
| Rate and extent of excretion | Predominantly faeco-biliary with urine as a minor pathway<br>Rapid and essentially complete by 168 hours |
| Metabolism in animals | Predominantly hydroxylation then, in some cases, glucuronidation; the major enzymes involved in metabolism were not identified |
| Toxicologically significant compounds in animals and plants | Isotianil, DCIT, DCIT-acid, 2-aminobenzonitrile (anthranilonitrile) |
| **Acute toxicity** | |
| Rat, $LD_{50}$, oral | > 2000 mg/kg bw |
| Rat, $LD_{50}$, dermal | > 2000 mg/kg bw |
| Rat, $LC_{50}$, inhalation | > 5 mg/L |
| Rabbit, dermal irritation | Not irritating |
| Rabbit, ocular irritation | Not irritating |
| Guinea pig, dermal sensitization | Sensitizing (Magnussen & Kligman) |
| **Short-term studies of toxicity** | |
| Target/critical effect | Liver (dog) |
| Lowest relevant oral NOAEL | 5.22 mg/kg bw per day (dog) |
| Lowest relevant dermal NOAEL | 1000 mg/kg bw per day (rat) |
| Lowest relevant inhalation NOAEC | > 5mg/L |
| **Long-term studies of toxicity and carcinogenicity** | |
| Target/critical effect | Lung alveolar wall bronchiolization in male rats |
| Lowest relevant NOAEL | 79.2 mg/kg bw per day (rat) |
| Carcinogenicity | Not carcinogenic[a] |
| **Genotoxicity** | Unlikely to be genotoxic |
| **Reproductive toxicity** | |
| Target/critical effect | Reductions in preweaning body weight |
| Lowest relevant parental NOAEL | 590 mg/kg bw per day (rat) |
| Lowest relevant offspring NOAEL | 68.2 mg/kg bw per day (rat) |
| Lowest relevant reproductive NOAEL | 590 mg/kg bw per day (rat) |
| **Developmental toxicity** | |
| Target/critical effect | Reduced fetal and placental weights |
| Lowest relevant maternal NOAEL | 300 mg/kg bw per day (rabbit) |
| Lowest relevant embryo/fetal NOAEL | 300 mg/kg bw per day (rabbit) |
| **Neurotoxicity** | |
| Acute neurotoxicity NOAEL | No data |
| Subchronic neurotoxicity NOAEL | No data |
| Developmental neurotoxicity NOAEL | No data |
| **Other toxicological studies** | |
| Immunotoxicity | No evidence from routine studies. |

| | |
|---|---|
| **Studies on toxicologically relevant metabolites** | |
| **DCIT** | Acute oral $LD_{50}$: no data<br>28-day NOAEL: no data<br>Not genotoxic (Ames, in vitro micronucleus test) |
| **DCIT-acid (3,4-dichloroisothiazole-5-carboxylic acid)** | Acute oral $LD_{50}$: > 300 < 2000 mg/kg bw (rat)<br>Skin irritancy: corrosive (in vitro epidermal reconstruct)<br>Eye irritancy: severe (in vitro ICE)<br>Skin sensitizer: EC3 = 3%<br>28-day NOAEL: 417 mg/kg bw per day (rat)<br>90-day NOAEL: 133 mg/kg bw per day (rat)<br>Not genotoxic (Ames, micronucleus in vitro)<br>Prenatal developmental:<br>Maternal NOAEL: 10 mg/kg bw per day<br>Developmental NOAEL: 50 mg/kg bw per day |
| **2-aminobenzonitrile (anthranilonitrile)** | Acute oral $LD_{50}$: > 300 < 2000 mg/kg bw<br>Not genotoxic (bacterial reverse mutation assay) |
| **Microbiological aspects** | No data submitted |
| **Human data** | No clinical cases or poisoning incidents have been recorded<br>No adverse effects in workers involved in the production of isotianil |

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

## Summary

| | Value | Study | Safety factor |
|---|---|---|---|
| ADI | 0–0.05 mg/kg bw [a] | One-year study of toxicity in dogs | 100 |
| ARfD | Unnecessary | | |

[a] Applies to isotianil and DCIT-acid, expressed as isotianil

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All unpublished reports were submitted to WHO by Bayer Research & Development, Crop Science, 40789 Monheim, Germany, who unless otherwise stated, also commissioned the study.


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# Mepiquat chloride

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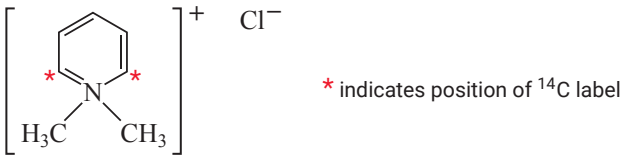
# Explanation

Mepiquat chloride is the International Organization for Standardization (ISO)-approved common name for 1,1-dimethylpiperidinium chloride (IUPAC), Chemical Abstracts Service number 24307-26-4. Mepiquat chloride is a quaternary ammonium compound which acts by inhibition of gibberellin biosynthesis, used as a plant growth regulator to restrict vegetative growth.

Mepiquat chloride has not previously been evaluated by Joint FAO/WHO Meeting on Pesticide Residue (JMPR) and was reviewed by the present Meeting at the request of the Codex Committee on Pesticide Residues (CCPR).

All critical studies contained statements of compliance with good laboratory practice (GLP), unless otherwise indicated. It is noted that due to the hygroscopic nature of the pure substance, a number of the studies were conducted using a test material of low and variable purity, in which the test substance was complexed with water. Limited additional information was identified in the literature that complemented the toxicological information submitted for the current evaluation.

***Figure 1. Chemical structure of [2,6-$^{14}$C]mepiquat chloride***




[2,6-$^{14}$C]mepiquat-chloside

# Evaluation for acceptable daily intake

## 1 Biochemical aspects

The absorption, distribution, metabolism and excretion (ADME) of mepiquat chloride were investigated in rats following the administration of $^{14}$C-labelled mepiquat chloride at different doses (high or low), by oral or intravenous route, after single or multiple dosing, and in the case of the dermal route after one single dose. A study in bile duct-cannulated rats with $^{14}$C-labelled mepiquat chloride was also available. In addition an in vitro comparative metabolism study was provided.

### 1.1 Absorption, distribution and excretion

#### (a) Oral route

Young adult male and female Sprague Dawley (SD) rats were given a single oral gavage dose of 1.2 or 12 mg/kg bw of [2,6-$^{14}$C]mepiquat chloride, or repeated daily oral gavage dosing of unlabelled mepiquat chloride (14 daily doses of 1.2 mg/kg bw) followed by a single oral 1.2 mg/kg bw dose of [2,6-$^{14}$C]mepiquat chloride. At first, dose levels of 12 mg/kg bw and 120 mg/kg bw were used for both single and repeat dose administration, however as there appeared mortality at the high dose, whilst at the 12 mg/kg bw dose level mild toxic effects were noted, the new, lower dose levels were adopted. In another single dosing study the distribution of mepiquat chloride into organs and tissues was also studied. SD rats were dosed with 1.2 or 12 mg/kg bw of [2,6-$^{14}$C]mepiquat chloride for 48 hours by gavage. In a third study, Wistar rats were dosed with 25.6 mg/kg bw for seven consecutive days by gavage with radiolabelled test substance.

Liquid scintillation counting (LSC) was employed to measure radioactivity. For urine, plasma and bile, direct measurements in the scintillation cocktail were made. Faeces were combusted in a sample oxidizer prior to determination of $^{14}$C radioactivity. All other samples were first subjected to tissue solubilization, scintillation cocktail prepared, and radiolabel content determined. Thin-layer chromatography (TLC) was used for chromatographic identification of metabolites.

*Study 1*

The plasma kinetics of [2,6-$^{14}$C]mepiquat chloride was examined. Nine groups of SD rats (five per sex) were administered [2,6-$^{14}$C]mepiquat chloride test substance (radiochemical purity greater than 98%; specific activity 616 MBq/mmol) at nominal dose levels of 1.2 mg/kg bw (low dose) or 12 mg/kg bw (high dose). A summary of the test procedures and groups in the rat metabolism studies is provided in Table 1.

***Table 1. Summary of dosing procedures***

| Group | Purpose | Dose (mg/kg bw) | Duration (hours) |
|---|---|---|---|
| 1 | Balance excretion | 1.2 | 168 |
| 2 | Balance excretion | 12 | 168 |
| 3 | Balance excretion | 12, multiple dosing | 168 |
| 4 | Pulmonary excretion | 12 | 48 |
| 5 | Biliary excretion | 1.2 | 24 |
| 6 | Biliary excretion | 12 | 24 |
| 7 | Pharmacokinetic | 1.2 | 96 |
| 8 | Pharmacokinetic | 12 | 95 |
| 9 | Tissue distribution | 12, multiple dosing | 168 |

Source: Holloway, 1987

Blood samples of 300–500 µL were taken, under anaesthesia, at 1, 2, 4, 8,12 and 24 hours following dosing, and thereafter once daily until termination at 96 hours post dosing. Following this, the sampling times were altered to include, additionally, sampling at 20 and 40 minutes post dosing for the low-dose group. The same dose levels were used for subsequent mass balance, biliary excretion and tissue distribution experiments.

In the single dose mass balance experiments, groups of five rats per sex were treated once with doses of 1.2 or 12 mg/kg bw [2,6-$^{14}$C]mepiquat chloride. Urine and faeces were collected during the acclimatization period in the metabolism cages prior to first dosing. After test substance administration, urine and faeces were collected in the intervals 0–12, 12–24, 24–48, 48–72, 72–96, and 96–168 hours. For mass balance estimates, the cage wash was also checked for radioactivity.

For the determination of exhaled $^{14}$C volatiles two male rats were housed in an airtight metabolism cage. Volatiles containing $^{14}$C, urine, and faeces were collected over a period of 48 hours post dosing. Tissues and organs were sampled as for other comparable treatment groups. It is notable that in the investigations of pulmonary elimination, food was available to rats immediately after dosing in the airtight metabolism cages, whereas with the remaining studies food was withheld for five hours after oral dosing.

In a further experiment, five males and five females received pretreatment with 1.2 mg/kg bw per day of unlabelled mepiquat chloride for 14 days prior to the administration of a single dose of [2,6-$^{14}$C]mepiquat chloride. The same pattern of sample collection was followed as for the single-dose trials described above.

The contribution of biliary excretion to total elimination was investigated in bile duct-cannulated rats. After recovery from surgery, three male and three female rats per group were administered the labelled test article at either 1.2 or 12 mg/kg bw. Bile was collected continuously with sampling intervals of one hour, up to terminal sacrifice at 24 hours after the final dose. Urine and faeces were also collected in the 24 hour period post administration, and the animals were dissected for measurement of residual radioactivity.

Tissue distribution was investigated with [2,6-$^{14}$C]mepiquat chloride after seven consecutive doses of 12 mg/kg bw per day of test article. At each of the time points 8, 24, 48, 96 and 168 hours one animal per sex was killed and samples from a wide range of organs and tissues assayed for radioactivity. The following organs and tissues were collected and weighed: gastrointestinal (GI) tract, bone, brain, fat, uterus, testes, heart, kidneys, liver, lungs, muscle, spleen, skin with fur, adrenals, bladder with contents, thyroids, and residual carcass.

The key toxicokinetic parameters determined are summarized in Table 2. The GI absorption of [2,6-$^{14}$C]mepiquat chloride following gavage administration of the test substance proved to be rapid, maximum blood and plasma profiles of radioactivity occurred within 40–60 minutes of application, with levels higher in females at the low dose and in males at the high dose. Furthermore, the maximum blood and plasma concentrations ($C_{max}$), and the areas under the concentration–time curves (AUCs) in the first 24 hours ($AUC_{t_0-t_{24}}$) for blood and plasma yielded similar values at each dose level, so that it can be deduced that the [2,6-$^{14}$C]mepiquat chloride is practically evenly distributed in the intra- and extracellular compartments of blood.

***Table 2. Toxicokinetic parameters measured in rat blood and plasma after single oral administration of [$^{14}$C]mepiquat chloride (mean values shown)***

| | | Oral dose (mg/kg bw) | $C_{max}$ (µg/g) | $T_{max}$ [minutes] | $AUC_{blood}$ [µg/g.equiv./h] |
|---|---|---|---|---|---|
| Blood | Males | 1.2 | 0.155 | 40 | 2.16 |
| | | 12 | 1.865 | 60 | 4.10 |
| | Females | 1.2 | 0.197 | 40 | 2.59 |
| | | 12 | 1.817 | 60 | 3.79 |
| Plasma | Males | 1.2 | 0.207 | 40 | 2.28 |
| | | 12 | 2.370 | 60 | 4.38 |
| | Females | 1.2 | 0.245 | 40 | 2.80 |
| | | 12 | 2.167 | 60 | 3.97 |

$C_{max}$: Maximum concentration achieved; $T_{max}$ Time to reach $C_{max}$; Source: Holloway, 1987

$AUC_{blood}$: Area under the concentration–time curve for blood

Mepiquat chloride was rapidly and extensively absorbed after an oral dose and eliminated predominantly in urine The mass balance experiments demonstrated the major route of excretion was via urine and represented 74.4%–87.7% of the administered dose (AD) within 168 hours following dosing (see Table 3). Approximately 70%–84% of the administered radiolabel was eliminated in urine within 48 hours of an oral dose of 1.2 or 12 mg/kg bw, and 52%–74% within the first 12 hours post dosing. Faecal elimination was of limited relevance, accounting for 8.2%–15% after an oral dose of 1.2 or 12 mg/kg bw by 168 hours post dosing, and this was mainly the result of the unabsorbed substance (Table 3). Pulmonary elimination was also insignificant and accounted for ca 0.2% of AD.

There were no significant differences between results obtained for male and female rats at the low-dose level. In the high-dose male group, a lower absorption was observed corresponding to the smaller proportion of radioactivity excreted via urine, compared to the same route for females at 12 mg/kg bw, and compared to both sexes at the lower dose of 1.2 mg/kg bw.

***Table 3. Excretion balance and organ/tissue residues at termination in rat experiments with [2,6-$^{14}$C]mepiquat chloride (percentage of administered dose shown)***

| Number of doses | | 1 × | | 1 × | | 14 × | |
|---|---|---|---|---|---|---|---|
| Dose | | 1.2 mg/kg bw | | 12 mg/kg bw | | 1.2 mg/kg bw | |
| Matrix/time period | Sex | Male | Female | Male | Female | Male | Female |
| Urine 0–12 hours | | 62 | 52 | 61 | 66 | 74 | 63 |
| Urine 12–24 hours | | 6.1 | 12 | 7.5 | 8.1 | 5.4 | 8.6 |
| Urine 24–48 hours | | 5.8 | 6.0 | 3.5 | 3.4 | 4.1 | 4.5 |
| Urine 48–72 hours | | 3.5 | 3.8 | 0.9 | 1.7 | 1.9 | 2.0 |
| Urine 72–96 hours | | 1.7 | 1.8 | 0.5 | 0.8 | 1.0 | 1.2 |
| Urine 96–168 hours | | 2.6 | 4.6 | 1.0 | 1.5 | 1.3 | 2.3 |
| Subtotal urine | | 81.7 | 80.2 | 74.4 | 81.5 | 87.7 | 81.6 |

| Number of doses | | 1 × | | 1 × | | 14 × | |
|---|---|---|---|---|---|---|---|
| Dose | | 1.2 mg/kg bw | | 12 mg/kg bw | | 1.2 mg/kg bw | |
| Matrix/time period | Sex | Male | Female | Male | Female | Male | Female |
| Cage wash (%) | | 3.2 | 7.7 | 3.1 | 2.3 | 2.2 | 7.1 |
| Faeces 0–12 hours[a] | | - | - | - | - | - | - |
| Faeces 12–24 hours[a] | | 13 | 7.7 | 14 | 12 | 6.8 | 6.8 |
| Faeces 24–48 hours | | 1.2 | 1.6 | 0.8 | 1.1 | 0.8 | 1.1 |
| Faeces 48–72 hours | | 0.2 | 0.4 | 0.2 | 0.2 | 0.2 | 0.4 |
| Faeces 72–96 hours | | 0.5 | 0.4 | 0.0 | 0.1 | 0.1 | 0.2 |
| Faeces 96–168 hours | | 0.3 | 0.4 | 0.1 | 0.3 | 0.3 | 0.3 |
| Subtotal Faeces | | 15.2 | 10.5 | 15.1 | 13.6 | 8.2 | 8.8 |
| Expired air 0–48 hours | | ND | ND | 0.2 | ND | ND | ND |
| Bile 0–24 hours | | 0.2 | 0.2 | 0.3 | 0.3 | ND | ND |
| Tissues, organs and carcass | | 0.1 | 0.2 | 0.0 | 0.1 | 0.1 | 0.1 |
| Total | | 97.2 | 91.1 | 90.0 | 95.5 | 96 | 90.5 |

[a] First sampling interval 0–24 hours; ND: Not determined; Source: Holloway, 1987

Radiolabel in bile recovered over 24 hours was insignificant (Table 4). The biliary excretion accounted for approximately 0.3% of the administered dose. According to the study report the maximum of the (mean) bile profiles occurred at about 2 hours post-administration. Reduced food consumption was seen in both dose groups in both female and male rats over the 24 hours that animals were in immobilization cages. There were no major differences between males and females or high and low dose in excretion % via urine, faeces and bile.

***Table 4. Excretion in bile duct-cannulated rats following a single dose of [2,6-$^{14}$C]mepiquat chloride, (percentage of administered dose shown)***

| | Dose of [2,6-$^{14}$C]mepiquat chloride | | | |
|---|---|---|---|---|
| | 1.2 mg/kg bw | | 12 mg/kg bw | |
| Matrix/sampling period | Males | Females | Males | Females |
| Urine, total, 0–24 hours | 53 | 44 | 44 | 47 |
| Faeces, total, 0–24 hours | 11 | 14 | 11 | 12 |
| Bile, total, 0–24 hours | 0.3 | 0.3 | 0.2 | 0.2 |
| Tissues, organs and carcass (%) | 14 | 22 | 15 | 9.2 |
| Total | 78.3 | 80.3 | 70.2 | 68.4 |

Source: Holloway, 1987

The quantitative results for residual radioactivities found in organs and tissues is presented in Table 5. Apart from the radiolabel in the GI tract contents (with greater than 15 µg/g mepiquat chloride equivalents) the levels in organs and tissues do not exceed 2 µg/g. Examination of the data for the rats sacrificed at 24 hours after the final dose reveal that, apart from GI tract and bladder, the equivalent concentrations in organs and tissues are below 1 µg/g in all cases. By 96 hours after the last dose of mepiquat chloride the equivalent concentrations of test substance were 0.11 µg/g or less in all organs and tissues. These investigations lead to the conclusion that elimination of radioactivity derived from the radiolabelled mepiquat chloride is rapid, with no noticeable concentration or accumulation in any tissue or organ.

*Table 5. Mean tissue concentration of radioactivity after single oral administration of [2,6-$^{14}$C]mepiquat chloride at a dose level of 12 mg/kg bw (results shown as µg/g equivalents)*

| | Sex/time after dosing (hours) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Males | | | | | Females | | | | |
| **Matrix/organ** | **8** | **24** | **48** | **72** | **96** | **8** | **24** | **48** | **72** | **96** |
| Blood | 0.05 | 0.01 | 0.01 | <LD | <LD | 0.15 | 0.01 | 0.01 | 0.02 | <LD |
| Plasma | 0.05 | 0.01 | <LD | <LD | <LD | 0.19 | 0.01 | <LD | <LD | <LD |
| Gastrointestinal tract | 17.10 | 1.46 | 0.12 | 0.05 | 0.01 | 25.41 | 0.97 | 0.08 | 0.40 | 0.09 |
| Bone | 0.04 | 0.01 | 0.01 | <LD | <LD | 0.10 | 0.01 | <LD | <LD | <LD |
| Brain | 0.02 | 0.01 | 0.01 | 0.01 | 0.01 | 0.04 | 0.01 | 0.01 | 0.01 | 0.01 |
| Fat | 0.20 | 0.04 | 0.04 | 0.02 | 0.02 | 0.38 | 0.05 | 0.02 | 0.02 | 0.02 |
| Uterus | - | - | - | - | - | 0.54 | 0.05 | 0.02 | 0.03 | 0.02 |
| Testes | 0.33 | 0.19 | 0.12 | 0.05 | 0.02 | - | - | - | - | - |
| Heart | 0.44 | 0.10 | 0.04 | 0.02 | 0.09 | 1.44 | 0.08 | 0.02 | 0.03 | 0.01 |
| Kidney | 1.11 | 0.55 | 0.24 | 0.06 | 0.07 | 1.75 | 0.19 | 0.05 | 0.10 | 0.02 |
| Liver | 0.65 | 0.18 | 0.09 | 0.03 | 0.03 | 1.61 | 0.10 | 0.04 | 0.05 | 0.02 |
| Lungs | 0.26 | 0.25 | 0.30 | 0.02 | 0.04 | 1.17 | 0.07 | 0.03 | 0.03 | 0.01 |
| Muscle | 0.78 | 0.56 | 0.33 | 0.15 | 0.05 | 0.76 | 0.53 | 0.22 | 0.10 | 0.03 |
| Spleen | 0.43 | 0.06 | 0.03 | 0.02 | 0.02 | 0.55 | 0.06 | 0.02 | 0.02 | 0.01 |
| Skin | 0.51 | 0.13 | 0.06 | 0.05 | 0.02 | 0.67 | 0.39 | 0.06 | 0.43 | 0.11 |
| Adrenals | 0.94 | 0.14 | 0.06 | 0.03 | 0.02 | 1.75 | 0.12 | 0.04 | 0.06 | 0.02 |
| Bladder | 0.59 | 1.79 | 0.27 | 0.03 | 0.01 | 0.75 | 0.09 | 0.03 | 0.03 | 0.02 |
| Thyroid | 0.91 | 0.27 | 0.12 | 0.03 | 0.04 | 1.85 | 0.27 | 0.10 | 0.08 | 0.02 |
| Carcass | 0.81 | 0.44 | 0.28 | 0.12 | 0.04 | 0.88 | 0.65 | 0.13 | 0.11 | 0.03 |

<LD: Value at or below the detection limit of 0.005 µg/g at 12 mg/kg bw; Source: Holloway, 1987

To conclude, $^{14}$C-labelled mepiquat chloride was rapidly and extensively absorbed, was widely distributed and nearly completely eliminated, mainly via urine. Toxicokinetic parameters were unaffected by exposure at doses up to 12 mg/kg bw, apart from a slightly lower oral absorption at the high-dose level. No significant differences were apparent in the patterns of absorption and elimination of radiolabel on the basis of sex, dosage (1.2 mg/kg bw compared with 12 mg/kg bw) or number of repeat administrations of the compound (Holloway, 1987).

It should be emphasized that the highest dose level used in the acceptable, in vivo, ADME study in rats does not cover the range of dose levels in the short-term, long-term and reproductive toxicity studies. In the residue section, the metabolite methyl piperidine was observed in hen muscle, and the hydroxylated metabolite (4-hydroxy-mepiquat chloride) was observed to be present in goat liver and milk in greater amounts relative to the parent compound and to total radioactivity. During in vivo rat ADME studies these metabolites were not observed.

***Study 2***

The distribution of mepiquat chloride into organs and tissues was studied after a single oral dose to groups of eight male and eight female SPF Sprague Dawley (Charles River) rats (Tables 6 and 7). The rats were administered by gavage [2,6-$^{14}$C]mepiquat chloride at nominal doses of 1.2 or 12 mg/kg bw. One untreated animal of each sex was also investigated. Two animals of each sex per dose group were sacrificed by exsanguination at the following times after dosing: 40 minutes, 6, 24 and 48 hours. Blood and plasma were collected at sacrifice. Treated and control animals were dissected, their tissues, organs and carcass were collected. Pooled liver and pooled kidney from two animals per sex at 40 minutes post administration were used for investigation of the metabolite pattern. No justification for the pooling of these organs was provided. Radioactivity in the organs and tissues was determined by LSC after homogenization.

At 48 hours after dosing, in low-dose animals, the highest mean equivalent concentrations of radiolabel in male animals were found in pituitary gland, urinary bladder, muscle, carcass, testes, and kidneys, and in female animals in the GI tract, muscle, carcass, pituitary gland, urinary bladder, bone, bone marrow, heart, and kidneys. In high-dose animals, the highest mean equivalent concentrations of radiolabel in male animals were found in the urinary bladder, muscle, carcass, pituitary gland, kidneys, testes and adrenals, and in female animals, the highest concentrations were found in the urinary bladder, muscle, carcass, GI tract and pituitary gland.

The measured radioactivity in tissues had almost the same distribution in both sexes, however, females had considerably lower levels in the urinary bladder (4.4 µg/g for low dose and 30.4 µg/g for high dose) compared to males (78.4 µg/g for low dose and 228.2 µg/g for high dose); this may potentially have been due to a larger amount of remaining urine in the male bladders. Conversely, there were higher levels of radioactivity measured in the GI tracts of females (0.0179 µg/g for low dose and 0.0990 µg/g for high dose) compared with the GI tracts of males (0.0027 µg/g for low dose and 0.0217 µg/g for high dose). When expressed as percentage of [2,6-$^{14}$C]mepiquat chloride administered, the largest amounts at 40 minutes following dosing were found in the liver, GI tract and carcass, both at low and high doses, and in males and females. At 48 hours the majority of remaining radioactivity was found in the carcass, for both low and high doses, and in males and females.

***Table 6. Concentrations of radioactivity in rat tissues following a single gavage oral dose of [2,6-$^{14}$C]mepiquat chloride at 1.2 mg/kg bw, (mean equivalent concentrations in µg/g shown)***

| | Sex/time after dosing | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Matrix/organ** | **40 min** | **6 h** | **24 h** | **48 h** | **40 min** | **6 hours** | **24 h** | **48 h** |
| Liver | 3.4664 | 0.1852 | 0.0099 | 0.0032 | 1.5671 | 0.0749 | 0.0053 | 0.0012 |
| Kidney | 5.8138 | 0.3150 | 0.0248 | 0.0044 | 1.8191 | 0.0753 | 0.0070 | 0.0019 |
| Fat | 2.692 | 0.0529 | 0.0019 | 0.0001 | 0.0509 | 0.0046 | ND | ND |
| Spleen | 0.3106 | 0.0684 | 0.0031 | 0.0008 | 0.1255 | 0.0477 | 0.0018 | 0.0005 |
| Heart | 1.6994 | 0.0848 | 0.0044 | 0.0009 | 0.5324 | 0.0497 | 0.0026 | 0.0028 |
| Bone | 0.343 | 0.0353 | 0.0048 | 0.0013 | 0.0610 | 0.0269 | 0.0059 | 0.0031 |
| Bone marrow | 0.4266 | 0.0829 | 0.0064 | 0.0036 | 0.1559 | 0.0566 | 0.0055 | 0.0029 |
| Lung | 1.7188 | 0.1081 | 0.0051 | 0.0009 | 0.9338 | 0.0526 | 0.0029 | 0.0005 |
| Brain | 0.0640 | 0.0027 | ND | ND | 0.0159 | 0.0010 | ND | ND |
| Muscle | 0.1655 | 0.0923 | 0.0364 | 0.0172 | 0.0655 | 0.0630 | 0.0271 | 0.0131 |
| Testes | 0.1621 | 0.0374 | 0.0118 | 0.0095 | - | - | - | - |
| Ovaries | - | - | - | - | 0.4509 | 0.0614 | 0.0034 | 0.0006 |
| Uterus | - | - | - | - | 0.1235 | 0.0636 | 0.0013 | 0.0002 |
| Pituitary gland | 1.5195 | 0.5073 | 0.0586 | 0.0363 | 0.2170 | 0.1318 | 0.0107 | 0.0075 |
| Thyroid | 2.6037 | 0.1910 | 0.0047 | 0.0008 | 0.7530 | 0.1393 | 0.0043 | 0.0006 |
| Adrenals | 1.1816 | 0.4142 | 0.0115 | 0.0017 | 0.6094 | 0.4183 | 0.0062 | 0.0009 |
| Pancreas | 0.4138 | 0.0904 | 0.0032 | 0.0008 | 0.1614 | 0.0597 | 0.0014 | 0.0004 |
| Urinary bladder | 78.3556 | 1.0659 | 0.1021 | 0.0339 | 4.4388 | 1.3640 | 0.0118 | 0.0043 |
| GI tract | 5.6067 | 0.7226 | 0.0865 | 0.0027 | 8.1535 | 3.1199 | 0.0357 | 0.0179 |
| Carcass | 0.6646 | 0.1192 | 0.0325 | 0.0135 | 0.1944 | 0.0810 | 0.0248 | 0.0092 |
| Plasma | 0.3330 | 0.0131 | 0.0006 | 0.0001 | 0.1729 | 0.0068 | 0.0003 | ND |
| Blood | 0.2516 | 0.0135 | 0.0010 | 0.0004 | 0.1206 | 0.0071 | 0.0007 | 0.0004 |

min: Minutes; h: Hours; ND: Not determined; Source: Jonas, 1992a

GI: Gastrointestinal

*Table 7. Concentrations of radioactivity in rat tissues following a single gavage oral dose of [2,6-$^{14}$C]mepiquat chloride at 12 mg/kg bw, (mean equivalent concentrations in µg/g shown)*

| | Sex/time after dosing | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| Matrix/organ | 40 min | 6 h | 24 h | 48 h | 40 min | 6 h | 24 h | 48 h |
| Liver | 21.5520 | 2.4715 | 0.0823 | 0.0379 | 14.2339 | 0.5544 | 0.0510 | 0.0392 |
| Kidney | 26.2244 | 2.4662 | 0.1496 | 0.0938 | 13.8332 | 0.7748 | 0.0664 | 0.0537 |
| Fat | 0.9686 | 0.0775 | 0.0122 | 0.0067 | 0.6876 | 0.0382 | 0.0030 | 0.0045 |
| Spleen | 1.0192 | 0.5940 | 0.0201 | 0.0095 | 1.3582 | 0.4274 | 0.0175 | 0.0107 |
| Heart | 3.0073 | 0.9222 | 0.0329 | 0.0124 | 6.2291 | 0.3758 | 0.0300 | 0.0195 |
| Bone | 0.3337 | 0.1767 | 0.0564 | 0.0152 | 0.4229 | 0.2188 | 0.0271 | 0.0165 |
| Bone marrow | 1.5223 | 0.7642 | 0.0464 | 0.0383 | 5.0215 | 0.4332 | 0.0251 | 0.0217 |
| Lung | 2.9691 | 0.6930 | 0.0323 | 0.0134 | 3.5480 | 0.5868 | 0.0253 | 0.0151 |
| Brain | 0.1114 | 0.0270 | 0.0026 | 0.0019 | 0.1390 | 0.0152 | 0.0023 | 0.0019 |
| Muscle | 0.4880 | 0.6826 | 0.3062 | 0.2209 | 0.4934 | 0.6292 | 0.2155 | 0.2116 |
| Testes | 0.4538 | 0.2598 | 0.1105 | 0.0801 | - | - | - | - |
| Ovaries | - | - | - | - | 4.0492 | 0.3297 | 0.0284 | 0.0173 |
| Uterus | - | - | - | - | 1.9897 | 0.4121 | 0.0180 | 0.0112 |
| Pituitary gland | 5.0711 | 1.7816 | 0.3806 | 0.1004 | 2.7044 | 0.8377 | 0.0979 | 0.0790 |
| Thyroid | 4.5346 | 1.7330 | 0.0457 | 0.0161 | 4.1942 | 0.9691 | 0.0290 | 0.0336 |
| Adrenals | 5.4170 | 7.0314 | 0.1022 | 0.0603 | 4.6953 | 4.0165 | 0.1249 | 0.0414 |
| Pancreas | 1.6008 | 0.6776 | 0.0198 | 0.0091 | 1.6227 | 0.3931 | 0.0142 | 0.0106 |
| Urinary bladder | 228.166 | 58.9698 | 2.6608 | 0.5209 | 30.3894 | 3.6494 | 0.4001 | 0.7211 |
| GI tract | 98.7995 | 18.6270 | 0.2735 | 0.0217 | 100.852 | 11.1002 | 0.5664 | 0.0990 |
| Carcass | 0.8022 | 0.7658 | 0.2723 | 0.1701 | 0.9719 | 0.4621 | 0.1991 | 0.1725 |
| Plasma | 1.7044 | 0.1060 | 0.0119 | 0.0028 | 1.8710 | 0.0506 | 0.0065 | 0.0032 |
| Blood | 1.1649 | 0.1143 | 0.0069 | 0.0030 | 1.3534 | 0.0550 | 0.0053 | 0.0036 |

min: Minutes; h: Hours; GI: Gastrointestinal; Source: Jonas, 1992a

The mean percentage of dose retained in organs and tissues is presented in Table 8. It was mentioned in the study report that the results of the animal sacrificed at 40 minutes post administration reflect a balance, since the losses because of excretion are low at this time point. The percentage retained was at minimum (83% of AD in low-dose females) at 40 minutes after dosing. Only residual tissue concentrations equal to a maximum level of 0.8 µg/g (0.64%–0.93% of AD) were present 48 hours after dosing.

*Table 8. Mepiquat chloride retained in organs and tissues up to 48 hours after oral administration, (mean percentage of dose retained shown)*

| | Dose administered/sex | | | |
|---|---|---|---|---|
| | 1.2 mg/kg bw | | 12 mg/kg bw | |
| Time point | Males | Females | Males | Females |
| 40 minutes | 89% | 83% | 93% | 84% |
| 6 hours | 16% | 31% | 30% | 16% |
| 24 hours | 2.4% | 1.5% | 1.7% | 1.7% |
| 48 hours | 0.64% | 0.56% | 0.78% | 0.93% |

Source: Jonas, 1992a

Based on the concentrations and recovery percentage at 48 hours it was concluded that the test substance is rapidly eliminated and does not accumulate in tissues. At 40 minutes the majority of the dose, as mean equivalent concentration, was found in the urinary bladder, GI tract, liver and kidneys at both doses, in males and females. (Jonas, 1992a, b).

***Study 3***

In a 1977 metabolic fate study (not GLP), five Wistar rats (sex not specified) were administered seven consecutive daily doses of 25.76 mg/kg bw of [2,6-$^{14}$C]mepiquat chloride orally by gavage. Faeces and urine from treated rats were pooled daily. No investigation of toxicity or reports of clinical signs were included in the study. Blood, muscle, kidney, liver, heart, brain, and fat samples were collected four hours after the last dose. Volatiles and expired carbon dioxide were collected. From the data on urine, faeces and expired [$^{14}$C]carbon dioxide the balance of elimination of radiolabel was calculated. Aliquots of liquid samples were either mixed with scintillation cocktail and directly counted, or dried and combusted before assaying for radioactivity. No information was provided on the reasons for treating samples differently. Subsamples of tissues and faeces were combusted, and the resulting [$^{14}$C] carbon dioxide radioactivity counted.

The faeces, urine, and expired carbon dioxide were collected for a material balance determination. Radioassay of these samples showed that approximately 48% of the total daily dose was excreted in urine (Table 9) and approximately 38% excreted in faeces. Pulmonary excretion was insignificant reaching about 0.02% of the total daily dose. Radioassay of homogenized samples provided the distribution of radiolabel in organs and tissues. Total residue in organs and sampled tissues was lower than 0.5 ppm equivalent in the heart, fat, blood and brain. In liver and muscle, the content was lower than 1.3 ppm equivalent four hours after the last of seven administrations. The highest concentration of radiolabel was found in kidney. Comparative TLC was used to characterize the extractable residue of the kidney, liver, muscle, heart, and fat, as well as the urine and faeces samples Approximately 86% of the administered daily doses were excreted. The extracts of the tissues, urine, and faeces samples contained only parent [2,6-$^{14}$C]mepiquat chloride which was not metabolized by Wistar rats (Clark & Kupelian, 1977).

***Table 9. Summary of the distribution of radiolabel in excreta and in organs and tissues of rats administered seven daily doses of 25.76 mg/kg bw [2,6-$^{14}$C]mepiquat chloride***

| Sample | Mepiquat chloride equivalents (ppm) | Percentage of daily dose |
|---|---|---|
| Kidney [a] | 1.750 | 0.06 |
| Liver [a] | 1.269 | 0.23 |
| Muscle [a] | 1.083 | 1.68 |
| Heart [a] | 0.444 | 0.01 |
| Fat [a] | 0.259 | 0.05 |
| Blood [a] | 0.162 | 0.04 |
| Brain [a] | 0.079 | - |
| Urine;<br>24 hours sampling interval [b] | - | 47.97<br>(average of daily dose) |
| Faeces;<br>(24 hours sampling interval [b] | - | 37.62<br>(average of daily dose) |
| Expired carbon dioxide;<br>total duration [c] | - | 0.02<br>(average of daily dose) |

[a] Collected 4 hours after the last dose; Source: Clark & Kupelian, 1977

[b] Urine and faeces were pooled daily;

[c] Starting with the first administration until terminal sacrifice, i.e., 4 hours after the last administration.

### (b) Intravenous route

This study was a subset of Study 1, discussed above. In the single dose, mass balance experiments, groups of five SD rats per sex were treated once with doses of 1.2 or 12 mg/kg bw [2,6-$^{14}$C]mepiquat chloride by the intravenous route. The elimination profile agreed well with the information obtained in the first study with [2,6-$^{14}$C]mepiquat chloride which followed the oral route, and 73%–84% of the intravenous dose was eliminated in urine within 12 hours of dosing. The major route of elimination was in urine, with 88.6%–94.2% of the radiolabel excreted in urine after either intravenous dosage (Table 10) (Holloway, 1987).

***Table 10. Excretion balance and organ/tissue residues at termination following intravenous administration in rat study with [2,6-$^{14}$C]mepiquat chloride, (percentage of administered radioactivity)***

| | | Dose of [2,6-$^{14}$C]mepiquat chloride | | | |
|---|---|---|---|---|---|
| | | 1.2 mg/kg bw | | 12 mg/kg bw | |
| **Matrix** | **Sample period** | **Males** | **Females** | **Males** | **Females** |
| **Urine** | 0–12 hours | 82 | 73 | 84 | 73 |
| | 12–24 hours | 5.7 | 5.6 | 4 | 4.8 |
| | 24–48 hours | 3.2 | 5.1 | 2.7 | 4.2 |
| | 48–72 hours | 1.5 | 2.1 | 1.3 | 2.8 |
| | 72–96 hours | 0.6 | 2.2 | 0.7 | 1.6 |
| | 96–168 hours | 1.3 | 2.5 | 1.5 | 2.2 |
| | Subtotal | 94.3 | 90.5 | 94.2 | 88.6 |
| **Faeces** | 0–12 hours | | | 0.3 | 0.2 |
| | 12–24 hours | 1.1[+] | 1.1[a] | 0.8 | 2.6 |
| | 24–48 hours | 1.0 | 2.0 | 0.3 | 1.2 |
| | 48–72 hours | 0.1 | 0.3 | 0.1 | 0.8 |
| | 72–96 hours | 0.1 | 0.4 | 0.1 | 0.5 |
| | 96–168 hours | 0.1 | 0.5 | 0.3 | 0.6 |
| | Subtotal | 2.4 | 4.3 | 1.9 | 5.9 |
| **Bile** | | ND | ND | ND | ND |
| Cage wash | | 2.1 | 3.3 | 4.3 | 6.5 |
| Expired air | All 0–24 hours | ND | ND | ND | ND |
| Tissues, organs and carcass | | 0.1 | 0.2 | 0.1 | 0.3 |
| **Total recovery** | | 98.9 | 98.3 | 100.5 | 101.3 |

[a] First sampling interval 0–24 hours; ND: Not determined; Source: Holloway, 1987

### (c) Dermal route

In an in vivo dermal absorption study in Han Wistar rats, a nominal dose of 0.038 mg/cm$^2$ (low dose) or 3.0 mg/cm$^2$ (high dose) corresponding to a 30% aqueous solution of [$^{14}$C]-mepiquat chloride or the 1/79 aqueous dilution of the 30% aqueous solution, were employed. These dilutions were applied under a semi-occlusive dressing to the clipped dorsal skin of eight male rats per dose (weight 291–321 g) for 10 hours. Solutions were prepared from a test formulation containing a nominal 60% mepiquat chloride concentration in tap water. After a 10-hour exposure period, four rats per dose were killed at 10 or 96 hours after the start of exposure. The amounts of radiolabel were determined in urine, faeces, blood cells, plasma, kidneys, liver, carcass, cage wash, treated skin site and area of skin surrounding the treated skin site. The skin washes and the protective cover were also checked for radioactivity.

Following single dermal administration there was very limited absorption through the skin, with more than 98% recovered from the skin wash at 10 hours at the high dose level, and 0.54% recovered from a second skin wash after 96 hours. At the low dose, recovery from the skin wash was 91.11% at 10 hours, and an additional 0.72% after 96 hours. Mean recoveries for all dose groups ranged from

97.9%–101.5% of the administered radiolabel, with extensive recovery from the protective cover, the application site, the surrounding skin and skin wash. Radioactivity absorbed was eliminated almost exclusively in urine. When tissue concentration of radioactivity is expressed as µg equiv./g, the highest amount of radiolabel was found in blood cells, except for the high dose group, after 10 hours when the highest concentration was found in the kidneys.

The findings do not suggest a substantial difference in the extent of absorption between the concentrated formulation and what represents an in-use dilution. At both dose levels, the main excretion route is via urine and the level in investigated tissues and organs is low at 96 hours (Beimborn & Leibold, 2003).

## 1.2 Biotransformation

### (a) In vivo

The metabolism of mepiquat chloride in the rat was investigated based on samples obtained in the three studies aforementioned. The parent compound and its metabolites were identified by comparison with reference standards and the use of TLC analysis. Matrices examined in these studies included urine, faeces, plasma, tissue samples and bile of animals from different study groups that had received either 1.2, 12 or 25.76 mg/kg bw.

The extracts of the tissues, urine, and faeces samples were found to contain only parent [2,6-$^{14}$C]mepiquat chloride (Clark & Kupelian, 1977).

No metabolites of mepiquat chloride were found in urine, faeces or tissue samples, and there was no evidence of biotransformation in urine, faeces or bile samples. No radioactivity was detected which could correspond to potential metabolites such as 1-methylpiperidine or piperidine, which were used as reference compounds (Holloway, 1987).

The metabolic fate of mepiquat chloride was evaluated by extracting the radioactivity with methanol from kidneys and livers of animals sacrificed 40 minutes after administration of the test substance and no metabolites could be found using four TLC systems (Jonas, 1992a, b)

### (b) In vitro

In an in vitro comparative metabolism study, final concentrations of 10 and 100 µM of [$^{14}$C]-mepiquat chloride were incubated for three hours with mouse, rat, dog and human cryopreserved hepatocytes prepared as a pool of male and female donors. Metabolite profiling was determined using a high-performance liquid chromatography (HPLC) method. Samples from all species were analyzed by liquid chromatography–mass spectrometry (LC-MS), using [$^{14}$C]-mepiquat chloride in methanol and the available hydroxylated metabolite of mepiquat chloride as reference compounds. Molecular masses of radiolabelled metabolites were investigated and correlated with peak identities in the radiochromatographic profiles. As a reference compound [$^{14}$C]-labelled 7-ethoxycoumarin was used to demonstrate metabolic activity of the different hepatocyte batches, and this gave the expected results.

The mepiquat chloride retention time was between 16.2 and 16.4 minutes in all species. At the dose level of 100 µM, in addition to mepiquat chloride, another radioactive component (not the parent [$^{14}$C]-mepiquat chloride) was observed below the level of quantification with rat hepatocytes at 4.8 minutes, and with dog hepatocytes at 4.9 minutes. This compound accounted for 0.7% of sample radioactivity. The component corresponded chromatographically to 4-hydroxy-mepiquat, although mass spectroscopic analysis failed to conclusively confirm the presence of this trace component. No other metabolism of [2,6-$^{14}$C]mepiquat chloride was seen in any species under the experimental conditions used. It was considered that all of the animal species gave results comparable results to those obtained with human hepatocytes and no metabolites unique to the human hepatocyte profiles were detected (Dawkins & Harrison, 2015).

## 1.3 Effects on enzymes and other biochemical parameters

No information was submitted on this topic.

## 2 Toxicological studies

All doses expressed refer to the active ingredient alone, unless otherwise specified.

### 2.1 Acute toxicity

The results of acute oral, dermal and inhalation toxicity studies with mepiquat chloride, along with the results of dermal and eye irritation, and skin sensitization studies are summarized in Table 11.

***Table 11. Acute toxicity of mepiquat chloride***

| Species | Strain | Sex | Route | Purity | Result | Reference |
|---|---|---|---|---|---|---|
| Mouse | NMRI | Male & Female | Oral | 57.9% | $LD_{50}$ : 450 mg ai/kg bw | Hildebrand & Kirsch, (1989a) |
| Rat | Wistar | Male & Female | Oral | 57.9% | $LD_{50}$: 270 mg ai/kg bw | Hildebrand & Kirsch, (1989b) |
| Rat | Wistar | Male & Female | Dermal | 57.9% | $LD_{50}$: > 2000 mg/kg bw | Hildebrand & Kirsch, (1989c) |
| Rat | Wistar | Male & Female | Inhalation | 57.9% | $LC_{50}$: > 4.89 mg/L | Gamer & Kirsch, (1991) |
| Rabbit | New Zealand White | Male & Female | Skin irritation | 94.0% | Not irritating | Leuschner, (1976) |
| Rabbit | Albino | Male & Female | Eye irritation | 94.0% | Not irritating | Leuschner, (1977) |
| Guinea pig | Pirbright White | Male | Skin sensitization | 99.8% | Not sensitizing | Grundler, (1979) |
| Mouse | CBA/CaO1aHsd | Female | Skin sensitization (LLNA) | 98.1% | Not sensitizing | Dony, (2019) |

$LD_{50}$: Median lethal dose; $LC_{50}$: Median lethal concentration; ai: Active ingredient

#### (a) Lethal doses

In an acute oral GLP study, groups of five NMRI mice of each sex were administered by gavage a single oral dose of mepiquat chloride (purity 57.9%, Batch no. WW 262/CP1490) in distilled water at dose levels of 0, 100, 200, 464, 1470, 2150 mg/kg bw (equivalent to 0, 57.9, 116, 268, 851 or 1250 mg/kg bw of active ingredient). No deaths or abnormal clinical signs were seen at 100 or 200 mg/kg bw. At 464 mg/kg bw and above, clinical signs noted included dyspnoea, apathy and staggering. Mortality was seen in, 2/5, 5/5 and 4/5 males, and 1/5, 3/5 and 4/5 females at 464, 1470 and 2150 mg/kg bw respectively. Under the conditions of the study, the acute oral $LD_{50}$ of the test substance was 780 mg/kg bw for both sexes (equivalent to 450 mg/kg bw) (Kirsch & Hildebrand, 1989a).

In an acute oral GLP study, groups of Wistar rats of each sex were administered by gavage a single oral dose of mepiquat chloride (purity 57.9%, Batch no. WW 262/CP1490) in distilled water at dose levels of 0, 100, 200, 464, 1470, 2150 mg/kg bw. No deaths or abnormal clinical signs were seen at 100 or 200 mg/kg bw. At 464 mg/kg bw and above clinical signs noted included dyspnoea, apathy and staggering. In males 2/5, 5/5 and 5/5 rats died at 464, 1470 and 2150 mg/kg bw respectively, whilst the corresponding deaths in females were 3/5, 5/5 and 5/5. Under the conditions of the study, the acute oral $LD_{50}$ of the test substance was ca 464 mg/kg bw for both sexes (equivalent to ca 270 mg kg bw of active ingredient (Kirsch & Hildebrand, 1989b).

In an acute dermal GLP study, mepiquat chloride (purity 57.9%, Batch no. WW 262/CP1490) was applied as an aqueous solution at 2000 mg/kg bw to groups of five Wistar rats of each sex. Application was to clipped skin of the dorsal and dorso/lateral trunk, and was covered with a semi-occlusive dressing for 24 hours, after which the application site was rinsed with warm water. There were no deaths and no abnormal clinical signs. The acute dermal $LD_{50}$ of the test substance in rats of both sexes was greater than 2000 mg/kg bw (equivalent to 1160 mg kg bw of active ingredient (Kirsch & Hildebrand, 1989c).

In an acute, nose-only, inhalation toxicity study, groups of five Wistar rats of each sex were exposed for four hours to an aerosol of mepiquat chloride (purity 57.9%; Batch no. WW 262/CP1490) diluted 50% (w/w) in distilled water, at mean analytical concentrations of 2.59 or 4.89 mg/L. The mass median aerodynamic diameters (MMADs) were 2.9 and 2.7 µm respectively. No deaths were observed at 2.59 mg/L. At 4.89 mg/L, 1/5 males and 2/5 females died on the day of exposure. During exposure, signs included irregular, accelerated or intermittent respiration, which continued only during test day 1 at 2.59 mg/L, but until test day 5 at 4.89 mg/L. Under the conditions of the study, the $LC_{50}$ was greater than 4.89 mg/L (Gamer & Kirsch, 1991).

**(b) Dermal irritation**

In a 1976 skin irritation study (not GLP-compliant), 0.5 g of mepiquat chloride (purity 94%; Reg. No. 85 559; LPT-batch) was tested undiluted and diluted (50% dilution, vehicle not reported). These test items were applied under an occlusive dressing to ca 6 $cm^2$ of intact and abraded clipped skin of three albino New Zealand White rabbits of each sex. No signs of mortality or toxicity were observed during the study. Mepiquat chloride did not produce any irritant reactions. The mean scores for dermal irritation over the period 24–72 hours were zero for both erythema and oedema. Under the conditions of the study, mepiquat chloride was not irritating to the skin of rabbits (Leuschner, 1976).

**(c) Ocular irritation**

In an eye irritation study (not GLP compliant), 0.1 g of mepiquat chloride (purity 94%; Reg. No. 85 559; LPT-batch) was applied to the conjunctival sac of the left eye of three New Zealand White rabbits of each sex. Twenty-four hours after instillation the eyes were treated with fluorescein and examined immediately, the process being repeated at 48 and 72 hours after instillation. There was no effect on cornea or iris at any time point. Conjunctival effects were restricted to redness grade 1 on day 1 after application. A Draize score of 0.5 (for 24–72 hours) was calculated. Under the conditions of the study, mepiquat chloride was not irritating to the eyes of rabbits (Leuschner, 1977).

**(d) Dermal sensitization**

In a skin sensitization study (not GLP compliant), mepiquat chloride (purity 99.8%; Batch no. 79/162) was tested on male Pirbright White Guinea pigs using the method of Landsteiner & Jacobs (1935), which resembles the Guinea pig maximization test (Magnusson & Kligman) in that it relied on intradermal injection. In the preliminary test on five animals per dose, 100 µL of the test substance was applied intracutaneously in physiological saline at concentrations of 10%, 25% and 50%. At concentrations of 10% no dermal irritation was seen, while at 25% and 50% pronounced changes were evident, with visible wheals, associated with death at 50%. The 25% solution caused necrotic skin changes at the application site and severe irritation in some cases. On the basis of the above findings a 10% solution of the test substance in physiological saline was chosen for the induction and challenge.

In the main phase of the study intracutaneous induction with a 10% test substance formulation in physiological saline caused slight erythema and oedema; occasionally necrosis was found at the injection site in various test animals. Intensification of the skin reaction, which would indicate a sensitizing effect even during the induction phase, was not detected. The intracutaneous challenge (10% test substance formulation in physiological saline) caused slight erythema and oedema both in the control and in the test animals. No sensitizing effect was detected for mepiquat chloride on the skin of Guinea pigs in a study conducted according to the method of Landsteiner (Grundler, 1979).

A GLP-compliant dermal sensitization test was conducted with mepiquat chloride (Purity 98.1%; Batch no. N60) using the mouse local lymph node assay (LLNA), and CBA/CaO1aHsd mice. Three concentrations of the test substance (10%, 25% and 50%) in ethanol and water mixture at 3 + 7 parts (v/v) were applied topically to five healthy female mice per group, on three consecutive days. Two vehicle control groups and a positive control group (five female mice per group) were maintained under the same environmental conditions and treated in the same manner as the test animals. Animals in the first vehicle control group were treated with the same ethanol and water vehicle as used with the test item. The second group received the vehicle used with the positive control item: acetone and olive oil mixture at 4 + 1 parts (v/v). The positive control group received a 25% (w/v) mixture of α-hexylcinnamaldehyde

(HCA) in the acetone:olive oil vehicle. Five days after the first application (day 6) all mice (five per group, including the control groups ) were given an intravenous injection containing 20 μCi of [$^3$H]methyl thymidine. Five hours later all animals were euthanized with carbon dioxide and the draining lymph nodes harvested and prepared for analysis in a scintillation counter. To measure cell proliferations the stimulation index (SI) was employed, that is, the ratio of proliferation in groups treated with the test item compared to proliferation in the vehicle controls.

There were no mortalities or any signs of systemic toxicity, including effects on body weight, over the duration of the study. No signs of local irritation were seen in the group tested at 10%, while at 25% and 50%, very slight erythema was seen on days 2 and 3. No changes in lymph node weight, ear weight or lymph node cell count were observed in any of the test item-treated groups. The indices determined for the lymph node cell count did not reach or exceed the lymph node cell count index threshold of 1.55, which is considered a positive response for BALB/c mice.

Treatment of mice with 10%, 25% and 50% mepiquat chloride resulted in SI values of 1.1, 1.2 and 1.2, respectively. The EC3 value (the amount of test substance needed to induce a three-fold increase in cell proliferation) could not be calculated, since none of the tested concentrations induced a SI greater than the threshold value of 3. An outlier was identified in the high-dose group, however, as exclusion of the outlier did not change the overall test result, the value in question was not excluded from the calculation. The mean SI determined for the concurrent positive control was 7.6 and for the lymph node cell count the value was 2.73, demonstrating the validity of the study. Furthermore, a statistically significant increase was observed for the ear and lymph node weights. Under the conditions of this study, mepiquat chloride was not a skin sensitizer in the LLNA assay at concentrations of less than, or equal to, 50% (Dony, 2019).

## 2.2 Short-term studies of toxicity

### (a) Oral administration

***Mouse***

In a 90-day oral toxicity study, mepiquat chloride (purity 57.9%; Batch no. WW 262/CP 1490) was administered in feed to five groups of 10 male and 10 female B6C3F1 mice at levels of 0, 300, 900, 2700 or 8100 ppm (equal to 0, 60, 186, 526 and 1731 mg/kg bw per day for males, 0, 83, 265, 705 and 2422 mg/kg bw per day for females).

All animals were observed for mortality and clinical signs during the study and their body weights and food consumption recorded. At termination of treatment all surviving animals were subjected to haematology, blood biochemistry, necropsy, organ weight analysis and histopathology.

Haematology and blood biochemistry revealed no treatment-related changes in male or female mice from any dose group. No treatment-related changes in gross finding at necropsy nor in organ weights were reported.

There was no evidence of mepiquat chloride-related toxicity in male or female mice fed a diet containing mepiquat chloride up to 8100 ppm.

The no-observed-adverse-effect level (NOAEL) for oral toxicity of mepiquat chloride in this 90-day study in mice was 8100 ppm (equal to 1731 mg/kg bw per day), the highest dose tested. (Schilling & Hilderbrand, 1992a)

***Rat***

*Study 1*

In a four-week oral toxicity study, four groups of five male and five female Wistar rats were fed diets incorporating mepiquat chloride (purity 57.9% in 44.3% water; Batch no. WW 262/CP 1490) at 0, 500, 2000 or 8000 ppm (equal to 0, 37.0–52.7, 149.4–200.9 and 531.0–696.5 mg/kg bw per day of active ingredient for males, 0, 43.7–54.3, 173.2–212.5, and 612.4–741.3 mg/kg bw per day of active ingredient for females). The health status of rats was checked daily. Food consumption and body weight were recorded weekly. The animals were checked at least daily for mortality. Additional inspection and palpation were performed once a week. Clinical chemistry and haematological examinations were

carried out after 29 days. All animals survived and were sacrificed at the end of the study for gross pathological and histopathological examinations.

No treatment-related changes were found in either male or female rats at 500 or 2000 ppm. At 8000 ppm, food consumption was reduced (by approximately 12% in males and 6% in females) and the body weight gain was retarded (by approximately 17% in males and 5% in females) at the end of the study for both sexes. The reduction in food consumption and body weight gain was more pronounced in males than females, but the shortfall reduced in both genders as the study continued. Relative to the controls, absolute liver weight, absolute kidney weight, and relative liver weight were lower (statistically significant) and the relative testes and adrenal gland weights higher (statistically significant) in males. In females absolute kidney weight was lower (statistically significant) compared to controls. The glucose, total protein, albumin, globulins, and triglyceride levels were decreased (statistically significant) and cholesterol increased (statistically significant) in males when compared to controls. However, these changes at 8000 ppm were secondary effects due to reduced food consumption, as the clinical chemistry and pathology effects that were observed were directly related to reduction in food consumption and there were no macroscopic nor microscopic findings in the organs examined.

The NOAEL for oral toxicity of mepiquat chloride in this four-week study in rats was 8000 ppm (equal to 531.0–696.5 mg/kg bw per day), the highest concentration tested (Schilling & Hildebrand, 1992b)

*Study 2*

In a 90-day oral toxicity study, mepiquat chloride (purity 94%; Batch no. LPT) was administered to 25 SD rats of each sex in the diet at the following dose levels: 0, 100, 300, 1000 or 3000 ppm (equal to 0, 9.2, 27.6, 91.8 and 275.5 mg/kg bw per day for males, 0, 8.9, 26.7, 91.2 and 278.8 mg/kg bw per day for females). The study was considered supportive as it was not GLP compliant and there are deviations from modern guidelines.

All animals were observed for mortality and clinical signs throughout the study. Body weights and food consumption were recorded. Ophthalmology, examinations for hearing and dentition, haematology, clinical chemistry, urinalysis, gross necropsy, organ weight analysis and histopathology were performed.

There were no mortalities, changes in behaviour or clinical signs of toxicity in the control or treated animals. Ophthalmological examination, noise test for hearing and examination of dentition did not reveal any significant changes. Urinalysis, haematology, clinical chemistry, and macroscopic inspection revealed no test substance-related adverse effects at any concentration.

At the highest dose (3000 ppm), food consumption declined during the second half of the study. Body weight gain was significantly inhibited, but the change in comparison to controls was within 10% for both males and females.

Organ weights of heart, lungs, spleen and kidney were all reduced to a statistically significant extent in males at 3000 ppm compared with controls, however the decreases were not considered of biological significance. Histological examinations revealed no treatment-related effects.

The NOAEL for oral toxicity of mepiquat chloride in this 90-day study in rats was 3000 ppm (equal to 275.5 mg/kg bw per day), the highest dose tested (Leuschner et al., 1977a).

*Study 3*

In a 90-day oral toxicity study, mepiquat chloride (purity 57.9%; Batch no. WW 262/CP 1490) was administered to five groups of 10 male and 10 female Wistar rats in the diet at dose levels of 0, 145, 579, 2316 or 4632 ppm (equal to 0, 10, 40, 163 and 319 mg/kg bw per day for males, 0, 12, 47, 188 and 372 mg/kg bw per day for females).

The animals were checked for mortality and state of health. Body weight and food consumption were recorded. Ophthalmology, haematology, clinical chemistry, urinalysis, gross pathology, histopathology, and organ weight evaluation were performed.

No mortalities or clinical signs of toxicity were observed in any of the dose groups. At the beginning of the study, the food consumption was reduced at the highest dose level in both sexes on day 7

(to 76% of controls in males, and 85% of controls in females), but food consumption was comparable to controls (difference from controls less than 10%) from day 14 onwards.

A retarded body weight gain occurred in the males in the highest dose group,observable on days 7 and 14 compared to control. The difference was however within 10%, as for the rest of the study period in all dose groups. For females there were no statistically significant differences in body weight and the differences from control were within 10% in all dose groups for the whole study period. The transient effects on food consumption and body weight were regarded as the results of reduced palatability rather than a sign of toxicity since no adverse effects were observed in urinary, haematological, clinical-chemical or pathological parameters.

Regarding haematology, clinical chemistry and ophthalmology parameters, no statistically significant differences between controls and test animals were found except for significantly decreased creatinine in the highest dose group females, which was unlikely to be an adverse effect in isolation from other findings.

Basophils were decreased in both sexes and all dose groups except for the females at 579 ppm, but the changes were not significant and values within three-fold compared to control. Platelets counts were increased in females of all dose groups, but were within 20% of controls. According to JMPR guidance document for WHO monographers and reviewers (JMPR, 2015) these changes are not considered adverse. There was a decrease compared to controls in polymorphonuclear neutrophils in females at all dose levels, with increased white blood cells. As this similar change was not observed in males its relevance seemed questionable.

In both males and females at all dose levels aspartate transaminase (AST) activity was clearly decreased compared to controls. A decrease in alanine transaminase (ALT) activity was also observed in some treatment groups, especially in females. The magnitude of the change was within 30% compared to controls and lacked any dose–response relationship. The magnitude of the change, its lack of relation to dose, its direction of change and the absence of liver pathology findings, all indicate that the activity effects are unlikely to represent an adverse finding and unlikely to be related to treatment. Reticulocytes in males were also reduced at 579 ppm and above but, without a clear dose–response relationship.

Masses of crystals were observed during urinalysis in 8/10 males (statistically significant) from the highest dose group but without corresponding pathological findings.

Terminal examinations, organ weight measurements, macroscopic and microscopic examination of organs and tissues did not reveal any statistically significant findings. The measured differences in body weights and organ weights were within 10% of controls in all dose groups of males and females.

Microscopical observations were made in some organs but were seen only sporadically and no clear dose–response relationship could be discerned.

The NOAEL for oral toxicity of mepiquat chloride in this 90-day study in rats was 4632 ppm (equal to 319 mg/kg bw per day), the highest dose tested (Schilling & Hilderbrand, 1992c).

*Study 4*

In a supplemental 90-day oral toxicity study aimed at identifying the dose level which caused clear evidence of systemic toxicity, mepiquat chloride (purity 57.9%; Batch no. WW 262/CP 1490) was administered to two groups of 10 male and 10 female Wistar rats at dose level of 0 or 12 000 ppm (equal to 0 and 826 mg/kg bw per day for males, 0 and 951 mg/kg bw per day for females).

The animals were observed for mortality and checked for signs of illness. Food consumption and body weight were recorded. Ophthalmology, urinalysis, clinical chemistry, haematology, neurofunctional tests, gross pathology, histopathology, and brain cholinesterase (ChE) examinations were carried out.

A dose level of 12 000 ppm caused systemic toxicity including reduced general state, impaired behaviour, tremors, impaired gait, ataxia, posture abnormalities, respiration abnormalities, vocalizations, faecal abnormalities, corneal clouding, increased clotting time, reduced grip strength, prolonged reaction time in the hot-plate test and changes in a number of clinical chemistry parameters.

Since there is only one dose group and systematic toxicities were observed in this group, no NOAEL could be identified. The LOAEL for oral toxicity of mepiquat chloride in this 90-day supplementary study in rats was 826 mg/kg bw per day, the only dose tested (Schilling & Hilderbrand, 1992d).

***Dog***

*Study 1*

In a four-week oral toxicity study, three groups of two male and two female beagle dogs were fed diets incorporating mepiquat chloride (purity: 57.2%; Batch no. WW 262/CP1490) at 0, 6000 and 12 000 ppm (equal to 0, 172.1–176.2 and 330.7–354.7 mg/kg bw per day for males, 0, 174.1–204.9, 329–359.0 mg/kg bw per day for females). The health condition and food consumption of dogs were checked daily, and body weight recorded weekly. Clinical chemical and haematological examination, and urinalyses were performed twice, once before and once at the end of the study. Neurofunctional observations for salivation were carried out every working day (five days each week). The animals were checked at least daily for mortality. At 12 000 ppm one female dog died on day 1. All the other animals survived and were sacrificed one day after the end of exposure for gross pathological and histopathological examinations. Salivation was observed in both sexes (all male animals, one female animal in each group) at 6000 and 12 000 ppm, but the salivation was reversible between daily feeds. According to the study authors, the female animals did not always finish the food within the observation period, and this may account for the differences in salivation between the sexes. No other treatment-related changes were found in either group. No abnormalities were observed in haematological investigations, clotting analysis, enzymes, blood chemistry or urinalysis. No treatment-related abnormalities were reported from pathological examinations.

No NOAEL could be determined, as salivation was observed at all doses However, it was noted that the toxicological significance of the observed salivation was not determined, as it may be related to receptor activation, or it may be related to local effects from the compound in the diet. The LOAEL for oral toxicity of mepiquat chloride in this four-week study in dogs was 6000 ppm (equal to 172.1–176.2 mg/kg bw per day), the lowest concentration tested (Mellert & Hildebrand, 1994a).

*Study 2*

In a 90-day oral toxicity study, five groups of four male and four female beagle dogs were administered mepiquat chloride (purity 94%; Batch no. LPT) in the diet for 13 weeks at dose level of 0, 100, 300, 1000 or 3000 ppm (equal to 0, 3.3, 9.8, 32.4 and 95.3 mg/kg bw per day for both sexes).

The three lower doses (100, 300 and 1000 ppm) were tolerated without reactions over three months. Among the 3000 ppm dose group sedation occurred from the first day onwards, reaching its maximum after 3–8 days and then disappeared within following three weeks. Later, it reappeared on 1–4 single days in 3/8 dogs and its return was accompanied by dogs lying on their abdomens or sides, as well as some observed tonoclonic spasms. Body weight gain was significantly inhibited, which was considered related to decreased food consumption. Haemoglobin (Hb) content, erythrocytes and haematocrit (Ht) values were lower compared to controls at the week 6 and 13 tests, mostly with statistical significance. Reticulocytes counts tended towards an increase after 13 weeks.

***Table 12. Comparison of haematological parameters in a 90-day dog study, mean values shown with [difference from control value] where significant***

| Test week | Group | Haemoglobin (mmol/L blood) | Erythrocyte count ($10^{12}$/L blood) | Haematocrit value (% of total blood) |
|---|---|---|---|---|
| Week 0 | Control | 9.6 | 6.5 | 44.9 |
| | 3000 ppm | 9.6 | 6.2 | 44.8 |
| Week 6 | Control | 9.6 | 6.5 | 45.5 |
| | 3000 ppm | 8.4 [−12.5%]* | 5.6 [−13.8%]* | 42.0 |
| Week 13 | Control | 9.5 | 6.4 | 45.0 |
| | 3000 ppm | 8.3 [−12.6%]* | 5.6 [−12.5%]* | 40.6 [−9.8%]* |

* Statistically significant at $p < 0.01$; Source: Leuschner et al., 1977b

The NOAEL for oral toxicity of mepiquat chloride in this 90-day study in dogs was 1000 ppm (equal to 32.4 mg/kg bw per day) based on signs of toxicity including sedation, reduction in body weight gain and haematological changes in red blood cell parameters at 3000 ppm (equal to 95.3 mg/kg bw per day) (Leuschner et al., 1977b).

*Study 3*

In a 12-month oral toxicity study, mepiquat chloride (purity 99.5% or higher; Batch no. N48) was administered in the diet to groups of six male and six female beagle dogs at 0, 200, 600 or 1800 ppm (equal to 0, 6.3, 19.9 and 58.4 mg/kg bw per day for both sexes). The dogs were weighed once a week and food consumption determined daily. Haematological and clinical chemistry analyses including urinalysis were carried out before initial dosing (day 11 for males, day 8 for females) and three times during the administration period. Ophthalmoscopy was performed prior to and at the end of the treatment period. Macroscopic examinations were performed on all dogs. Organs and tissues were examined histopathologically.

There were no unscheduled deaths in this study. A few animals showed decreased food consumption. Feed efficiency was calculated for each dose group at four-weekly intervals based on the body weight changes and the total amount of feed consumed during this period; no treatment-related changes in food efficiency were revealed. No effect of the test substance on the growth of the dogs was evident, and occasional clinical signs that were observed did not show dose–response relationship and were considered incidental.

Some slight haematological changes in Hb (on days 93 and 184), red blood cell (RBC) counts (on day184), Ht (on day 184) were observed in males at 200 ppm. Given the differences were minimal from controls (average Hb, RBC and Ht values increased by 6.2%, 6.6% and 6.6% respectively), the absence of a dose–response relationship and no consistent pattern of change across time, these changes were considered within normal biological variability. A statistically significant increase in reticulocytes was reported in males at 200 ppm and above on day 184, and a decrease in females at 1800 ppm on day 367. However, the reticulocyte values across the dose groups and in both sexes were variable and inconsistent (the coefficient of variation ranged from 15.4% to 25% for the males at 200 ppm and above, and was 100% for the females at 1800 ppm), and there was dose–response relationship, and these findings were therefore considered incidental to treatment. A statistically significant decrease in clotting values, was observed in males at 1800 ppm and females at 200 ppm on days 93 and 188 respectively. This reflected biological/analytical variability and was not considered indicative of a consistent, dose-related effect of treatment. Blood chemistry analysis results indicated statistically significant increase in total protein in males at 600 and 1800 ppm on days 184 and 367 respectively, and a statistically significant increase in globulins in males at 600 ppm. There was a slightly increased level of iron pigment storage in the spleen and liver of males at 1800 ppm, seen in the pathology findings. In a preceding three-month feeding study, statistically significant signs of anaemia were observed at 3000 ppm, with no effects observed at 1000 ppm. The increased iron pigment storage in the spleen and liver at 1800 ppm may be a sign of a transient anaemia in this study (Table 13). No treatment-related changes were found in body weight, food consumption, food efficiency, ophthalmology or urinalysis.

***Table 13. Incidence and grading of iron pigment in liver and spleen of male dogs in a 12-month oral toxicity study***

| | | Dose level (ppm) | | | |
|---|---|---|---|---|---|
| | | **0** | **200** | **800** | **1800** |
| **Number of dogs** | | 6 | 6 | 6 | 6 |
| **Spleen:** | Incidence | 6 | 6 | 6 | 6 |
| | Grade: minimal | 6 | 5 | 6 | 3 |
| | slight | 0 | 1 | 0 | 3 |
| **Liver:** | Incidence | 6 | 6 | 6 | 6 |
| | Grade: minimal | 6 | 6 | 6 | 4 |
| | slight | 0 | 0 | 0 | 2 |

Source: Hellwig & Hilderbrand, 1989

The NOAEL for oral toxicity of mepiquat chloride in this 12-month study in dogs was 1800 ppm (equal to 58.4 mg/kg bw per day), the highest dose tested (Hellwig & Hilderbrand, 1989).

*Study 4*

In the 12-month study, mepiquat chloride (purity 56.05%; batch no. 91-2 PCP 01826) was administered in the diet to groups of six male and six female beagle dogs at 0 or 6000 ppm (equivalent to 0 and 166 mg/kg bw per day for males, 0 and 173 mg/kg bw for females). The test substance/water solutions were prepared generally at two to three weekly intervals.

The study was initially started with a dosage of 0 and 8000 ppm of the active ingredient. The administration of the test substance at 8000 ppm was discontinued when on day 1, one male and two female dogs died. The surviving animals were allowed to recover for five days of a treatment-free period. The animals which had died, were replaced by three new animals and treatment restarted at dietary dose levels of 0 and 6000 ppm.

Animals were observed daily for changes in behaviour and external appearance. Body weight was recorded weekly and food consumption daily. The dogs were examined for salivation four times a day generally each working day (five days each week) and from study day 91 onwards at weekly intervals. Ophthalmological examination was performed before the start of treatment and at termination. Haematology and clinical chemistry examinations as well as urinalysis were carried out once before treatment commenced and after about 3, 6 and 12 months of the study.

One female dog receiving 6000 ppm was sacrificed in a moribund state on study day 17. The main sign of toxicity was salivation in treated animals. It was reported that salivation was observed from two hours after feeding onwards, starting at a slight degree after two hours, advancing to reach moderate to severe until 4–6 hours after feeding (Table 14). Findings were reported to be reversible until the next day's feeding session.

***Table 14. Twelve-month study in dogs: summary of clinical observations***

| | Sex, dose level (ppm) | | | |
|---|---|---|---|---|
| | Males | | Females | |
| **Clinical sign (number of dogs)** | **0** | **6000** | **0** | **6000** |
| Animals investigated | 6 | 6 | 6 | 6 |
| Mortality | 0 | 0 | 0 | 1[a] |
| Weakness of the hind limbs, lateral position, extension spasms, ataxia of the hind limbs, subnormal body temperature, reduced general state | - | - | 0 | 1[a] |
| Vomiting (observed on day 7) | - | - | 0 | 1 |
| Salivation: | | | | |
| slight | 0 | 2 | 0 | 0 |
| moderate | 0 | 2 | 0 | 2 |
| severe | 0 | 2 | 0 | 4 |

[a] Female No. 72:1: sacrificed moribund on study day 17; clinical signs observed on study days 7, 16 and 17.

Source: Mellert & Hildebrand, 1994b

During the study, individual females showed decreased appetite, which could be regarded as an incidental finding, but there was no evident effect of the test substance on food consumption of the animals. Body weight gain of the dogs at the 6000 ppm paralleled that of the control group, except for one female at 6000 ppm on the day 7, which was not statistically significant, and this finding in female dogs was considered incidental not related to treatment.

Ophthalmoscopic examinations at the end of the study showed serous discharge, reddish mucosae, and entropion in both eyes in one female from the 6000 ppm dose group; this was considered to be an incidental finding. Otherwise, no abnormalities were detected. In the clinical chemistry examinations, total bilirubin was decreased, and eosinophils increased compared to controls at all examinations of females. In females the mean corpuscular volume (MCV) values were increased compared to controls. Elevated values of white blood cells were observed for both males and females (Table 15).

*Table 15. Twelve-month study in dogs: summary of haematology and clinical chemistry results,* [*bracketed numbers are percentage of control value*]

| | Sex, dose level (ppm) | | | |
|---|---|---|---|---|
| | Males | | Females | |
| Time point | 0 | 6000 | 0 | 6000 |
| **Haematology results** | | | | |
| **White blood cells ($10^9$/L)** | | | | |
| Day 91 | 8.21 | 10.26* [125%] | 10.80 | 12.15 [113%] |
| Day 186 | 10.02 | 10.95 | 9.45 | 11.31** [120%] |
| Day 361 | 8.83 | 11.06* [125%] | 8.94 | 13.97 [156%] |
| **Platelets ($10^9$/L)** | | | | |
| Day 91 | 330 | 294 [89%] | 359 | 383 |
| Day 186 | 363 | 325 | 384 | 433 [113%] |
| Day 361 | 364 | 291* [80%] | 387 | 429 [111%] |
| **Haematocrit (L/L)** | | | | |
| Day 91 | 0.426 | 0.413 | 0.414 | 0.450 |
| Day 186 | 0.464 | 0.467 | 0.464 | 0.473 |
| Day 361 | 0.465 | 0.469 | 0.445 | 0.505* [113%] |
| **Neutrophilic bands (%)** | | | | |
| Day 91 | 2.83 | 1.67 [59%] | 2.33 | 4.00 [172%] |
| Day 186 | 0.67 | 1.33 [199%] | 1.33 | 0.60 [45%] |
| Day 361 | 1.33 | 1.17 [88%] | 1.33 | 0.80 [60%] |
| **Monocytes (%)** | | | | |
| Day 91 | 7.33 | 6.50 [89%] | 7.67 | 8.40 |
| Day 186 | 8.33 | 8.50 | 9.50 | 9.60 |
| Day 361 | 6.17 | 7.00 [113%] | 9.00 | 6.20 [69%] |
| **Eosinophils (%)** | | | | |
| Day 91 | 2.83 | 2.67 | 1.83 | 3.60 [197%] |
| Day 186 | 2.67 | 3.17 [119%] | 2.67 | 4.20 [157%] |
| Day 361 | 3.67 | 2.33 [63%] | 2.17 | 2.40 [111%] |
| **Reticulocytes (‰)** | | | | |
| Day 91 | 4 | 3 | 4 | 5 |
| Day 186 | 7 | 5 | 4 | 9 |
| Day 361 | 8 | 4 | 4 | 11* |
| **Clinical chemistry results** | | | | |
| **AST (µkat/L)** | | | | |
| Day 91 | 0.38 | 0.49** [129%] | 0.44 | 0.54 [123%] |
| Day 186 | 0.41 | 0.44 | 0.44 | 0.46 |
| Day 361 | 0.53 | 0.61 [115%] | 0.49 | 0.57 [116%] |
| **ALP (µkat/L)** | | | | |
| Day 91 | 2.55 | 2.54 | 3.36 | 2.61 [78%] |
| Day 186 | 2.01 | 2.39 [119%] | 2.63 | 2.60 |
| Day 361 | 2.22 | 2.98* [134%] | 3.08 | 2.58 [84%] |

| | | | | |
|---|---|---|---|---|
| **Glucose (mmol/L)** | | | | |
| Day 91 | 6.15 | 6.09 | 5.71 | 6.14 |
| Day 186 | 5.58 | 5.95 | 5.44 | 5.95 |
| Day 361 | 6.09 | 5.95 | 5.66 | 6.37** [113%] |
| **Urea (mmol/L)** | | | | |
| Day 91 | 5.29 | 5.92 [112%] | 4.86 | 3.78 [78%] |
| Day 186 | 5.66 | 6.07 | 5.64 | 5.87 |
| Day 361 | 4.79 | 4.35 | 4.86 | 3.78 [78%] |
| **Total bilirubin (µmol/L)** | | | | |
| Day 91 | 2.23 | 2.62 [117%] | 2.79 | 2.13 [76%] |
| Day 186 | 2.87 | 2.90 | 3.24 | 2.64 [81%] |
| Day 361 | 3.78 | 3.32 [114%] | 3.87 | 2.78 [72%] |
| **Triglycerides (mmol/L)** | | | | |
| Day 91 | 0.33 | 0.37 [112%] | 0.48 | 0.47 |
| Day 186 | 0.48 | 0.47 | 0.54 | 0.58 |
| Day 361 | 0.39 | 0.45 [115%] | 0.46 | 0.47 |
| **Cholesterol (mmol/L)** | | | | |
| Day 91 | 4.96 | 4.65 | 4.79 | 4.17 [87%] |
| Day 186 | 5.65 | 5.50 | 5.26 | 5.90 [112%] |
| Day 361 | 4.55 | 4.61 | 5.35 | 4.59 [86%] |

Statistically significant at: * $p < 0.05$; ** $p < 0.02$; Source: Mellert & Hildebrand, 1994b

At 6000 ppm the absolute weight of adrenal glands was increased, and the relative weight of kidneys reduced in males, both to a statistically significant extent. For both males and females, the relative weight of the thyroid gland was reduced compared to controls (Table 16).

***Table 16. Twelve-month study in dogs: body weights and organ weights***

| | | Sex, dose level (ppm) | | | |
|---|---|---|---|---|---|
| | | Males | | Females | |
| **Parameter** | | **0** | **6000** | **0** | **6000** |
| **Body weight gain, mean** ± standard deviation **(g)** | | | | | |
| Time point: | Day 7 | 0.1 ± 0.2 | 0.0 ± 0.2 | 0.3 ± 0.1 | 0.1** ± 0.0 |
| | Day 49 | 0.5 ± 0.5 | 0.4 ± 0.6 | 1.0 ± 0.4 | 0.6 ± 0.7 |
| | Day 91 | 0.9 ± 0.7 | 1.0 ± 0.7 | 1.6 ± 0.8 | 1.2 ± 1.0 |
| | Day 182 | 1.7 ± 0.8 | 2.0 ± 1.1 | 2.3 ± 0.9 | 2.4 ± 1.0 |
| | Day 252 | 1.8 ± 0.9 | 2.0 ± 1.2 | 2.3 ± 1.0 | 26. ± 1.4 |
| | Day 364 | 1.1 ± 1.2 | 1.7 ± 1.3 | 1.9 ± 0.9 | 2.2 ± 1.4 |
| **Absolute organ weights, [as % of control]** | | | | | |
| Adrenal glands (mg) | | 1221.333 | 1397.833* [114%] | 1396.5 | 1451.6 |
| **Relative organ weights, (% relative to body weight) [as % of control]** | | | | | |
| Kidneys | | 0.492 | 0.466* [95%] | 0.481 | 0.439 |
| Adrenal glands | | 0.009 | 0.011 [122%] | 0.012 | 0.012 |
| Thyroid gland | | 0.006 | 0.005 [83%] | 0.007 | 0.006 [86%] |
| Liver | | 2.813 | 2.634 | 2.811 | 2.511 [89%] |

Statistically significant at: * $p < 0.05$; ** $p < 0.02$; Source: Mellert & Hildebrand, 1994b

Histopathological examination revealed vacuolation in the kidneys of male and female animals. Increased severity of haemosiderin storage was observed in the spleens of males (Table 17).

***Table 17. Twelve-month study in dogs: findings from histopathological examination of the kidneys and spleen***

| | Sex, dose level (ppm) | | | |
|---|---|---|---|---|
| | Males | | Females | |
| | 0 | 6000 | 0 | 6000 |
| **Number of animals per group** | **6** | **6** | **6** | **6** |
| | **Incidence and grading of kidney vacuolation** | | | |
| Number of kidneys examined | 12 | 12 | 12 | 12 |
| Incidence | 1 | 7 | 4 | 9 |
| Minimal | 1 | 5 | 4 | 1 |
| Slight | – | 2 | – | 8 |
| | **Incidence and grading of haemosiderin storage in the spleen** | | | |
| Number of spleens examined | 6 | 6 | 6 | 6 |
| Incidence | 6 | 6 | 6 | 6 |
| Minimal | 6 | 2 | 3 | 4 |
| Slight | – | 4 | 3 | 2 |

Source: Mellert & Hildebrand, 1994b

Since there is only one dose group and kidney vacuolation was observed in both sexes in this group, no NOAEL could be identified. The LOAEL for oral toxicity of mepiquat chloride in this 12-month study in dogs was 6000 ppm (equal to 166 mg/kg bw per day), the only dose tested. (Mellert & Hildebrand, 1994b)

**(b) Dermal application**

In a four-week dermal toxicity study, mepiquat chloride (purity 58.1%; Batch no. 2000-1) was applied repeatedly for 28 consecutive days to the skin of Wistar rats (10 of each sex per group) for six hours per day, five days per week at dose levels of 0, 100, 300 and 1000 mg/kg bw per day under a semi-occlusive dressing. Doubly distilled water was used as the vehicle. Food consumption and body weight were recorded weekly. Signs of toxicity and mortality were checked daily. Clinical chemical and haematological examinations, and urinalyses were performed at the end of the study. All animals survived and were sacrificed at the end of the study for gross pathological and histopathological examinations. No treatment-related changes were found at any dose levels.

The NOAEL for dermal toxicity of mepiquat chloride in this four-week study in rats was 1000 mg/kg bw per day, the highest dose tested (Mellert et al. 2002).

**(c) Exposure by inhalation**

No data was submitted for this topic.

## 2.3 Long-term studies of toxicity and carcinogenicity

The long-term toxicity and carcinogenic potential of mepiquat chloride has been evaluated in a 24-month carcinogenicity study and a 104-week study in mice, and in a 24-month carcinogenicity, a 24-month chronic study, and a 104-week study in rats. The 104-week rat study included a 52-week long-term toxicity phase.

***Mouse***

*Study 1*

In a carcinogenicity study, groups of 50 male and 50 female B6C3F1 mice were administered mepiquat chloride (purity: 57.9% ; Batch no. WW 262/CP 1490) in the diet for up to 104 weeks at

concentrations of 0, 500, 2000 and 7500 ppm (equal to 0, 74, 297 and 1140 mg/kg bw per day for males, 0, 85, 328 and 1348 mg/kg bw per day for females). Satellite groups of 10 male and 10 female mice were similarly treated for 12 months at concentrations of 0, 83, 314, and 1249 mg/kg bw per day for males and 0, 95, 414 and 1607 mg/kg bw per day for females. Food consumption and body weights were determined weekly for 14 weeks and every four weeks thereafter. The general state of animal health and mortality were checked daily; furthermore, comprehensive clinical examinations of the animals were performed once a week. Blood was collected for differential blood smears from the satellite group animals (day 369) and at study termination from all surviving animals of the main groups. Only the blood smears of the control and the high-dose groups were evaluated. In addition, blood smears were also prepared and evaluated from all animals killed in extremis during the study. After 12 months of treatment all surviving mice of the satellite group were sacrificed. After 24 months the surviving mice in the main groups were sacrificed. All animals were subjected to complete gross pathology examinations. Selected organs (brain, liver, kidneys, adrenal glands, testes) were weighed. Histopathological examinations were carried out on all animals in control and high-dose groups, in addition these organs, as well as lungs, liver, kidneys, adrenal glands, colon, urinary bladder and preputial glands were examined from all animals in the low- and mid-dose groups. Gross lesions were examined from all animals so affected. Animals that died intercurrently, or were sacrificed in a moribund state, were investigated histopathologically as outlined for the control animals.

There were no treatment-related mortalities or clinical signs in either males or females. Body weight was slightly decreased (statistically significant) in males at 500 ppm on days 91 and 98, and at 7500 ppm from day 63 to day 210 (except on day 77) and also on day 350. The percentage to which body weight was depressed compared to controls ranged from 3.8% to 7.0% in males at 500 ppm and above. In females receiving 500 ppm, body weight compared to controls was increased on day 35, and in the 2000 ppm female group it was decreased on day 42. In the satellite groups, body weight was decreased in females at 500 ppm (on day 98), at 2000 ppm (on days 49, 56 and 77) and at 7500 ppm (on days 49, 56, 63, 70, 77, 91 and 98), achieving statistical significance (Table 18). There was no effect on food consumption or food efficiency in either the main or satellite groups. There were no relevant differences between treated animals and controls in the type or incidence of tumours, or in the number of animals bearing tumours. In addition, in the tissues where slight numerical differences in incidence are highlighted, there was no clear evidence of a dose–response relationship, and there were no indications of any associated preneoplastic lesions, leading to a lack of biological plausibility for these tumours being a treatment-related effect. No treatment-related changes were observed in haematology parameters, nor in pathology results from this study.

***Table 18. Twelve-month carcinogenicity study in mice: mean body weights in main groups at selected time points (11 out of 37 observations)***

| | Sex, dose level (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | 0 | 500 | 2000 | 7500 | 0 | 500 | 2000 | 7500 |
| Treatment day | Mean body weight (g) | | | | | | | |
| 0 | 23.4 | 23.3 | 23.3 | 23.1 | 19.0 | 19.1 | 19.1 | 18.7 |
| 7 | 24.6 | 24.6 | 24.6 | 24.5 | 19.2 | 18.9 | 19.6 | 19.6 |
| 35 | 27.4 | 27.2 | 27.0 | 26.9 | 21.4 | 22.1** | 21.5 | 21.9 |
| 91 | 32.0 | 30.4* | 30.8 | 30.1** | 25.3 | 25.2 | 25.1 | 25.6 |
| 182 | 36.2 | 33.8* | 35.5 | 33.7** | 28.5 | 29.2 | 30.1 | 30.0 |
| 294 | 37.9 | 36.4 | 37.1 | 36.0 | 33.0 | 33.8 | 35.3 | 34.4 |
| 378 | 39.1 | 37.9 | 39.0 | 37.7 | 33.9 | 34.4 | 36.3 | 34.9 |
| 462 | 39.3 | 37.6 | 38.9 | 37.4 | 34.6 | 35.6 | 37.1 | 36.2 |
| 546 | 40.1 | 38.8 | 39.5 | 38.3 | 36.2 | 36.7 | 37.9 | 36.7 |
| 658 | 38.7 | 37.6 | 37.6 | 36.2 | 35.9 | 35.0 | 35.9 | 35.6 |
| 714 | 38.0 | 36.7 | 36.9 | 35.6 | 34.9 | 35.0 | 35.7 | 34.9 |

Statistically significant at: * $p < 0.05$; ** $p < 0.01$; Source: Mellert et al., 1994

Since the statistical significance was transient and the magnitude of change in males was minimal, and there was no treatment-related increase in tumour incidence when compared to controls, the NOAEL for both oral toxicity and for carcinogenicity of mepiquat chloride in this 104-week study in mice was 7500 ppm (equal to 1140 mg/kg bw per day), the highest dose tested (Mellert et al., 1994).

*Study 2*

In a carcinogenicity study, groups of 100 (control) or 50 (test groups) NMRI mice were administered mepiquat chloride (purity: 94%; Batch no. LPT) in the diet for 104 weeks at concentrations of 0, 100, 300, 1000 or 3000 ppm (equal to 0, 16, 48.9, 169.4 and 513.5 mg/kg bw per day for males, 0, 21.7, 65.3, 226.1 and 689.4 mg/kg bw per day for females). Food consumption was monitored daily and body weights were determined and recorded at weekly intervals. The average body weights of animals in each group were recorded at four-week intervals. Animals were also monitored daily for mortality, behaviour and general appearance throughout the administration period. Faeces were collected and examined. All animals were palpated once a week to examine potential tumour growth from week 26 onwards. Ophthalmological, auditory acuity and dentition inspections were performed at the end of the administration period. Haematology, clinical chemistry and urinalysis investigations were carried out on 10 animals of each sex per group at 26, 52, and 104 weeks after the start of treatment. All animals which died prior to scheduled sacrifice and those that were killed at the termination of treatment, were subjected to a full necropsy. The weight of heart, liver, lungs, spleen, kidney, thymus, gonads and brain were recorded. Organs and tissues (heart, liver, lungs, spleen, adrenal, thymus, pituitary, gonads, trachea, thyroid, brain, urinary bladder) underwent histopathological examination.

There were no treatment-related clinical signs (including behaviour, external appearance and faeces) seen in males or females. Individual animals died from week 11 onwards, with mortality increasing during the second year of the study. There was no dose–response relationship to the number of animals dying. Before dying, some animals showed poor appetite and apathy, and ataxia or cachexia and neglect of coat was noted in individual cases. Mortality was not affected by the treatment and ranged between 48% and 64% for males and females of the different treatment groups (Table 19). The body weight of males receiving 1000 and 3000 ppm was slightly lower than that of the controls, but without reaching statistical significance (Table 20). There were no treatment-related food consumption differences in either males or females.

***Table 19. Carcinogenicity study (104-weeks) in mice: mortality rates***

| Dose level (ppm) | Mortality rate in % | | |
|---|---|---|---|
| | Males | Females | Males + females |
| 0 | 49 | 61 | 55 |
| 100 | 48 | 64 | 56 |
| 300 | 48 | 62 | 55 |
| 1000 | 54 | 60 | 57 |
| 3000 | 50 | 60 | 55 |

Source: Leuschner et al., 1979a

***Table 20. Carcinogenicity study (104-weeks) in mice: mean body weight (g) ± standard deviation***

| | Sex, dose level (ppm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Male | | | | | Female | | | | |
| Time point | 0 | 100 | 300 | 1000 | 3000 | 0 | 100 | 300 | 1000 | 3000 |
| Start | 18.2 ± 0.7 | 18.3 ± 0.8 | 18.3 ± 0.6 | 18.3 ± 0.8 | 18.3 ± 0.7 | 18.2 ± 0.8 | 18.3 ± 0.8 | 18.2 ± 0.8 | 18.3 ± 0.8 | 18.2 ± 0.6 |
| Week 52 | 46 ± 4.4 | 35.3 ± 4.5 | 44.7 ± 4.4 | 35.9 ± 3.1 | 46.3 ± 5 | 37.3 ± 4.2 | 43.2 ± 4.9 | 36 ± 3.3 | 43.5 ± 5.6 | 35 ± 3.8 |
| Week 104 | 42.3 ± 4.1 | 36.3 ± 3.3 | 43.5 ± 4.7 | 37.3 ± 3.1 | 42.5 ± 5 | 38.7 ± 5.4 | 40.8 ± 5.4 | 37.1 ± 4.9 | 42.2 ± 5.9 | 37.5 ± 4.9 |

Source: Leuschner et al., 1979a

In the haematology analysis, the numbers of leucocytes were significantly higher than for controls in males at 100 ppm after 52 weeks. This observation may be considered a spurious finding, as it was not related in severity to the dose level and was even less marked at the end of the study. In the clinical biochemistry analysis, a slight increase in free cholesterol in the females at 3000 ppm after 104 weeks was noted. As no true dose–response relationship was observed, both these findings were considered incidental rather than treatment-related. Microscopic evaluations indicated no carcinogenic effect due to the test compound. An 8% rate of lymphoma (compared with 2% in controls) in females at 3000 ppm was considered to be unrelated to treatment as there was relationship to dose. In addition, it was also found that there was a 27% rate of leukaemia (compared with 56.5% in controls) at 3000 ppm (Table 21). No treatment-related changes were apparent as a result of ophthalmology, urinalysis, organ weights, or pathological examinations.

***Table 21. Carcinogenicity study (104-weeks) in mice: selected tumour rates***

| | **Sex, dose level (ppm)** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Males** | | | | | **Females** | | | | |
| | **0** | **100** | **300** | **1000** | **3000** | **0** | **100** | **300** | **1000** | **3000** |
| Number of mice examined | 100 | 50 | 50 | 50 | 50 | 100 | 50 | 50 | 50 | 50 |
| Tumour-bearing mice | 67 | 26 | 23 | 18 | 19 | 71 | 35 | 27 | 36 | 32 |
| **Reticulohistio-cytary system/blood** | | | | | | | | | | |
| Leukaemia | 54 | 15 | 8 | 12 | 13 | 59 | 28 | 20 | 33 | 14 |
| Lymphoma | 1 | 1 | 2 | 1 | 2 | 1 | 1 | 2 | 0 | 4 |
| Lymphogranulomatosis | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |

Source: Leuschner et al., 1979a

The NOAEL for oral toxicity and carcinogenicity of mepiquat chloride in this 104-week study in mice was 3000 ppm (equal to 513.5 mg/kg bw per day), the highest concentration tested (Leuschner et al., 1979a)

***Rat***

*Study 1*

A chronic study was performed in Wistar rats over a duration of 24 months. In the main study, groups of 20 animals of each sex were fed mepiquat chloride (purity 57.9%; Batch no. WW 262/CP 1490) at concentrations of 0, 290, 2316 or 5790 ppm (equal to 0, 13, 106 and 268 mg/kg bw per day for males, 0,18,146 and 371 mg/kg bw per day for females, adjusted for purity). It is noted that this was a small sample size for a 24 month study.

Food consumption and body weight were determined weekly for 14 weeks and every four weeks thereafter. The general state of animal health was checked daily, with comprehensive clinical examinations of the animals performed once a week. The first 10 animals of each group were subjected to neurofunctional observations before, and ca 1, 2, 3 and 6 months after the start of the administration period. The functional observation battery (FOB) consisted of 31 qualitative parameters such as behaviour, convulsions, salivation, vocalization, papillary reflex, vision, audition, as well as three quantitative parameters (grip strength of fore- and hind-limbs and hotplate test). Haematological as well as clinical chemistry examinations and urinalysis were conducted after ca 3, 6, 12, 18 and 24 months. Ophthalmological examinations were carried out once before the start of the study and toward the end of the administration period, just on control and high-dose groups. A check was made for dead and moribund animals twice a day. After 24 months of treatment all surviving rats were sacrificed. All animals were subjected to complete gross pathological examination. Adrenal glands, brain, kidneys, liver and testes were weighed. Histopathological examinations were performed on all animals in the control and high-dose groups. In addition, lungs, liver and kidneys were examined in all animals from the low- and mid-dose groups, and any gross abnormalities that were identified were examined. Any animals in the low- and mid-dose groups that died or were killed in extremis, underwent a histopathological examination of all organs as detailed for the high-dose group.

The cumulative mortality of males up until day 728 at 0, 290, 2316 and 5790 ppm was 25%, 30%, 5% and 30% respectively. For females corresponding values were 25%, 25%, 30% and 0% ; the absence of mortality at 5790 ppm for females demonstrated a lack of dose–response relationship. To conclude, there were no deaths and no clinical signs that could be attributed to treatment.

Mean body weights (Table 22) were impaired in high-dose males and females; from day 7 to day 728 in males, and day 98 to day 574 in females. At the end of the administration period the value was about 12% below control values. Consistent with this, the values of body weight change were about 17% and 20% below control values in high-dose males and females respectively. The adverse effect on body weight and its development at the high dose may be attributed to a lower intake of energy and nutrients since food consumption was generally lower than for controls, generally by about 10%. However, since these weight differences were different by more than 10% compared to controls the reduction may be considered biologically relevant. In other groups the values were within the biological range of variation. This data suggests that the adverse effects on body weight and its gain cannot be explained by a decrease in food intake and is considered a clear sign of toxicity.

***Table 22. Chronic study in males and female rats (24-months): selected body weights (for 10 out of 37 time points), mean weight (g), [bracketed numbers are percentage of control value]***

| | Sex, dose level (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| Study day | 0 | 290 | 2316 | 5790 | 0 | 290 | 2316 | 5790 |
| 7 | 51.6 | 51.3 | 50.3 | 33.9** [66%] | 25 | 26.5 | 24.4 | 21.1* [84%] |
| 35 | 186.1 | 184.5 | 181.2 | 150.5** [81%] | 80.2 | 84 | 79.3 | 72.9 |
| 63 | 258.8 | 257.7 | 251.7 | 214.0** [83%] | 114.6 | 117.5 | 113.3 | 103.9* [91%] |
| 91 | 306.2 | 307.8 | 296.5 | 253.9** [83%] | 130 | 136.8 | 129.7 | 120.1 |
| 126 | 341.3 | 345.1 | 331 | 284.0** [83%] | 149.9 | 155.9 | 145 | 131.4** [88%] |
| 182 | 367.6 | 377.8 | 360 | 313.6** [85%] | 155.3 | 160.1 | 148.7 | 137.2* [88%] |
| 378 | 464.7 | 467.6 | 447.4 | 401.9* [86%] | 192 | 198.7 | 185.1 | 159.8** [83%] |
| 546 | 525.1 | 520.9 | 500.4 | 446.4* [85%] | 219.8 | 224 | 207.8 | 176.0** [80%] |
| 714 | 541.5 | 541.8 | 476.4 [88%] | 430.2* [79%] | 241.5 | 242.2 | 230.3 | 206.3 [85%] |
| 728 | 521.2 | 510.8 | 466 [89%] | 434.3* [83%] | 235.6 | 216.9 | 230.6 | 188.6 [80%] |

Statistically significant at: * $p < 0.05$; ** $p < 0.02$; Source: Mellert & Hildebrand,1994c

Results of the limited haematological and clinical chemistry information obtained from blood smears did not show substance-related changes in either sex. Urinalysis revealed a significant and consistent alteration in the urine sediments of the high-dose males, consisting of increased crystals, especially triple phosphate (struvite) crystals during the first 12 months of the study. Although there was only a statistically significant increase in urinary crystals after three months of test substance administration and it was found only in one sex, this finding was considered to be substance-related. However, it is unclear to which mechanism the increase in urinary crystals is due.

Gross pathological examination, (Table 23) did not reveal treatment-related adverse findings except for a statistically significant decrease in terminal body weight and absolute brain weight in high-

dose males. The decrease in brain weight was not considered to be an indirect effect as a consequence of the decreased l body weight. No other weight parameter varied significantly between groups.

***Table 23. Chronic study (24-month) in rats: incidence of notable findings***

| | Sex, dose level (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Parameter** | **0** | **290** | **2316** | **5790** | **0** | **290** | **2316** | **5790** |
| Terminal body weight: | | | | | | | | |
| mean | 693.6 | 670.6 | 632.6 | 598.2 | 360.1 | 344.3 | 356.4 | 316.72 |
| standard deviation | ± 118.3 | ± 91.96 | ± 81.40 | ± 67.39 | ± 55.96 | ± 69.47 | ± 59.173 | ± 37.2 |
| Brain weight: mean | 2.324 | 2.272 | 2.269 | 2.221 | 2.066 | 2.023 | 2.078 | 2.051 |
| standard deviation | ± 0.143 | ± 0.103 | ± 0.082 | ± 0.099 | ± 0.097 | ± 0.103 | ± 0.102 | ± 0.07 |
| **Incidence of primary neoplasms** | | | | | | | | |
| Number of rats examined | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| Brains examined | 50 | 13 | 15 | 50 | 50 | 22 | 22 | 50 |
| Glioma | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| Oligodendroglioma | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| Granular cell tumour | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| Glioblastoma | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| **Incidence of microscopic findings** | | | | | | | | |
| Number of rats examined | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 |
| Brains examined | 20 | 8 | 2 | 20 | 20 | 10 | 10 | 20 |
| Compression | 2 | 1 | 2 | 1 | 9 | 8 | 8 | 5 |
| Ventriculardilation | 1 | 2 | 0 | 1 | 8 | 4 | 2 | 1 |
| Haemorrhage | 3 | 0 | 0 | 1 | 4 | 1 | 2 | 0 |
| Meningitis | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| Meningioma | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Oligodendroglioma | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 |
| Granular cell tumour | 4 | 1 | 0 | 0 | 1 | 0 | 0 | 0 |
| Schwannoma | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |

Source: Mellert & Hildebrand,1994c

The incidence of mammary gland adenocarcinoma in females was 10% (2/20) in the control, low-, mid- and high-dose groups after adjustment of the correct denominator for calculation of the tumour incidence. The historical control incidence of mammary adenocarcinoma from the same laboratory for studies conducted five years prior and five years after this mepiquat chloride study, indicate a mean control incidence of 9.3% with a range of 0–25%, indicating that the 10% incidence seen could not be attributed to treatment.

Histopathologically, no substance-related effects were obtained with any of the dose groups. The incidence and severity of all histopathological lesions recorded in this study were comparable across all groups and they did not vary remarkably among survivors and decedents. All lesions were considered to be incidental and typical of the background pathology encountered in rats of this strain and age.

The NOAEL for oral toxicity of mepiquat chloride in a 24-month study in rats was 2316 ppm (equal to 106 mg/kg bw per day), based on reduced food consumption, reduction in body weight and body weight gain and apparent increase in urinary crystals observed, all at 5790 ppm (equal to 268 mg/kg bw per day) (Mellert & Hildebrand,1994c).

The 24-month study above is not suitable as a stand-alone evaluation of carcinogenic potential as the group size of 20 rats per sex does not provide sufficient statistical power for the reliable detection of a carcinogenic effect. Therefore, this chronic toxicity study is not suitable to establish a NOAEL for carcinogenicity based on differences in tumour incidence between treated and control animals.

*Study 2*

A carcinogenicity study was performed in Wistar rats over a duration of 24 months. In the main study, groups of 50 rats of each sex per dose were fed mepiquat chloride (purity 57.9%; Batch no. WW 262/CP 1490) at concentrations of 0, 290, 2316, or 5790 ppm (equal to 0, 13, 106 and 268 mg/kg bw per day for males, 0,18,146 and 371 mg/kg bw per day for females).

Food consumption and body weight were determined weekly for 14 weeks and every four weeks thereafter. The general state of animal health and mortality were checked twice daily from Monday to Friday and once daily on weekends and public holidays. Detailed clinical examinations of the animals were performed once a week. Blood was collected for differential blood smears at the termination of the study from control and high-dose animals, and additionally from all animals killed in extremis during the study. At the end of the study all surviving rats were killed and a complete necropsy performed on rats from all groups. The weights of the brain, liver, kidneys, adrenal glands and testes were recorded before fixation. Altogether 43 organs and tissues were preserved in 4% formalin for histopathological examination. Histopathological examinations were performed on all animals from control and high-dose groups. In addition, potential key target organs were examined from all groups, including the lungs, liver and kidneys, as were any gross lesions that were identified. Any animals from low- and mid-dose groups that died, or were killed in extremis, were treated histologically in the same way as high-dose animals, and all organs examined.

The cumulative mortalities in males up to day 728 were 46%, 24%, 28% and 18% in the control, low-, mid- and high-dose groups respectively. In females the corresponding values were 34%, 22%, 20% and 26% respectively. Thus it is demonstrated that mortality was not affected by the administration of mepiquat chloride.

In general, mean body weights were consistently lower at all doses for males and at high-dose for females from the beginning of the administration period onwards when compared with control weights. In high-dose males and females the depressions in mean body weight were statistically significant during the overall administration period, with a reduction by 10%–20% compared to controls. In males from the 2316 ppm group, body weight was depressed to a statistically significant extent on several days, especially in the second year of administration. By the end of the study, body weight differences at this dose ranged from 11%–23% in a dose-related manner. In female rats receiving 290 ppm or 2316 ppm, no significant deviations from control values occurred. Regarding body weight change, the values for high-dose groups represented a reduction towards termination by about 23% in males and 29% in females in comparison to the controls, and this was considered biologically relevant. Again, the lowered values seen in males, 15% and 11% for mid- and low-dose groups respectively, were statistically significant (Table 24). At the mid- and low-dose mean body weights were lower than for controls only in the male groups to an extent that was statistically significant. However, when comparing the male body weight data of the present carcinogenicity study with the body weight data of the parallel running chronic toxicity study that used the same dose levels, the growth curves for mid- and low-dose males were comparable between the studies, and it was obvious that they did not differ if compared with the controls from the chronic toxicity study. The statistically significant differences between control group and the low-dose or mid-dose group resulting from this (numerical) increase in control group weights towards the end of the study are therefore not biologically relevant and were assessed as incidental. The highest dose led to a clear impairment in food consumption and body weight in both sexes. However, in males of the mid- and low-dose groups, mean body weights were statistically lower than for controls.

*Table 24. Carcinogenicity study (24-month) in male and female rats: selected body weights (10 out of 37 observations), mean weight (g), [bracketed numbers are percentage of control value]*

| | Sex, dose level (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | 0 | 290 | 2316 | 5790 | 0 | 290 | 2316 | 5790 |
| Study day | Body weight (g) | | | | | | | |
| 7 | 57.6 | 56.2 | 54.6* | 37.8** [66%] | 26.3 | 28.4 | 27.3 | 21.5** [82%] |
| 35 | 197.9 | 194.3 | 187.7* | 159.1** [80%] | 82.3 | 87.6 | 85.9 | 72.9** [89%] |
| 63 | 277.8 | 272.2 | 265.3 | 225.6** [81%] | 120 | 122.8 | 122.3 | 102.6** [86%] |
| 91 | 331.4 | 323.2 | 314.4* | 267.4** [81%] | 144.7 | 145.3 | 141.6 | 120.6** [83%] |
| 126 | 377.3 | 367.6 | 358.1* | 306.5** [81%] | 161 | 158.1 | 155.9 | 132.4** [82%] |
| 182 | 412.7 | 402.2 | 392.3 | 337.0** [82%] | 170.4 | 166.8 | 164.9 | 140.3** [82%] |
| 378 | 520.1 | 502.2 | 481.6** [93%] | 416.8** [80%] | 208.6 | 205.8 | 196.9 | 163.5** [78%] |
| 546 | 586.8 | 547.0* | 538.1** | 454.8** [78%] | 248.6 | 234.8 | 228.6 | 186.2** [75%] |
| 714 | 585.2 | 536.2 | 510.6** [87%] | 466.0** [80%] | 274.3 | 269.2 | 256 | 184.3** [67%] |
| 728 | 597.3 | 531.8** [89%] | 508.4** [85%] | 459.8** [77%] | 259.2 | 269.3 | 253 | 184.0** [71%] |

Statistically significant at: * $p < 0.05$; ** $p < 0.01$; Source: Mellert & Hildebrand,1994d

Haematology did not reveal any changes in red or white blood cell parameters that could be attributed to treatment. At the end of the administration period no substantial differences were seen between animals of the control and the highest dose group (5790 ppm) from the results of the differential white blood cell count or in the red blood cell morphology, this being the case in both sexes. Any changes seen in the white blood cell count of the prematurely sacrificed animals of both sexes from all test groups were spontaneous, incidental or age-dependent and could not be associated with the test substance administered. Changes observed in the red blood cell morphology were noted both in the animals of the control group and in the animals of the dose groups, therefore, these changes are assessed as being spontaneous or age-dependent.

Significant differences in absolute or relative organ weights were recorded in brain, liver and kidneys. In the brain, an increase in the relative group mean value was recorded in both sexes receiving the highest dose. A decrease in absolute liver weight was recorded for males of the 2316 ppm group, and for both sexes receiving 5790 ppm (decreased by 17% and 16% in males and females, respectively). The kidneys from the 5790 ppm dose group showed a depressed absolute weight in males, whilst the relative kidney weight was increased in females, with respect to controls. All these organ weight changes were considered to be indirectly related to treatment, but only as a consequence of the decrease in terminal body weight (Table 25). Decreased absolute and relative adrenal weights were seen at all doses in males, however the results were highly variable, and did not achieve statistical significance.

*Table 25. Carcinogenicity study (24-month) in rats: mean organ weights at termination after 24 months*

| | Sex, dose level (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Parameter** | **0** | **290** | **2316** | **5790** | **0** | **290** | **2316** | **5790** |
| | **Absolute weight (g), [% of control value]** | | | | | | | |
| Terminal body weight | 747.4 | 681.4** | 657.2** [88%] | 611.5** [82%] | 380.6 | 383.3 | 372.1 | 305.6** [80%] |
| Liver | 21.322 | 19.963 | 19.394* | 17.607** [83%] | 12.088 | 11.809 | 11.295 | 10.149** [84%] |
| Kidneys | 4.55 | 4.338 | 4.288 | 3.89** [85%] | 2.773 | 2.774 | 2.781 | 2.669 |
| Adrenal glands (mg) | 199.296 | 94.895 [48%] | 93.778 [47%] | 88.512 [44%] | 205.758 | 141.615 [69%] | 205.9 | 233.811 [114%] |
| | **Relative weight, (% of body weight), [relative weight as % of control value]** | | | | | | | |
| Brain | 0.306 | 0.355 [116%] | 0.353 [115%] | 0.365* [119%] | 0.562 | 0.543 | 0.56 | 0.672** [120%] |
| Testes | 0.936 | 1.041 [111%] | 0.922 | 1.086 [116%] | – | – | – | – |
| Kidneys | 0.619 | 0.648 | 0.664 | 0.64 | 0.746 | 0.737 | 0.759 | 0.89** [119%] |
| Adrenal glands | 0.029 | 0.014 [48%] | 0.015 [52%] | 0.015 [52%] | 0.054 | 0.037 [69%] | 0.057 | 0.085 [157%] |

Statistically significant at: * $p < 0.05$; ** $p < 0.01$; Source: Mellert & Hildebrand,1994d

Histopathologically, there was no morphological evidence of any oncogenic or systemic toxic potential from the test substance. There were a few non-neoplastic findings; these included alveolar mineralization and alveolitis in the lungs, vacuolated foci and eosinophilic foci in the liver, ground cell infiltration with pelvic mineralization, and tubular atrophy in the kidney. These all occurred with greater frequency in high-dose rats than in controls and were considered related to the reduced physical condition of these rats, being therefore of no toxicological significance. On gross examination, the adrenals of control animals appeared larger than normal. There were no adverse histopathological findings in the adrenals of males. As for neoplastic findings, the incidences of mammary gland adenocarcinoma, urothelial papilloma in the urinary bladder, glioblastoma in the brain, stromal polyp in the uterus/cervix, stromal polyp in the uterus/cervix and lung carcinoma metastases were all age-related and within the historical control range (Table 26), so these tumours were considered not to be related to treatment. There was no evidence of carcinogenicity.

*Table 26. Carcinogenicity study (24-month) in rats: summary of primary neoplasms at 104-weeks*

| | Sex, dose level (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Observations** | **0** | **290** | **2316** | **5790** | **0** | **290** | **2316** | **5790** |
| Number of rats per group | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| Lungs: number examined | 50 | 13 | 15 | 50 | 50 | 22 | 22 | 50 |
| Carcinoma | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Squamous carcinoma | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Urinary bladder: number examined | 50 | 15 | 17 | 50 | 50 | 11 | 9 | 50 |
| Urothelial papilloma | 1 | 0 | 2 | 3 | 0 | 0 | 0 | 0 |
| Leiomyosarcoma | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |

| | Sex, dose level (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Observations** | **0** | **290** | **2316** | **5790** | **0** | **290** | **2316** | **5790** |
| Uterus/cervix: number examined | 0 | 0 | 0 | 0 | 50 | 19 | 24 | 50 |
| Adenoma | - | - | - | - | 1 | 0 | 0 | 0 |
| Adenocarcinoma | - | - | - | - | 0 | 0 | 1 | 2 |
| Squamous carcinoma | - | - | - | - | 0 | 0 | 0 | 1 |
| Stromal polyp | - | - | - | - | 2 | 1 | 4 | 4 |
| Adenomatous polyp | - | - | - | - | 0 | 0 | 1 | 0 |
| Fibroma | - | - | - | - | 0 | 0 | 0 | 1 |
| Leiomyoma | - | - | - | - | 0 | 1 | 0 | 1 |
| Schwannoma | - | - | - | - | 2 | 1 | 4 | 0 |
| Haemanqiosarcoma | - | - | - | - | 0 | 0 | 1 | 2 |
| Haemangiopericytoma | - | - | - | - | 0 | 0 | 0 | 1 |
| Stromal sarcoma | - | - | - | - | 2 | 0 | 0 | 1 |
| Mammary gland: number examined | 0 | 0 | 0 | 2 | 47 | 16 | 14 | 50 |
| Adenoma | - | - | - | 0 | 0 | 0 | 1 | 0 |
| Cystadenoma | - | - | - | 0 | 0 | 2 | 1 | 1 |
| Fibroadenoma | - | - | - | 1 | 6 | 3 | 3 | 5 |
| Adenocarcinoma | - | - | - | 1 | 4 | 1 | 3 | 6 |
| Fibroma | - | - | - | 0 | 0 | 0 | 2 | 0 |
| Haemangiosarcoma | - | - | - | 0 | 1 | 0 | 0 | 0 |

Statistically significant at: * $p < 0.05$; ** $p < 0.01$; Source: Mellert & Hildebrand,1994d

The NOAEL for chronic toxicity in this study was 2316 ppm (equal to 106 mg/kg bw per day) based on reductions in body weight at 5790 ppm (equal to 268 mg/kg bw per day) (Mellert & Hildebrand,1994c).

The NOAEL for carcinogenicity in this study was 5700 ppm (equal to 268 mg/kg bw per day), the highest dose tested (Mellert & Hildebrand,1994d).

*Study 3*

In a combined toxicity and carcinogenicity study, groups of 35, 50, 55 or 105 male and female Sprague Dawley rats were administered mepiquat chloride in the diet for up 104 weeks at concentrations of 0, 100, 300, 1000, 3000 and 9000 ppm (equal to 0, 6, 18, 62, 186 and 684 mg/kg bw per day for males, 0, 7, 21, 72, 212 and 670 mg/kg bw per day for females). Groups of five male and five female animals from the 0, 3000 and 9000 ppm dose groups were designated for an interim kill at 52 weeks. No rationale for the group sizes was presented.

Animals were observed daily throughout the administration period for changes in behaviour and general appearance. All animals were palpated once a week from week 26 onwards to examine for potential tumour growth. Food consumption was monitored daily and body weights measured weekly; however both were only recorded at four-week intervals.

Haematology investigations (Hb content, erythrocytes, leucocytes, differential count and Ht value) were carried out on 10 animals of each sex per dose before the start of the study and at weeks 6, 13, 26, 52, 78 and 104 after the start of treatment, with the exception of thromboplastin time, blood clotting time, platelets and reticulocyte counts, which were not determined on weeks 6 and 78.

Clinical chemistry examinations consisted of measuring the activities of ALT, alkaline phosphatase (ALP), and levels of blood urea and glucose. These assays were performed before first administration, then after 6, 13, 26, 52, 78 and 104 weeks of treatment using 10 animals of each sex per

dose. After 13, 26, 52 and 104 weeks of treatment, sodium, potassium, calcium, chloride, AST, uric acid, total protein, total bilirubin and creatinine were determined. After 26, 52 and 104 weeks of treatment, albumin and globulin were determined, and after 52 and 104 weeks of treatment, free cholesterol, total cholesterol, nonesterified fatty acids, esterified fatty acids, total lipids, phosphatides, triglycerides and iodine in hormone were determined. Urinalysis, including colour, specific gravity, protein, glucose, bilirubin, Hb, ketone bodies and pH, was carried out on 10 animals of each sex per dose before the start of the study and at 6, 13, 26, 52, 78 and 104 weeks after the start of treatment.

Ophthalmological, auditory and dental testing was performed on five animals of each sex at dose levels 0, 3000 and 9000 ppm after 52 weeks of treatment, and then at the end of the study for all surviving animals. At the end of 52 weeks an intermediate dissection was performed on five animals of each sex at 0, 3000 and 9000 ppm; this was accompanied by a gross examination, determination of organ weights (heart, liver, lungs, spleen, kidney, adrenal, thymus, pituitary, gonads, thyroid, brain) and a histopathological examination of 31 organs from each animal. At termination of the treatment, all surviving animals were euthanized and subjected to necropsy, organs were weighed, and histopathological examinations performed in the same way as in the 52-week intermediate study. With respect to animals found dead during the treatment period, all such animals were subjected to necropsy and histopathological examination.

Occasionally deaths occurred from the fourth test month on, increasingly during the last two months. After 104 weeks the mortality rates were as shown in Table 27.

***Table 27. Carcinogenicity study in rats; mortality rates after 104 weeks at various dose levels***

| | Mortality rate in % | | |
|---|---|---|---|
| **Dose level (ppm)** | **Males** | **Females** | **Males + females** |
| 0 | 28 | 19 | 23.5 |
| 100 | 30 | 10 | 20.0 |
| 300 | 22 | 18 | 20.0 |
| 1000 | 20 | 18 | 19.0 |
| 3000 | 22 | 22 | 22.0 |
| 9000 | 20 | 20 | 20.0 |

Source: Leuschner et al.,1979b

There were no treatment-related effects on behaviour or external appearance in any group of either sex. The faeces were normal. However, in most cases there was a prelethal period lasting several days in which apathy and a lack of appetite was observed. One rat showed cachexia, and almost all of the animals that died prematurely had a dishevelled coat. According to observations from this study, tumours and pulmonary, cardiac, gastric, renal and hepatic lesions were found to be the main causes of death. With respect to nature, extension and frequency, the tumours were within the normal range for aging Sprague Dawley rats of this strain (Table 28). Thus, the mortality was not attributed to the administration of mepiquat chloride.

***Table 28. 104-week combined toxicity and carcinogenicity study in rats: Tumour rate of mepiquat chloride in different concentration groups***

| | Tumour rate as % (referring to tumour bearing animals) | | |
|---|---|---|---|
| **Dose level (ppm)** | **Males** | **Females** | **Males + females** |
| 0 | 49 | 32 | 40 |
| 100 | 46 | 30 | 38 |
| 300 | 42 | 36 | 39 |
| 1000 | 44 | 36 | 40 |
| 3000 | 46 | 34 | 40 |
| 9000 | 47 | 30 | 38 |

Source: Leuschner et al.,1979b

During 104 weeks of treatment with mepiquat chloride in the food at 100, 300 and 1000 ppm body weight (Table 29) did not differ from those of the controls. At a dose of 3000 ppm body weight gain was inhibited in both sexes months 3–4 onwards, but all results remained within limits that denied them any statistical significance. The highest dose of 9000 ppm inhibited body weight gain to a greater extent, also from the months 3–4 onwards. The surviving males and females at terminal sacrifice showed significantly lower weights in comparison to the untreated rats, males by ca 11%, females by ca 14%. Corresponding to the changes in body weight, the three groups fed lower doses of mepiquat chloride (100, 300 and 1000 ppm) maintained a normal consumption of food and drinking water throughout the study. At the beginning of the experiment, rats in the two highest dose groups (3000 and 9000 ppm) fed in a relatively normal manner. However, from the beginning of month 3–4, appetite was suppressed in both sexes at these higher doses.

***Table 29. Combined toxicity and carcinogenicity study (104 weeks) in rats: selected body weights for males and females, (mean weight (g) ± standard deviation)***

| | Sex, time point (weeks) | | | | | |
|---|---|---|---|---|---|---|
| | Males | | | Females | | |
| Dose level (ppm) | 0, Start | 52 | 104 | 0, Start | 52 | 104 |
| 0 | 101.4 ± 1.5 | 543.8 ± 52.6 | 564.2 ± 79.3 | 101.9 ± 1.4 | 355.1 ± 33.4 | 372.8 ± 50.3 |
| 100 | 101.8 ± 1.5 | 551.2 ± 59.6 | 585.6 ± 82.8 | 102.0 ± 1.5 | 355.4 ± 33.2 | 379.6 ± 61.9 |
| 300 | 102.2 ± 1.3 | 564.8 ± 50.2 | 583.3 ± 82.0 | 101.9 ± 1.3 | 352.7 ± 30.0 | 369.1 ± 64.8 |
| 1000 | 101.8 ± 1.2 | 549.7 ± 47.9 | 570.2 ± 74.5 | 102.0 ± 1.2 | 349.8 ± 32.1 | 384.2 ± 52.5 |
| 3000 | 101.8 ± 1.4 | 518.3 ± 44.6 | 569.8 ± 64.8 | 101.7 ± 1.1 | 323.6 ± 36.0 | 372.6 ± 47.6 |
| 9000 | 101.5 ± 1.4 | 495.7 ± 63.0 | 500.8 ± 88.8 | 101.5 ± 1.4 | 325.4 ± 34.3 | 320.1 ± 38.5 |

Source: Leuschner et al.,1979b

The limited haematological information obtained from blood smears did not point to adverse effects. No definite changes in the blood profile (Hb content, red blood cell, white blood cell and differential counts) were observed in the treated rats during the 104 weeks. Meanwhile there were occasional and transient alterations in some clinical chemistry parameters, but very few were presumed treatment-related, given that they were highly variable and reversible. In the group of females receiving 100 ppm, α1-immunoglobulin was increased after 52 weeks. This finding is obviously an incidental one, as normal values were present at all other sampling times and at all higher dose concentrations. In addition, the activities of the serum ALT and AST were within the range of natural variation, whilst that of ALP was significantly increased in the female rats at 3000 ppm after 52 weeks. Since ALP values varied very greatly and the increase was only slight, it cannot be determined whether any difference was caused by treatment or falls within the bounds of natural variation.

***Table 30. Combined toxicity and carcinogenicity study (104 weeks) in rats: selected clinical biochemistry values for males and females***

| | Sex, time point (weeks) | | | | | |
|---|---|---|---|---|---|---|
| | Male | | | Female | | |
| Dose level (ppm) | 0, Start | 52 | 104 | 0, Start | 52 | 104 |
| | ALT activity (U/L) | | | | | |
| 0 | 65.6 ± 9.3 | 64.2 ± 10.3 | 60.3 ± 8.2 | 65.1 ± 8.9 | 64.7 ± 8.6 | 62.4 ± 10.5 |
| 100 | 63.4 ± 6.8 | 62.4 ± 7.7 | 61.7 ± 9.2 | 64.8 ± 9.4 | 62.8 ± 11.7 | 59.7 ± 10.6 |
| 300 | 66.0 ± 7.8 | 57.5 ± 7.1 | 61.1 ± 11.1 | 63.9 ± 9.0 | 61.9 ± 8.1 | 54.2 ± 11.4 |
| 1000 | 54.0 ± 10.6 | 63.1 ± 9.3 | 60.9 ± 8.9 | 63.2 ± 9.2 | 59.7 ± 11.7 | 65.4 ± 7.7 |
| 3000 | 63.0 ± 9.2 | 58.4 ± 11.6 | 65.3 ± 14.4 | 62.6 ± 5.9 | 61.1 ± 9.4 | 62.1 ± 12.3 |
| 9000 | 64.6 ± 9.8 | 59.6 ± 9.4 | 59.8 ± 10.9 | 60.9 ± 11.6 | 60.0 ± 5.6 | 59.6 ± 10.0 |

| | Sex, time point (weeks) | | | | | |
|---|---|---|---|---|---|---|
| | Male | | | Female | | |
| Dose level (ppm) | 0, Start | 52 | 104 | 0, Start | 52 | 104 |
| | ALP activity (U/L) | | | | | |
| 0 | 219.1 ± 21.3 | 86.2 ± 11.5 | 76.1 ± 15.0 | 182.5 ± 20.9 | 69.5 ± 13.3 | 61.0 ± 4.2 |
| 100 | 224.6 ± 27.8 | 92.5 ± 17.5 | 76.0 ± 20.2 | 177.9 ± 13.9 | 78.2 ± 18.3 | 75.1 ± 9.8 |
| 300 | 226.4 ± 23.4 | 91.7 ± 12.6 | 76.3 ± 15.1 | 184.4 ± 11.0 | 73.6 ± 7.5 | 62.0 ± 12.6 |
| 1000 | 219.6 ± 22.8 | 95.1 ± 14.4 | 80.0 ± 19.3 | 183.0 ± 21.2 | 89.3 ± 8.7 | 65.8 ± 12.9 |
| 3000 | 219.4 ± 21.3 | 91.8 ± 10.3 | 68.6 ± 13.8 | 181.2 ± 12.0 | 98.0 ± 18.4 | 75.1 ± 15.5 |
| 9000 | 208.7 ± 17.3 | 80.2 ± 12.6 | 69.7 ± 10.3 | 177.2 ± 16.8 | 66.7 ± 9.4 | 66.4 ± 10.1 |
| | AST activity (U/L) | | | | | |
| 0 | 86.6 ± 8.9 | 79.4 ± 12.2 | 69.9 ± 11.9 | 84.2 ± 10.2 | 78.1 ± 11.5 | 76.9 ± 12.9 |
| 100 | 83.7 ± 11.0 | 76.9 ± 12.0 | 66.7 ± 9.3 | 85.3 ± 11,9 | 84.1 ± 13.0 | 68.3 ± 11.5 |
| 300 | 83.3 ± 7.0 | 86.7 ± 7.4 | 72.3 ± 11.6 | 86.3 ± 9.7 | 90.4 ± 8.5 | 74.9 ± 12.4 |
| 1000 | 87.1 ± 9.4 | 86.1 ± 9.2 | 76.4 ± 15.4 | 83.8 ± 13.4 | 83.8 ± 10.8 | 77.1 ± 11.6 |
| 3000 | 87.1 ± 6.9 | 83.7 ± 10.8 | 74.9 ± 15.9 | 86.4 ± 13.0 | 84.6 ± 12.8 | 74.3 ± 13.2 |
| 9000 | 76.0 ± 9.9 | 71.8 ± 9.1 | 73.5 ± 11.6 | 72.3 ± 11.7 | 67.7 ± 10.0 | 71.6 ± 15.0 |

Source: Leuschner et al.,1979b

There were no treatment-related effects on behaviour or external appearance in any group of either sex.

At necropsy, there were no treatment-related changes in gross findings. Single findings in individual organs obviously fell within the bounds of natural variation.

Comparison of organ weights with controls revealed a reduction in the thymus weight of females receiving 100 ppm, furthermore heart, liver and kidney were in most cases significantly decreased in females of the 9000 ppm group. The findings at this highest concentration must be viewed in parallel with the decrease observed in body weight. Male rats showed increased heart weight at 100, 300 and 1000 ppm in comparison with concurrent controls. No historical control information was provided. Apart from this, reductions were recorded in thyroid weight (at 100, 1000 and 3000 ppm), adrenal weight (1000, 3000 and 9000 ppm), and the brain (at 9000 ppm). Histopathological investigation of the adrenal did not show substance-related changes. Kidney weight in 3000 ppm males was increased compared to controls. Altogether the values showed the typical high deviation expected for aged Sprague Dawley rats.

The NOAEL for chronic toxicity was 3000 ppm (equal to 186 mg/kg bw per day) based on the adverse effect on body weight and body weight gain in both sexes at the highest dose of 9000 ppm. (equal to 684 mg/kg bw per day) (Leuschner et al., 1979b).

It should be emphasized that Leuschner et al., (1979b) was conducted before mandatory GLP requirements and many of the requirements in the modern guidelines were not met. In addition, there were too few animals in 9000 ppm, high-dose group.

## 2.4 Genotoxicity

The results of studies on the genotoxicity of mepiquat chloride are summarized in Tables 31 and 32.

### (a) In vitro studies

A range of in vitro studies was conducted to assess the genotoxicity of mepiquat chloride at purities ranging from 60.0% to 99.86%, and to assess its potential for inducing chromosomal aberration and damage, and gene mutation (summarized in Table 31). There was no evidence of genotoxicity.

*Table 31. Overview of in vitro genotoxicity tests with mepiquat chloride*

| End-point | Test object | Concentration/ doses tested | Purity | Results | Reference |
|---|---|---|---|---|---|
| Bacterial reverse mutation | *Salmonella typhimurium* TA1535, TA1537, TA1538, TA98, TA100 | 4, 20, 100, 500 and 2500 µg/plate in distilled water; ± S9. | 99.8% | Negative | Engelhardt & Zeller, 1979 |
| Bacterial reverse mutation | *Salmonella typhimurium* TA1535, TA100, TA98, TA1537<br>*Escherichia coli* WP2 *uvrA* | 156.3, 312.5, 625, 1250, 2500 and 5000 µg/plate in distilled water; ± S9 | 99.6% | Negative | Nishibe & Kanaguchi, 1990a |
| DNA damage repair | *Bacillus subtilis* (H17 Rec$^+$), (M45 Rec$^-$) | 2484, 4968, 9935, 19 870 and 39 740 µg/disk in distilled water; −S9<br>1242, 2484, 4968, 9935 and 19 870 µg/disk in distilled water; +S9 | 99.6% | Negative | Nishibe & Kanaguchi, 1990b |
| Mammalian gene mutation | Chinese hamster ovary (CHO) cells | Experiment 1: 162.5, 325, 650, 1300 and 2600 µg/mL in aqueous solution: +30% S9 in the mix<br>Experiment 2: As Experiment 1 but +10% S9 in the mix. | 617.6 g/L [a] | Negative | Engelhardt & Leibold, 2002a |
| Chromosomal aberration | CHO cells | 2.0, 3.0, 4.0 and 5.0 mg/mL in water; ±S9 | >99% | Negative | Taalman, 1987 [b] |
| Unscheduled DNA synthesis | Rat hepatocytes | Trial 1: 2 hours treatment; 25.6, 51.2, 102, 256, 512 and 1020 µg/mL in culture medium.<br>Trial 2: 2 hours treatment; 1000, 2000 and 3000 µg/mL in culture medium. | 99.86% | Negative | Cifone, 1987 |
| Micronucleus induction | Human lymphocytes | 4 hours treatment; 0, 375, 750 and 1500 µg/mL; ± S9<br>24 hours treatment: 0, 375, 750 and 1500 µg/mL; ± S9: | 60% in aqueous solution | Neither clastogenic nor aneugenic | Morris, 2019 [c] |

[a] See certificate of analysis, dated August 21, 2000 (not provided);

[b] This is a very old study, with limited relevance;

[c] Compliant with current OECD standards;

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*

### (b) In vivo studies

A micronucleus assay was performed in mice to assess the potential of mepiquat chloride to induce clastogenic or aneugenic changes in vivo (Table 32). A dominant lethal assay in mice was also provided. There was no evidence of genotoxicity.

*Table 32. Overview of in vivo genotoxicity tests with mepiquat chloride*

| End-point | Test object | Concentration tested | Purity | Results | Reference |
|---|---|---|---|---|---|
| Micronucleus assay in mice | Male NMRI mice bone marrow cells | Two administrations 24 hours apart at 0, 250, 500, 750 and 1000 mg/kg bw in distilled water (oral administration); 24 hour sampling time | 617.6 g/L[a] | Negative | Engelhardt & Leibold, 2002b |
| Dominant lethal assay | Male NMRI mice | 0, 100, 300, 1000, 3000 ppm (equal to 0, 26.1, 78.5, 268.2 and 801.6 mg/kg bw) in the diet (oral administration) | 94% | Negative | Leuschner, 1977 |

[a] See certificate of analysis, dated August 21, 2000 (not provided); bw: Body weight

Mepiquat chloride was tested in an adequate range of studies and overall, the outcome of these studies fulfils the basic requirements to cover the three genetic end-points (gene mutations and structural and numerical chromosome aberrations) and it was concluded that mepiquat chloride is unlikely to be genotoxic.

## 2.5 Reproductive and developmental toxicity

### (a) Multigeneration studies

*Study 1*

In a non-guideline screening study investigating the effects of mepiquat chloride on male and female reproduction, mepiquat chloride (purity 59.6%; batch FRE-002008) was administered at 0, 305, 1220 or 4880 ppm mepiquat chloride (corrected for purity and actual administration) in the diet to 10 Han Wistar rats of each sex per group (equal to 0, 20, 115 and 420 mg/kg bw per day for males, 0, 34, 134 and 474 mg/kg bw per day for females (premating), for 14 days prior to mating, and until study day 28 for males or until lactation day (LD) 13 for females. Animals were observed for general health and mortality twice daily, with a detailed clinical examination conducted once daily throughout the study. Animals were weighed twice weekly, with females weighed on gestation days (GDs) 1, 4, 7, 11, 14, 17 and 20 and on LDs 1, 4, 7, 10, 13 and 14. Food consumption was measured until cohabitation, and female food consumption was recorded on GDs 0, 4, 7, 11, 14, 17 and 20 and LDs 1, 4, 7, 10 and 13. Blood samples were taken from five rats of each sex per group at scheduled necropsy, on which haematology and serum chemistry examinations were conducted. Blood samples were also collected for thyroid hormone analysis. Litters were observed for mortality twice daily. A detailed clinical observation of pups (F1) was performed on postnatal days (PNDs) 1, 4, 7, 10 and 13. Pups were sexed on days 0, 4 and 13, and weighed on days 1, 4, 7, 10 and 13. Anogenital distance was measured on day 1. On day 13, male pups were evaluated for the presence of nipples and areolae. After scheduled sacrifice, a complete gross pathology examination was made of all dams. Organs were weighed, and tissues collected and preserved.

All parental animals survived,and no substance-related clinical observations were noted at the daily examinations. Lower mean body weight gains were seen at the high dose in both males and females, resulting in male body weight at 4880 ppm being 11% lower than control group on study day 28, and female body weight at 4880 ppm being 15.5% lower than controls on GD 20. Body weight gains for females at the high dose were comparable to controls throughout lactation, however mean absolute body weight remained between 10.3% and 13.4% lower than control weight during LDs 1–13. These effects on body weight in both sexes were considered treatment-related and correlated with decreased food consumption in these groups.

There were no effects on reproductive performance, gestation length, parturition, haematology, serum chemistry, or thyroid hormone levels at any dose. No treatment-related findings were observed on gross pathology or on microscopic examination of organs, and there were no effects on organ weight. The mean number of implantation sites was lower in females fed at 4880 ppm (10.2 sites per female) in comparison to control groups (12.0 and 13.0 sites per female), resulting in a lower mean number of pups born in the highest dose group. While pup body weights during PNDs 1–4 were comparable between groups, lower mean pup body weight gain at 4880 ppm was observed during PNDs 4–13, resulting in

lower pup body weight compared to controls (by up to 17%). No effects were observed on anogenital distance or areolae/nipple anlagen retention, and no effects on thyroid hormones in pups.

Overall, the NOAEL for parental toxicity in this study was 1220 ppm in the diet (equal to 115 mg/kg bw per day) based on decreased body weight, body weight gain and food consumption. No NOAEL for male reproductive effects was established, as the dosing period was insufficient, while the NOAEL for reproductive effects in females was 1220 ppm in the diet (equal to 115 mg/kg bw per day), based on decreased a number of implantation sites and live pups at 4880 ppm (equal to 134 mg/kg bw per day) (Millard, 2022).

*Study 2*

In a multigeneration reproduction study, mepiquat chloride (purity: 57.9%; Batch no. WW 262/CP 1490) was administered to Wistar rats in the diet at nominal concentrations of 0 (control), 500, 1500 or 5000 ppm mepiquat chloride (based on 864, 2591 and 8636 ppm of test substance) using 25 rats of each sex per group with two matings for the first generation and one for the second. These doses resulted in exposure to the test substance being, in the F0 generation:

0, 51.2, 153 or 500 mg/kg bw per day for F0 males;

0, 54, 164 or 530 mg/kg bw per day for premating F0 females;

0, 42–46, 128–137 or 433–460 mg/kg bw per day for F0 females during gestation;

0, 70–80, 198–228 or 625–662 mg/kg bw per day for F0 females during lactation (days 0–14 postpartum only).

In the F1 generation, substance intake was:

0, 48.6, 147 or 575 mg/kg bw per day for F1 males,

0, 53.3, 162 or 627 mg/kg bw per day for premating F1 females;

0, 45.6, 135 or 486 mg/kg bw per day for F1 females during gestation;

0, 72.7, 224 or 715 mg/kg bw per day for F1 females during lactation (days 0–14 only).

F0 generation rats were treated continuously, with dosing commencing at least 70 days prior to mating. At least 10 days after weaning of the F1a generation at postpartum day (PPD) 21, F0 parental animals were mated again (avoiding mating of the same partners used in the first generation). Following weaning of the F1b generation at PPD 21, F0 parental animals were sacrificed and examined. Prior to weaning, 25 rats of each sex per group of the F1a generation were selected as the basis for the F1 generation parental animals. They were exposed continuously at the relevant dose and were mated at least 98 days after weaning. Resulting pups (the F2 generation) were raised until weaning. Standardization of all litters was done on PPD 4 to ensure, where possible, that each litter contained four male and four female pups. Where this was not possible, litters were standardized to eight pups per litter with as near equal sex distribution as possible.

Food consumption was determined weekly for both sexes, for days 0–7, 7–14 and 14–20 postconception for females, and again over PPDs 0–4. 4–7 and 7–14. Food consumption was not determined between days 14 and 21 after parturition, as it was reasoned that food might be consumed by both dams and pups. Body weight was measure weekly premating and during gestation, and in dams on PPDs 4, 7, 14 and 21. Parental animals were scrutinized daily for signs of toxicity. Nesting, littering and lactation behaviour were evaluated daily, with littering behaviour checked in the afternoon. Neurofunctional examinations were carried out premating on all parental (F0 and F1) animals, on all dams during lactation and after weaning, utilizing a functional observation battery (FOB), including general state, tremors, convulsions, piloerection, lacrimation/secretion of pigmented tears, salivation, pupil size, faeces, vocalization, paresis, paralysis, ataxia, body tone, posture, appearance, activity, respiration, urine, skin colour, righting response, behaviour, grip strength, pupillary reflex, sensitivity of the body surface and pain perception. Reproduction performance was assessed in both males and females. Clinical chemistry analysis, haematology and urinalysis were carried out for 12 rats of each sex per group of F0 and F1 parental animals.

Pups were examined as soon as possible on day of birth to determine total pup numbers and the number of live- and stillborn pups, as well as their sex. Litters were checked for dead or moribund pups twice daily and for clinical signs of toxicity daily. Pups were weighed on PPDs 1, 4, 7, 14 and 21, and

checked on PPDs 4, 13 and 15 for achievement of particular development stages. Behavioural tests were done on day 13 (gripping reflex) and day 21 (hearing test and pupillary reflex). Necropsy examinations on pups culled, stillborn and pups dying up to weaning, included external examinations and macroscopic examination of organs, with any abnormalities further investigated.

The mean food consumption of F0 males and females was reduced at 5000 ppm during the premating period, and for females during gestation and lactation in both F1a and F1b litters, along with decreased mean body weights compared with controls. There were no abnormal clinical signs during premating or gestation, however during lactation tremors were observed in 17/25 F1a females and 20/25 F1b females at 5000 ppm. Among the F1a and F1b pups during lactation, tremor was seen in four and ataxia in two at 5000 ppm. No other abnormalities were observed on neurofunctional examination. Increased creatinine concentrations were detected in females receiving 5000 ppm.

There were no effects on female mating indices or duration until sperm was detected. Fertility was slightly lower at 500 ppm (92%) and at 5000 ppm (80%) in the F1a mating interval, however for the F1b mating interval no treatment-related effects were observed, with one female at 500 ppm failing to become pregnant (96%). The duration of gestation was slightly shorter in the 5000 ppm group compared to concurrent and historical controls. There were no differences in mean pup number or live birth index.

During lactation increased mortality or cannibalization was seen at 5000 ppm, particularly during PPDs 0–7, resulting in a decreased survival rate. There were no effects on sex distribution or sex ratios of pups either on the day of birth or PPD 21 postpartum. Pup body weight gain was reduced throughout lactation at 5000 ppm, with weights lower than for controls on PPD 21. Pups at 5000 ppm were slower to reach morphological development stages such as pinna unfolding, auditory canal opening and eye opening than were controls. At 5000 ppm, decreased relative liver weights in males and increased relative kidney weights were seen in females, as well as decreased lipid storage in the livers of both sexes. One pup in the F1b litter at 1500 ppm showed both anophthalmia and hydrocephaly. These abnormalities were seen at a low level in historical controls, and while it cannot be definitively determined, the absence of effects at the high dose suggests that this was not directly related to treatment.

Among the F1 generation parental animals, there were two mortalities in the high-dose group before the beginning of the premating period. Apart from this there were no treatment-related clinical signs for males or females during premating or gestation. During lactation, tremors and hypersensitivity were seen between days 7 and 28 of the lactation/postweaning period. Decreased grip strength was observed at 5000 ppm in F1 males (not statistically significant), and in females at 5000 ppm before mating, during lactation and, to a lesser extent, after weaning of the F2 pups. Decreased food consumption was observed at 5000 ppm in both males and females premating, and in females during gestation and lactation, along with decreased body weights. During lactation, tremors and hypersensitivity were seen between days 7 and 28 of the lactation/postweaning period. Decreased grip strength was observed at 5000 ppm in F1 males (not statistically significant), and in females at 5000 ppm before mating, during lactation and, to a lesser extent, after weaning of the F2 pups. There were no effects on mating indices for the F1 parental animals, however the fertility index for F1 females at 5000 ppm was lower than concurrent controls (although within the historical control range). Decreased ALT activity in males, and decreased globulin concentrations in females were detected at 5000 ppm compared to controls.

The mean number of delivered F2 pups per dam was lower at 5000 ppm than for controls, with no effects on the percentage of liveborn pups, viability or sex ratio. Mean body weight gain was impaired at 5000 ppm. There were no abnormal clinical signs at any dose, however signs of delayed development were seen at 5000 ppm. A small number of pups at 500 or 1500 ppm showed slightly delayed development but this was within the historical control range. In behavioural tests, pups at 5000 ppm showed a lower gripping reflex response than controls.

***Table 33. Mean gestation time (days) of F0 female rats in a multigeneration study***

| | Dietary dose (ppm) | | | | |
|---|---|---|---|---|---|
| **Parameter** | **0** | **500** | **1500** | **5000** | **Historical controls** |
| Duration of gestation (F1a) | 22.0 | 22.0 | 22.0 | 21.6** | 21.7–22.5 |
| Duration of gestation (F1b) | 21.9 | 21.9 | 21.8 | 21.6* | |

Statistically significant at: * $p < 0.05$; ** $p < 0.01$; Source: Hellwig, 1993

*Table 34. Pup body weight changes in F1a and F1b in a multigeneration rat study*

| | Generation/dietary dose group (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | F1a | | | | F1b | | | |
| **Parameter** | **0** | **500** | **1500** | **5000** | **0** | **500** | **1500** | **5000** |
| | | | | **Males** | | | | |
| Day 1 weight (g)<br>% of control | 6.5 | 6.6 | 6.5 | 6.1*<br>94% | 6.6 | 6.8 | 6.6 | 6.2*<br>94% |
| Day 14 weight<br>% of control | 33.2 | 33.0 | 33.2 | 24.1**<br>73% | 33.9 | 34.6 | 33.5 | 24.2**<br>71% |
| Days 1–4 weight gain (g)<br>% of control | 2.7 | 2.8 | 2.7 | 1.3**<br>48% | 2.9 | 3.1 | 2.7 | 1.6**<br>55% |
| Days 4–21 weight gain (g)<br>% of control | 45.5 | 44.9 | 44.2 | 27.5**<br>60% | 46.3 | 46.6 | 44.9 | 29.1**<br>63% |
| | | | | **Females** | | | | |
| Day 1 weight (g)<br>% of control | 6.2 | 6.3 | 6.2 | 5.8<br>94% | 6.2 | 6.4 | 6.3 | 5.9<br>95% |
| Day 14 weight (g)<br>% of control | 33.2 | 32.4 | 32.7 | 23.5**<br>71% | 33.0 | 33.8 | 32.6 | 23.4**<br>71% |
| Days 1–4 weight gain (g)<br>% of control | 2.6 | 2.7 | 2.6 | 1.2**<br>46% | 2.8 | 3.0 | 2.8 | 1.6**<br>57% |
| Days 4–21 weight gain (g)<br>% of control | 43.2 | 43.4 | 41.7 | 26.4**<br>61% | 43.8 | 44.5 | 42.8 | 28.0**<br>64% |

Statistically significant at: * $p < 0.05$; ** $p < 0.01$; Source: Hellwig, 1993

*Table 35. Pup body weight changes in the F2 generation in a multigeneration rat study*

| **Parameter** | **F2 generation/dietary dose group (ppm)** | | | |
|---|---|---|---|---|
| | **0** | **500** | **1500** | **5000** |
| | | | **Males** | |
| Day 1 weight (g)<br>% of control | 6.4 | 6.5 | 6.5 | 6.3<br>98% |
| Day 14 weight<br>% of control | 31.7 | 30.4 | 31.6 | 23.9**<br>75% |
| Days 1–4 weight gain (g)<br>% of control | 2.4 | 2.4 | 2.6 | 1.6*<br>67% |
| Days 4–21 weight gain (g)<br>% of control | 43.1 | 41.0 | 41.9 | 27.2**<br>63% |
| | | | **Females** | |
| Day 1 weight (g)<br>% of control | 6.1 | 6.3 | 6.1 | 6.1<br>100% |
| Day 14 weight<br>% of control | 30.7 | 30.4 | 30.3 | 23.7**<br>77% |
| Days 1–4 weight gain (g)<br>% of control | 2.5 | 2.4 | 2.5 | 1.7*<br>68% |
| Days 4–21 weight gain (g)<br>% of control | 40.7 | 40.1 | 39.5 | 26.1**<br>64% |

Statistically significant at: * $p < 0.05$; ** $p < 0.01$; Source: Hellwig, 1993

Historical control data (from volume IV of the study report) are available for viability and lactation index and have been added to Table 36 below.

*Table 36. Summary of F1a and F1b litter data in a rat multigeneration study*

| Parameter | Generation/dietary dose (ppm) | | | | | | | | Historical control data |
|---|---|---|---|---|---|---|---|---|---|
| | F1a | | | | F1b | | | | |
| | 0 | 500 | 1500 | 5000 | 0 | 500 | 1500 | 5000 | |
| Total number of litters | 25 | 23 | 25 | 20 | 25 | 24 | 25 | 25 | |
| Viability index | 96 | 95 | 94 | 89 | 95 | 98 | 94 | 96 | 89–99 |
| Lactation index | 95 | 99 | 100 | 91 | 100 | 99 | 99 | 97 | 95–100 |

Source: Hellwig, 1993

With one exception, the values for lactation and viability index in the table above were within the historical control ranges and were not different from concurrent controls to any statistically significant extent. However it was noted that the total number of litters at 5000 ppm was smaller than concurrent controls by 20%. Although lactation index was marginally lower than controls for the F1a litter at 5000 ppm, this was not evident in the F1b litter. The large number of pups cannibalized and higher number of pup deaths on days 1–4 at the top dose of 5000 ppm were considered to be a secondary consequence of marked maternal toxicity, including decreased body weight and an increased incidence of tremors during lactation. The lower total number of pups delivered at the top dose in the F1a and F2 litters reflects the lower total number of litters in these groups, and no differences were seen in these end-points in the F1b litter.

The NOAEL s for parental and offspring toxicity were 1500 ppm of mepiquat chloride (equal to 155 mg of active ingredient/kg bw per day) based on decreased food consumption and lowered body weight, as well as tremors in dams during lactation, all at 5000 ppm of mepiquat chloride, (equal to 550 mg of active ingredient per day). The NOAEL for reproductive toxicity was 5000 ppm mepiquat chloride (equal to 550 mg of active ingredient per day), the highest dose tested (Hellwig, 1993).

**(b) Developmental toxicity**

***Rat***

*Study 1*

In a non-GLP developmental toxicity study, Sprague Dawley, Crl:CD®(SD)BR rats were administered mepiquat chloride (purity not stated, Batch no. XXVI/408) at dose levels of 0, 100, 300, 1000 or 3000 ppm of technical material in the diet (equivalent to 0, 6.6, 20, 66 and 200 mg/kg bw per day). Dosing was from day 0 to day 20 after mating (25 rats per group) or from mating until PPD 21 (10 rats per group). Test compound concentration and stability in the food was not determined.

Clinical signs and food consumption was determined daily, and rats were weighed three times per week. Following scheduled sacrifice, a macroscopic assessment of all animals was done. Conception rate, number of corpora lutea and implantation sites were counted, and the number of viable fetuses was established, and their sex determined. Dead implants and early and intermediate resorptions were determined. Fetuses were examined macroscopically, weighed and measured, followed by an examination of soft tissue and skeleton. For animals maintained for postpartum examination, delivery behaviour and delivery date were determined, and the number and sex of the fetuses delivered was established. Pups were examined for clinical signs and developmental signs and were weighed on the day of birth and then on PPDs 7, 14 and 21. Behavioural status was evaluated by behavioural studies, including swimming test and testing on a rotating bar. On PPD 21 animals were sacrificed and examined. The heart, liver, kidneys and spleen were weighed. Visceral and skeletal examinations of the pups were made.

There were no mortalities of parental animals, or abnormal clinical signs observed throughout the study. There were no differences in food consumption or body weight between groups. Conception rate, mean number of corpora lutea and viable implants, as well as the percentage of dead implants were unaffected. There were no effects on placental weight, length and weight of fetuses at any dose level or effects on malformations, variations, or delayed development of fetal organs and skeletons. For the animals retained for postnatal investigation, the date of delivery, delivery behaviour and litter

size showed no test effects related to treatment. The conception rate, mean number of corpora lutea and viable implants, as well as the percentage of dead implants, was unaffected. There were no differences in organ weights compared to controls. The body weight and body weight gain of the pups, as well as their development was unaffected by treatment, nor did the test substance affect viability or behaviour. The pups showed no macroscopic effects of treatment. Organ weights, absolute and relative to body weight were in the normal range, and there were no effects on malformations, variations or delays in development of fetal organs and skeletons.

The NOAELs for maternal and fetal toxicity were 3000 ppm in the diet (200 mg/kg bw per day), the highest dose tested. (Hofmann & Peh, 1977)

*Study 2*

In a developmental toxicity study, groups of 25 mated female Wistar rats were administered mepiquat chloride by gavage (purity 57.9%, Batch no. WW 262/CP 1490) in distilled water at dose levels of 0 (control) 50, 150 or 300 mg/kg bw per day (as active ingredient), from day 6 to day 15 (inclusive) of presumed gestation (post insemination). Analysis of the test substance was confirmed in a later study by Hellwig & Hildebrand (1997). Rats were examined for mortality twice daily and clinical signs at least once per day, and food consumption and body weight were determined daily. On day 20, dams were sacrificed and a macroscopic examination performed. Adjusted body weight was determined, and the uterus and ovaries were examined. The number of corpora lutea, implantation sites, live and dead fetuses and early and late resorptions were determined. Fetuses were weighed, sexed and examined macroscopically. After fixation, either soft tissue or skeletal examination was carried out, and identified abnormalities classified as malformations, variations, retardations or unclassified.

At 300 mg/kg bw per day, clinical signs, including tremor, piloerection, unsteady gait and hypersensitivity were see within the first two hours after dosing, and resolved within two hours after dosing. No mortalities were seen during the study. The food consumption and body weight gain were reduced for the majority of the dosing period (days 6–13 post coitum). Compared with controls, food consumption was reduced by 11% at the high dose over days 6–15 (not statistically significant), and by up to 19% on the first days of dosing (days 6–8), while body weight change was suppressed by about 16% ($p < 0.01$) on days 6–15. Body weight change was also suppressed by 17% on days 6–8, which in this case was not associated with decreased food consumption. At other dose levels, no treatment-related maternal effects were noted.

There were no treatment-related macroscopic findings at necropsy in dams, and gravid uterus weights were not affected by treatment. There were no effects on placental or fetal weights. There were no notable findings on external examination of the fetuses, however one fetus (0.3%) at 150 mg/kg bw per day had unilateral anophthalmia (the historical control value for which was less than 0.01%), and one high-dose fetus (0.3%) displayed microglossia, the historical control value for which was also less than 0.01%. On soft tissue and skeletal examination, several malformations were detected in all groups, and these were not therefore considered treatment-related.

The NOAEL for maternal toxicity was 150 mg/kg bw per day based on clinical signs of toxicity and impaired body weight gain at 300 mg/kg bw per day. The NOAEL for fetal toxicity and teratogenicity was 300 mg/kg bw per day, based on the absence of any adverse findings at the highest test dose in this study (Hellwig & Hildebrand, 1992).

***Table 37. Body weight and food consumption (g) and clinical findings, prenatal toxicity study in rats***

| | Dose (mg/kg bw per day) | | | |
|---|---|---|---|---|
| **Parameter** | **0** | **50** | **150** | **300** |
| Number of animals assessed | 25 | 25 | 25 | 25 |
| Food consumption: day 6–15 post coitum (g) | 25.1 | 25.9 | 25.0 | 22.4 |
| Body weight change (g): days 6–8 | 6.3 | 7.2 | 4.6 | 2.2** |
| days 8–10 | 10.9 | 9.5 | 10.9 | 7.3** |
| days 10–13 | 18.0 | 18.3 | 17.9 | 14.3* |
| day 6–15 post coitum | 45.1 | 48.5 | 45.1 | 37.8** |

| | | | | |
|---|---|---|---|---|
| Tremor during treatment (*n*) | 0 | 0 | 0 | 22 |
| Unsteady gait during treatment (*n*) | 0 | 0 | 0 | 22 |
| Hypersensitivity during treatment (*n*) | 0 | 0 | 0 | 24 |
| Ataxia during treatment (*n*) | 0 | 0 | 0 | 4 |

*n*: Total number of animals; Source: Hellwig & Hildebrand, 1992

Statistically significant at: * $p < 0.05$; ** $p < 0.01$

***Table 38. Summary of selected fetal findings in rat developmental toxicity study with mepiquat chloride, (percentage fetal and litter incidences shown)***

| | | Dose (mg/kg bw per day) | | | | Historical control data | |
|---|---|---|---|---|---|---|---|
| **Finding** | | **0** | **50** | **150** | **300** | **Mean** | **Range** |
| Hydrocephaly: | fetal | 1.6 | 0.7 | 0 | 1.3 | 0.2 | 0–1.6 |
| | litter | 10.0 | 4.5 | 0 | 8.7 | 1 | 0–10 |
| Malformation of great vessels: | fetal | 0 | 0 | 0.7 | 0 | 0.03 | 0–0.8 |
| | litter | 0 | 0 | 4.3 | 0 | 0.2 | 0–5.3 |
| Dilatation of right ventricle: | fetal | 0.8 | 0.7 | 0 | 0 | 0.03 | 0–0.8 |
| | litter | 5.0 | 4.5 | 0 | 0 | 0.2 | 0–5.0 |
| Total soft tissue malformations: | fetal | 1.6 | 0.7 | 0.7 | 1.3 | Comparable HCD not available in study report | |
| | litter | 10 | 4.5 | 4.3 | 8.7 | | |
| Total soft tissue variations: | fetal | 11 | 11 | 13 | 14 | 28.8 | |
| | litter | 50 | 50 | 57 | 61 | 71.9 | |
| Dilated renal pelvis: | fetal | 11 | 11 | 13 | 14 | 28.6 | 7.1–66.9 |
| | litter | 50 | 50 | 57 | 61 | 71.9 | 36.4–100 |
| Hydroureter: | fetal | 6.2 | 4.7 | 2.8 | 6.6 | 6.8 | 1.0–17.9 |
| | litter | 35 | 23 | 17 | 35 | 27.5 | 6.5–64 |
| Total skeletal malformations: | fetal | 2.2 | 4.3 | 7.1 | 4.3 | NA | |
| | litter | 15 | 26 | 26 | 25 | | |
| Total skeletal variations: | fetal | 50 | 42 | 37 | 35 | 40.5 | NA |
| | litter | 100 | 96 | 96 | 83 | 91.3 | |

NA: HCD data not available; Source: Hellwig & Hildebrand, 1992

***Rabbit***

In a developmental toxicity study, groups of 15 inseminated female Himalayan rabbits were administered mepiquat chloride by gavage (purity 56.7%; Batch No. CP028814) in distilled water at dose levels of 0, 50, 100 or 150 mg/kg bw active ingredient per day, from days 7–19 (inclusive) of presumed gestation (post insemination). Animals were checked at least once daily for mortality and clinical signs, and food consumption was determined daily. Animals were weighed on days 0, 2, 4, 7, 9, 11, 14, 16, 19, 21, 24, 25 and 29 post insemination. On day 29, dams were sacrificed and examined. The weight of the unopened uterus, number of corpora lutea and implantation sites were determined. The fetuses were examined macroscopically, weighed, and a visceral and skeletal examination was made.

One female at the top dose delivered early (day 28) and the data from this doe were excluded from the study. There were no other mortalities during the study. Two high-dose rabbits showed blood in the bedding on some days between days 23 and 27. Food consumption by the high-dose rabbits was reduced to a statistically significant extent throughout the treatment period, by 42% in comparison with controls. At 100 mg/kg bw per day, statistically significant reductions in food consumption were seen between days 11 and 14, with a reduction by about 16% compared with controls over the whole period, while the food consumption of low-dose rabbits was not influenced by treatment. A loss of absolute body weight was seen in rabbits receiving 150 mg/kg bw per day between days 7 and 16, however there was compensatory weight gain thereafter. At 100 mg/kg bw per day, there was a reduced mean body weight gain between days 11 and 14, with weight gain over the whole period between 21% lower than controls. At 50 mg/kg bw per day, body weight gain was not affected.

There were no effects on gravid uterus weight. One high-dose female had multiple erosions of the stomach mucosa. No other treatment-related necropsy findings were recorded. There were no effects on the sex distribution of fetuses, and no effects on placental or fetal weights. There were no external malformations. Pseudoanklyosis was seen at all doses and was not considered treatment-related. Hydrocephaly was seen in male fetuses exposed to the low and high doses, but within (or at the top end of) the historical control values. The incidence of skeletal variations at all doses was within the historical control range. Although the incidence of accessory thirteenth ribs was significantly higher than for concurrent controls and outside the historical control range at 150 mg/kg bw per day, there was no evidence of a dose–response relationship and considerable variability in incidence was evident in the control, 10 and 100 mg/kg bw per day dose groups, strongly suggesting that the observed difference at 150 mg/kg bw per day were not treatment-related.

The NOAEL for maternal toxicity was 50 mg of active ingredient/kg bw per day based on reduction in body weight gain during the treatment period at 100 mg of active ingredient/kg bw per day. The NOAEL for fetal toxicity and teratogenicity was 150 mg of active ingredient/kg bw per day, the highest dose tested, (Bachmann et al., 1998).

***Table 39. Mean maternal body weights and body weight change during gestation in a developmental toxicity study in rabbits***

| | Dose (mg/kg bw per day) | | | |
|---|---|---|---|---|
| **Time point/time period** | **0 (control)** | **50** | **100** | **150** |
| Mean body weight (g) | | | | |
| Day 0 | 2642 | 2654 | 2633 | 2639 |
| Day 7 | 2669 | 2683 | 2669 | 2696 |
| Day 11 | 2643 | 2667 | 2628 | 2653 |
| Day 14 | 2684 | 2700 | 2655 | 2628 |
| Day 16 | 2716 | 2729 | 2688 | 2620 |
| Day 19 | 2714 | 2748 | 2705 | 2646 |
| Day 21 | 2712 | 2758 | 2736 | 2655 |
| Mean body weight change over period (g) | | | | |
| Days 11–14 | 41.7 | 33.6 | 0.4** | −24.7** |
| Days 14–16 | 31.4 | 28.9 | 33.7 | −8.4* |
| Days 19–21 | −1.5 | 10.5 | 31.7** | 8.7 |

Source: Bachmann et al., 1998

Statistically significant using Dunnett's test (2-sided) test, at: * $p < 0.05$; ** $p < 0.01$;

***Table 40. Summary of selected fetal findings in rabbit developmental toxicity studies with mepiquat chloride, (percentage fetal and litter incidences shown)***

| | | Dose (mg/kg bw per day) | | | | Historical control data | |
|---|---|---|---|---|---|---|---|
| | | **0** | **50** | **100** | **150** | **Mean** | **Range** |
| Total soft tissue malformations | fetal | 2.2 | 3.1 | 2.8 | 2.9 | 1.7 | 0–3.5 |
| | litter | 14.0 | 20.0 | 20.0 | 14.0 | 10.4 | 0–23.5 |
| Total soft tissue variations | fetal | 25 | 14 | 20 | 21 | 20.9 | 6.3–50.6 |
| | litter | 86 | 60 | 67 | 86 | 69 | 33.3–100 |
| Pseudoanklyosis | fetal | 3.3 | 1.0 | 1.9 | 0 | 1.4 | 0–5.3 |
| | litter | 21.0 | 6.7 | 13.0 | 0 | 6.4 | 0–15.4 |
| Hydrocephaly | fetal | 0 | 1.0 | 0 | 2.0 | 0.3 | 0–1.9 |
| | litter | 0 | 1.0 | 0 | 2.0 | 1.5 | 0–7.7 |
| Total skeletal malformations | fetal | 3.3 | 2.1 | 1.9 | 1.0 | 1.6 | 0– 4.5 |
| | litter | 14 | 13 | 6.7 | 7.1 | 8.3 | 0–28.6 |

| | | Dose (mg/kg bw per day) | | | | Historical control data | |
|---|---|---|---|---|---|---|---|
| | | 0 | 50 | 100 | 150 | Mean | Range |
| Total skeletal variations | fetal | 17 | 20 | 22 | 25 | 14.4 | 4.1–24.8 |
| | litter | 64 | 87 | 73 | 86 | 60.1 | 28.6–86.7 |
| Splitting of skull bones | fetal | 3.3 | 2.1 | 6.6 | 5.9 | 2.3 | 0–7.9 |
| | litter | 21 | 13 | 33 | 36 | 12.6 | 0–42.9 |
| Epactal bone between nasal & frontal | fetal | 2.2 | 6.3 | 3.8 | 6.9 | 1.9 | 0–5.8 |
| | litter | 14 | 40 | 20 | 43 | 10.4 | 0–30 |
| Accessory 13th rib | fetal | 5.4 | 2.1 | 5.7 | 8.8 | 2.2 | 0–6.2 |
| | litter | 29 | 13 | 27 | 50 | 10.7 | 0–26.7 |
| Percentage females | | 51.1 | 54.2 | 55.7 | 65.7 | 49%; (1033/2102 fetuses) | |
| Percentage males | | 48.9 | 45.8 | 44.3 | 34.3 | 51%; (1069/2102 fetuses) | |

Source: Bachmann et al., 1998

## 2.6 Special studies

### (a) Neurotoxicity

*Study 1*

In an acute neurotoxicity study, groups of 10 male and 10 female Wistar rats were administered by gavage mepiquat chloride (purity 617.6 g/L; Batch no. 00/0772-1) in distilled water as a single oral dose at dose levels of 0 (vehicle control), 100, 300 or 1200 mg of test substance/kg bw in a dose volume of 10 mL/kg bw. This corresponded to active ingredient concentrations of 0, 58, 174, and 697 mg of active ingredient/kg bw. The animals were observed for up to two weeks after dosing. The NOAEL was 58 mg of active ingredient/kg bw based on signs of toxicity in the qualitative FOB of and motor activity tests, specifically observations of decreased motor activity in males at 174 mg of active ingredient/kg bw in the early observation period. No abnormalities were detected at any dose during detailed histopathological examination of the nervous system. The clinical effects observed were considered to be related to reactivity with nicotinergic and muscarinergic receptors, however a full mode of action framework analysis had not been conducted. In vitro studies indicated a reversible binding to receptors rather than irreversible neurotoxicity (Mellert, Kaufmann & van Ravenzwaay, 2002a).

*Study 2*

In another study, groups of 10 male and 10 female Wistar (CrlGlxBrlHan:WI) rats received mepiquat chloride (purity 617.6 g/L; Batch no. 00/0772-1) in the diet for 13 weeks at concentrations of 0, 1625, 6500 or 13 000 ppm of the test substance (equivalent to 0, 943, 3770 and 7540 ppm of active substance). This corresponded to daily intakes of 65.6, 259.0 and 516.6 mg of active ingredient/kg bw per day for males and 79.4, 366.9 and 616.5 mg of active ingredient/kg bw per day for females. The NOAEL was 7540 ppm of active substance (516.6 mg of active ingredient/kg bw per day for males, 616.5 mg of active ingredient/kg bw per day for females), based on the absence of neurotoxicity at the highest test dose, including an absence of histopathological findings. However, the overall NOAEL in the three-month dietary study with neuropathological assessments was 943 ppm of active substance (65.6 mg of active ingredient/kg bw per day for males, 79.4 mg of active ingredient/kg bw per day for females) based on impaired body weight gain at 3770 ppm
(Mellert, 2003; Mellert, Kaufmann & van Ravenzwaay, 2002b; amendment in 2002c).

*Study 3*

Test substance BAS 083 W (mepiquat chloride, purity 56.7%; Batch No. CP028814) was tested for its effect on the embryonic, fetal and postnatal development of the nervous system in Wistar rats. The test substance solutions were administered to 40 presumed-pregnant female rats per dose group by gavage at doses of 0, 15, 30 or 60 mg/kg bw in double-distilled water from day 6 post coitum until PPD 10. After parturition only those litters which consisted of at least eight pups, and for which the date of littering (PPD 0) was on one of four consecutive days, were selected for further examination. From PPD 11 to PPD 21 the same dose levels of 0, 15, 30 and 60 mg/kg bw were administered to 23, 22, 30, and 28 litters from each respective group. A standard dose volume of 10 mL/kg bw was administered to both dams and pups.

There were no test substance-related findings in dams at any dose level. Specifically, clinical observations, open field observations, body weights, food consumption, gestational parameters, reproductive performance, and litter size were not affected in the dams. There were no test substance-related findings in pups from PPD 0 to 10. After direct dosing to pups commenced on PPD 11, 22 of 224 pups from 17 litters (9.8% of dosed pups) died at the 60 mg/kg bw per day dose level. The majority of pups died between PPDs 11 and 14 and seven pups died on PPDs 15–21. These deaths were attributed to the direct acute toxicity of mepiquat chloride. There were no pup deaths at the 15 or 30 mg/kg bw per day dose level. There were no treatment-related effects on body weight, clinical observations (including those made every two hours after dosing for six hours), open field observations, sexual maturation, motor activity, auditory startle habituation, learning and memory, neuropathology or morphometric measurements of brain areas. In addition, in utero and postnatal exposure to mepiquat chloride did not result in a differential effect when pups were pharmacologically challenged with a dose of nicotine when they were approximately 70 days old.

None of the pups at PNDs 22 and 62 showed any treatment-related neuropathology or brain morphometry changes. In addition, the nicotine probe study revealed that in utero and postnatal exposure to mepiquat chloride did not result in differential effects to pharmacological challenge with nicotine at approximately 70 days old.

The NOAEL for developmental neurotoxicity was 60 mg/kg bw per day, the highest dose tested. For systemic postnatal pup toxicity, the NOAEL was 30 mg/kg bw per day based on lethality following direct gavage doses of 60 mg/kg bw per day (Schneider et al., 2006a).

*Study 4*

In an acute study on preweaning rats, mepiquat chloride (purity 56.7%; Batch no. CP028814) was tested to determine the effect of direct exposure on pups from PPD 11 until PPD 21 without prior developmental exposure of the pup via the dam. Mepiquat chloride was administered as an aqueous solution to the offspring of 10 female Wistar rats at doses of 30, 60 or 120 mg of active ingredient/kg bw per day. Additionally the offspring of two female Wistar rats were administered 200 mg of active ingredient/kg bw per day to confirm effects seen in previous studies. The parental females were not exposed to the test substance. The dams and pups were observed daily. Food consumption and body weight of dams was measured regularly through gestation and lactation. Pups were sexed and examined on the day of birth, and weighed on PPDs 1, 4, 7 and 11–21. Pups that died or were sacrificed during lactation were examined, particularly for signs of gavage errors. On day 22, animals were sacrificed and discarded without further examination. At 200 mg/kg bw per day all pups died within 2–4 hours of dosing. At 120 mg/kg bw per day, 43/78 pups died within four hours of, with a further seven pups dying during days 11–15. Lateral position and tremors were observed in almost all pups within six hours of dosing. At 60 mg/kg bw per day, 3/74 pups died after dosing, with no other substance-related findings, while at 30 mg/kg bw per day no test-related findings were observed. Maternal food consumption was comparable between control and treated groups during gestation. Based on the limited evaluation conducted in this study, particularly given the lack of postmortem examinations, an overall NOAEL could not be established (Schneider & van Ravenzwaay, 2006).

*Study 5*

The action of mepiquat chloride (purity 99%; Batch no. WW 285) on nicotinic acetylcholine receptors was examined using interosseal muscles from the feet of adult mice, and concentrations up to 1000 µM. Interosseal muscles were excised and placed in a dish, following by superfusion with physiological saline containing collagenase, then by trypsin. The dissociated muscles were placed into culture dishes and maintained with a physiological solution. Electophysiological measurements were performed using the patch clamp technique, with single channel current records and a recording period of 10 seconds. Mepiquat chloride was applied at 10, 100 or 1000 µM to five cell-attached patches and three outside-out patches. The frequency of channel openings was determined by dividing the number of channel openings by the time recorded, with single channel openings identified by a computer program as square current pulses. Acetylcholine was used as a reference substance. Under the conditions of this test, mepiquat chloride has a specific (although slight) effect on nicotinic acetylcholine activated receptors, with effects at 1000 µM mepiquat chloride comparable to those observed with acetylcholine at 10 µM (Franke & Mellert, 1991).

*Study 6*

The action of mepiquat chloride (purity 99%; Batch no. WW 285) on muscarinic acetylcholine receptors was examined using membranes of bovine cerebral cortex, rat heart and rat submaxillary glands, comparing effects with specific reference substances selected for their high affinity for the muscarinic receptors under investigation (pirenzepine, methoctramine and 4-DAMP, as well as atropine). Membranes were prepared from bovine cerebral cortex by placing brains into sucrose solution, following by homogenization and extraction of a pellet of tissue. Rat heart membranes were prepared by homogenizing freshly extracted rat hearts, filtering and extracting a pellet, as were rat submaxillary glands. The membranes were incubated with radioligand aliquots of the membrane fraction and buffer, both with and without competitor substances. Nonspecific binding was determined in parallel for each radiolabelled ligand concentration. After incubation, the membranes were collected on glass fibre filters, transferred into scintillation vials and the radioactivity measured. The resulting saturation and competition curves were evaluated, and the displacement of radiolabel by mepiquat chloride compared with the positive control substances. Under the conditions of the test, mepiquat chloride had measurable, but very low and nonselective, affinity for muscarinic receptors, with affinity only in the high micromolar dose range, in comparison to the positive controls which had affinities in the nanomolar concentration range, that is around five orders of magnitude more dilute than mepiquat chloride. (Weifenbach, 1991).

**(b) Immunotoxicity**

Mepiquat chloride (purity 64.8%; Batch no. 13023729T0) was administered in the diet to groups of 10 male Wistar rats at 0, 972, 2981 or 9000 ppm of active ingredient (equal to 0, 82, 258 and 744 mg/kg bw per day) for four weeks. Cyclophosphamide monohydrate (positive control) was administered at 4.5 mg/kg bw daily by gavage for four weeks. Rats were immunized six days before blood sampling with 0.5 mL sheep red blood cells (sRBCs) by intraperitoneal injection. Animals were observed daily for signs of toxicity. Food consumption and body weight were determined weekly, and detailed clinical examinations in an open field were conducted at the same times. At the end of the study, all animals were assessed for gross pathology, and the spleen and thymus were weighed. At 9000 ppm, decreased mean body weight was observed from day 7, resulting in a decrease by 18.2% in comparison with controls by the end of the study. No other treatment-related signs were observed. In the positive control group, reduced food consumption and decreased body weight gain were observed, and decreased sRBC IgM antibody titres were apparent. Mepiquat chloride was not immunotoxic under the conditions of this test (Stark et al., 2013),

**(c) Studies on metabolites**

Metabolite 4-hydroxy-mepiquat chloride (Reg no 281319; purity 98.1%; Batch no. 00345-208) was administered to three female Wistar rats by single oral gavage at 464 mg/kg bw to determine acute oral toxicity. No mortalities, abnormal clinical signs, effects on body weight or gross pathological abnormalities were observed. The $LD_{50}$ of 4-hydroxy-mepiquat chloride was greater than 464 mg/kg bw. (Gamer & Hellwig, 2005)

Metabolite 4-hydroxy-mepiquat chloride (Reg no 281319; purity 98.1%; Batch no. 00345-208) was negative for mutagenicity in *Salmonella typhimurium* strains TA1535, TA100, TA1537, TA98, and in *Escherichia coli* strain WP2 *uvr*A (Engelhardt & Leibold, 2005).

An in silico comparison of mepiquat chloride and 4-hydroxy-mepiquat chloride was carried out using DEREK Nexus (v. 6.0.1) and OECD QSAR Toolbox (v. 4.2). All SAR alerts identified for 4-hydroxy-mepiquat chloride were also present for mepiquat chloride (Pellizzaro & Beevers, 2019).

The compound *N*-methylpiperidine is a residual impurity detected in mepiquat chloride at up to 0.27%. *N*-Methylpiperidine was tested for mutagenic potential in an Ames test (*Salmonella typhimurium* strains TA1535, TA100, TA537, TA98 and *Escherichia coli* strain WP2 *uvr*A) in the presence and absence of metabolic activation. Under the conditions of the test *N*-methylpiperidine is not mutagenic. (Engelhardt, 1998)

The compound *N*-methylpiperidine, an impurity detected in mepiquat chloride, was assessed for acute oral and inhalation effects using DEREK Nexus (v. 6.0.1) and the OECD QSAR Toolbox (v. 4.2).

Two alerts were identified for *N*-methylpiperidine not present in mepiquat chloride. The first was for DNA binding, which was not considered to be of concern based on previously identified negative Ames test for *N*-methylpiperidine. The second alert related to irritation of mucous membranes, which is consistent with identified irritation and sensitization effects of *N*-methylpiperidine. No additional alerts were identified (Pellizzaro & Beevers, 2019).

**(d) Endocrine disruption activity**

An in vitro test was conducted in line with OECD TG 458 to evaluate the ability of mepiquat chloride to activate or suppress androgen receptor-dependent transcription, using the stably transfected transactivation assay. Mepiquat chloride (purity 59.6%; Batch no. FRE-002008) was dissolved in ethanol and diluted with cell culture medium. The responsiveness of the test system was verified using an androgen (5α-dihydrotestosterone, DHT), a week androgen (mestanolone) and as a negative compound, di(2-ethylhexyl)phthalate (DEHP), tested in triplicate and in parallel to the test cultures. The agonist assay was conducted at concentrations from 1000 µM to 0.001 µM, and the antagonist assay at concentrations from 1000 µM to 0.01 µM. The agonist test was negative, with the maximum effects less than 10% of the response for the positive control. The antagonist test was negative as the inhibitory effect was less than 30% (Roedig, 2021a).

An in vitro test was conducted in line with OECD TG 456 to evaluate the ability of mepiquat chloride to induce or inhibit the production of testosterone and estradiol using H295R cells. Mepiquat chloride (purity 59.6%; Batch no. FRE-002008) was dissolved in ethanol and diluted with cell culture medium. The responsiveness of the test system was verified using a positive control (forskolin) and negative control (prochloraz), as well as testing for cytotoxicity. Under the conditions of the test mepiquat chloride did not induce or inhibit the production of testosterone in two independent experiments, and did not induce or inhibit the production of 17β-estradiol in two independent experiments. Mepiquat chloride was judged to be negative for both testosterone and estrogen induction (Roedig, 2021b).

An in vitro aromatase assay (human recombinant) was conducted in line with EPA test guideline OPPTS 890.1200: *Aromatase (human recombinant) in human recombinant microsomes containing CYP19 and P450 reductase*. Mepiquat chloride (purity 59.6%; Batch no. FRE-002008) was dissolved in ethanol. Radiolabelled and nonradiolabelled androstenedione were used at a concentration of 100 nM. Controls for full enzyme activity, background activity were used, and a positive control (4-hydroxyandrostendione, a known aromatase inhibitor) was included. Under the conditions of the test, mepiquat chloride was classified as not inhibiting the catalytic activity of aromatase (Roedig, 2021c).

In an adequate array of tests designed to comply with the 2018 ECHA/EFSA joint Guidance for the identification of endocrine disruptors (ECHA, 2018), mepiquat chloride showed no endocrine-related adversity or activity, so does not meet the criteria as an endocrine disruptor.

In order to fulfil EU guidance specifications for sufficient testing, the following GLP and guideline compliant studies were newly submitted: a Hershberger assay, a stably transfected human androgen receptor transcriptional activation assay, a steroidogenesis assay, and an aromatase assay. From these studies, a clear conclusion was made based on sufficient data that mepiquat chloride displays no endocrine-related activity.

In a dose range-finding study male Crl:CD Sprague Dawley rats were treated with mepiquat chloride (purity 59.6%; Batch no. FRE-002008) by oral gavage at 0, 100, 200 or 300 mg of active ingredient/kg bw per day for 10 days. Rats were examined twice daily for mortality and once daily for detailed clinical observations. Food consumption and body weight were determined daily. At the end of the dosing period, animals were killed, and gross necropsy examinations were conducted. One rat died after receiving four doses of 300 mg/kg bw per day. There were no other deaths, and no abnormal clinical signs throughout the study. Body weights were reduced at 300 mg/kg bw per day by up to 15.8% compared to controls, while at 200 mg/kg bw per day lower mean body weight gains than for controls were seen immediately after the initiation of dosing, and also for the entire dosing period, with reductions of up to 9.3% (not statistically significant). At 100 mg/kg bw, depressed mean body weight gain was observed, however decreases were only by 5.5% and not statistically significant. Food consumption was lower at 300 and 200 mg/kg bw per day than for controls, and slightly lower at 100 mg/kg bw per day. No abnormalities were found at terminal examination.

Based on this study, doses of 0, 10, 30 and 100 mg/kg bw were selected for the Hershberger Assay (Davis, 2021).

In a Hershberger assay, based on OECD TG 441, mepiquat chloride (purity 59.6%; Batch no. FRE-002008) was administered to peripubertal orchidoepididymized male CRL:CD Sprague Dawley rats once daily via oral gavage at 0, 10, 30 or 100 mg of active ingredient/kg bw per day. Positive control substances were administered, testosterone propionate by subcutaneous injection and flutamide by oral gavage. Mepiquat chloride was also administered in combination with testosterone propionate. Rats were examined twice daily for mortality and once daily for detailed clinical observations and to be weighed. At the end of the study rats were necropsied, blood samples were collected and organs (adrenal glands, bulbourethral (Cowper's) glands, glans penis, kidneys, liver, the levator ani/bulbocavernosus muscle group, seminal vesicles with coagulating glands, and ventral prostate) were weighed. Samples of these organs were collected and preserved for possible future analysis.

There were no mortalities or treatment-related clinical signs. Mean body weight in the testosterone propionate group was higher than for vehicle controls (statistically significant) over the whole treatment period, resulting in slightly higher (by 6%) body weight at the end of the test period. No other effects were seen in the test substance-treated groups. There were no test-substance related gross macroscopic findings. Effects of the positive and negative controls on organ weights were as anticipated. No effects of androgen agonism or antagonism on organ weights were seen in rats treated with mepiquat chloride, either alone or in combination with testosterone propionate. Under the conditions of this test, mepiquat chloride demonstrated no androgenic or anti-androgenic effects (Herbeth, 2021).

A summary of endocrine data for mepiquat chloride is given in Table 41 below.

*Table 41. In vivo endocrine disrupting data for mepiquat chloride*

| Study/doses | NOAEL* | LOAEL* | Findings at LOAEL | Reference |
|---|---|---|---|---|
| Non-guideline human deiodinase enzyme inhibition assay;<br>200 µM | - | - | Inactive | |
| Reproduction/ developmental toxicity screening study in rat<br>0, 305, 1220 and 4880 ppm | ***Parental F0 systemic toxicity:***<br>1220 ppm (115 mg/kg bw/day for males and 134 mg/kg bw/day for females prior to mating)<br>***Reproductive toxicity:***<br>4880 ppm (420 mg/kg bw/day for males and 474 mg/kg bw/day for females prior to mating)<br>***Postnatal F1 toxicity:***<br>1220 ppm (115 mg/kg bw/day for males and 134 mg/kg bw/day for females) | ***Parental F0 systemic toxicity:***<br>4880 ppm (420 mg/kg bw/day for males and 474 mg/kg bw/day for females prior to mating)<br>***Reproductive toxicity:***<br>> 4880 ppm (420 mg/kg bw/day for males and 474 mg/kg bw/day for females prior to mating)<br>***Postnatal F1 toxicity:***<br>4880 ppm (420 mg/kg bw/day for males and 474 mg/kg bw/day for females) | ***Parental F0 systemic toxicity:***<br>Reduced:<br>absolute body weight<br>body weight gain<br>food consumption<br>and efficiency<br>No effects on T3, T4 and TSH levels<br>***Parental F0 reproductive toxicity:***<br>Reduced number of implantation sites<br>(No other effects)<br>***Postnatal F1 toxicity:***<br>Reduced:<br>number of live pups<br>pup absolute body weight<br>pup body weight gain<br>No effects on T3, T4 and TSH levels | Millard, 2022 |
| Developmental neurotoxicity study in rats<br>0, 15, 30 and 60 mg/kg bw per day | ***Neurotoxicity:***<br>60 mg/kg bw/day<br>***Toxicity:***<br>30 mg/kg bw/day | ***Neurotoxicity:***<br>> 60 mg/kg bw/day<br>***Toxicity:***<br>60 mg/kg bw/day | ***Neurotoxicity:***<br>no developmental neurotoxicity at any dose level<br>***Toxicity:***<br>Raised mortality in pups | Schneider et al., 2006a |

## 3. Microbiological aspects

There was no information available in the public domain and no experimental data were submitted that addressed the possible impact of mepiquat chloride residues on the human intestinal microbiome.

## 4. Observations in humans

No information was provided on the health of workers involved in the manufacture or use of mepiquat chloride. No information on accidental or intentional poisoning in humans was available.

## Comments

Mepiquat chloride was rapidly and extensively absorbed after an oral dose of [*2,6*-$^{14}$C]mepiquat chloride at either 1.2 or 12 mg/kg body weight (bw). The maximum concentration ($C_{max}$) at 1.2 mg/kg bw was reached 40 minutes after dosing, and 60 minutes after dosing at 12 mg/kg bw. Levels were slightly higher (18%–20%) in both the blood and plasma of females at the low dose than in that of males. Radiolabel was identified mainly in the gastrointestinal (GI) tract after eight hours, with levels dropping over subsequent time periods. Levels were also high in kidney after eight hours, and the urinary bladder at 24 hours. Levels in other tissues were low in all time periods. Mepiquat chloride was eliminated predominantly in urine, in part by active secretion, with up to 87.7% of the radiolabel eliminated via this route after a low oral dose of 1.2 mg/kg bw, or 81.5% of the radiolabel after a higher oral dose of 12 mg/kg bw. Elimination via the faeces accounted for around 10%–15% of the radiolabel. Less than 1% of the radiolabel was excreted in bile over 24 hours (Clark & Kupelian, 1977).

Metabolism of mepiquat chloride was extremely limited. No metabolites of mepiquat chloride were detected in urine, faeces or tissue samples during in vivo studies. In an in vitro comparative metabolism study with mouse, rat, dog and human liver microsomes, trace evidence of a hydroxylated metabolite was seen at the highest concentration in the rat and dog, and no unique human metabolites were identified (Clark & Kupelian, 1977; Dawkins & Harrison, 2015; Holloway, 1987; Jones et al., 2013).

### Toxicological data

The acute oral median lethal dose ($LD_{50}$) of mepiquat chloride in mice was 780 mg/kg bw (Hildebrand & Kirsch, 1989a), and in rats it was 464 mg/kg bw (Hildebrand & Kirsch, 1989b); the dermal $LD_{50}$ was greater than 2000 mg/kg bw (Hildebrand & Kirsch, 1989c). The inhalation median lethal concentration ($LC_{50}$) of mepiquat chloride was greater than 4.89 mg/L (Gamer & Kirsch, 1991). Mepiquat chloride was not irritating to skin or eyes of rabbits (Leuschner, 1976, 1977), and it was not skin sensitizing in either the mouse local lymph node assay (LLNA; Dony, 2019), nor in a non-guideline Guinea pig maximization test (Grundler, 1979).

In repeat-dose dietary studies in mice, rats and dogs, effects in mice and rats were limited to decreased body weight gain, associated with decreased food consumption. In dogs, salivation, sedation and neurological signs were seen at high doses, with deposition of iron pigment in the liver and spleen observed after 12 months.

In a 90 day dietary study in mice, mepiquat chloride was administered at 0, 300, 900, 1700 or 8100 ppm (equal to 0, 60, 186, 526 and 1731 mg/kg bw per day for males, 0, 83, 265, 705 and 2422 mg/kg bw per day for females). The no-observed-adverse-effect level (NOAEL) was 8100 ppm in the diet (equal to 1731 mg/kg bw per day), the highest dose tested (Schilling & Hilderbrand, 1992a).

In a four-week dietary toxicity study in rats, mepiquat chloride was administered at 0, 500, 2000 or 8000 ppm (equal to 0, 44, 175 and 633 mg/kg bw per day for males, 0, 48, 191 and 688 mg/kg bw per day for females). The NOAEL was 8000 ppm (equal to 633 mg/kg bw per day), the highest dose tested (Schilling & Hildebrand, 1992b).

In a 90-day dietary toxicity study (not GLP) in rats, mepiquat chloride was administered at 0, 100, 300, 1000 or 3000 ppm (equal to 0, 9.2, 27.6, 91.8 and 275.5 mg/kg bw per day for males, 0, 8.9, 26.7, 91.2 and 278.8 mg/kg bw per day for females). The NOAEL was 1000 ppm (equal to 91.2 mg/kg bw per day) based on reduced food consumption and body weight gain at 3000 ppm (equal to 275.5 mg/kg bw per day) as well as decreased weight of heart, lungs, spleen and kidney in males (Leuschner et al., 1977a).

In a second 90-day dietary toxicity study in the same facility, mepiquat chloride was administered at 0, 145, 579, 2316 or 4632 ppm (equal to 0, 10, 40, 163 and 319 mg/kg bw per day for males, 0, 12, 47, 188 and 372 mg/kg bw per day for females). The NOAEL was 4632 ppm (equal to 319 mg/kg bw per day), the highest dose tested (Schilling & Hilderbrand, 1992c).

In a subsequent 90-day toxicity study in rats with doses of mepiquat chloride of 0 and 12 000 ppm, (equal to 0 and 826 mg/kg bw per day), systemic toxicity was seen at 12 000 ppm, and effects included impaired behaviour, tremors, impaired gait, ataxia, posture abnormalities, abnormal respiration and

vocalizations. Effects were considered to be related to effects on the nicotinic acetylcholine-activated receptors. The lowest-observed-adverse-effect level (LOAEL) in this study was 12 000 ppm (equal to 826 mg/kg bw per day) the only dose tested (Schilling & Hilderbrand, 1992d).

Overall, the NOAEL for short-term effects in rats was 4632 ppm (equal to 319 mg/kg bw per day), a value that incorporated all available short-term studies in rats, based on a LOAEL of 12 000 ppm (equal to 826 mg/kg bw per day).

In a 90-day dietary toxicity study in dogs, mepiquat chloride was administered at 0, 100, 300, 1000 or 3000 ppm (equal to 0, 3.3, 9.8, 32.4 and 95.3 mg/kg bw per day). The NOAEL for toxicity was 1000 ppm (equal to 32.4 mg/kg bw per day) based on signs of sedation progressing to periodic lateral positioning and tonic–clonic spasms, as well as decreases in haemoglobin, erythrocytes and haematocrit after six and 13 weeks at 3000 ppm (equal to 95.3 mg/kg bw per day) (Leuschner et al., 1977b).

In a 12-month dietary study in dogs at dose levels of 0, 200, 600 or 1800 ppm (equal to 0, 6.3, 19.9 and 58.4 mg/kg bw per day) the NOAEL was 600 ppm (equal to 19.9 mg/kg bw per day) based on the deposition of iron pigment in the spleen and liver at 1800 ppm (equal to 58.4 mg/kg bw per day) (Hellwig & Hildebrand, 1989).

In a second 12-month dietary study in dogs, mepiquat chloride was initially administered in the diet at 0 or 8000 ppm, reduced to 6000 ppm (equal to 0 and 166 mg/kg bw per day). The LOAEL was 6000 ppm (equal to 166 mg/kg bw per day) based on salivation, haematological changes, weight changes in kidneys, thyroid and adrenal glands, along with histopathological findings in the kidneys and spleen (Mellert & Hildebrand, 1994b). It was noted that the haematological effects observed in the 90-day study and the increase in iron pigment stored in the spleen and liver in the 12-month study were likely to have been due to related effects. Overall, the NOAEL for effects in dogs was 1000 ppm (equal to 32.4 mg/kg bw per day) based on the deposition of iron pigment in spleen and liver at 1800 ppm (equal to 58.4 mg/kg bw per day).

In a chronic toxicity and carcinogenicity dietary study, mice were fed mepiquat chloride for up to 104 weeks at 0, 500, 2000 or 7500 ppm (equal to 0, 74, 297 and 1140 mg/kg bw per day in males, 0, 85, 328 and 1348 mg/kg bw per day in females). No differences in the type or incidence of tumours were observed during the study, and there were no treatment-related changes in haematology or pathology. The NOAEL for chronic toxicity was 2000 ppm (equal to 297 mg/kg bw per day) based on a decrease in body weight at 7500 ppm. The NOAEL for carcinogenicity was 7500 ppm (equal to 1140 mg/kg bw per day) the highest dose tested (Mellert et al., 1994).

In a second toxicity and carcinogenicity study, mice were administered the test article at 0, 100, 300, 1000 or 3000 ppm (equal to 0, 16, 48.9, 169.4 and 513.5 mg/kg bw per day for males, 0, 21.7, 65.3, 226.1 and 689.4 mg/kg bw per day for females). The NOAELs for toxicity and carcinogenicity were both 3000 ppm (equal to 513.5 mg/kg bw per day), the highest dose tested (Leuschner, et al., 1979a).

The overall NOAEL for chronic toxicity in mice was 3000 ppm (equal to 513.5 mg/kg bw per day). The overall NOAEL for carcinogenicity was 7500 ppm (equal to 1140 mg/kg bw per day), the highest dose tested.

In a chronic study in rats, mepiquat chloride was fed for 24 months at dietary levels of 0, 290, 2316 or 5790 ppm (equal to 0, 13, 106 and 268 mg/kg bw per day for males, 0, 18, 146 and 371 mg/kg bw per day for females). The NOAEL for toxicity was 2316 ppm (equal to 106 mg/kg bw per day) based on reduced food consumption, decreased body weight and body weight gain and an increase in urinary crystals at 5790 ppm (equal to 268 mg/kg bw per day). While there were no signs of carcinogenicity at any dose, the study was not considered a suitable one from which to draw conclusions on carcinogenicity because of the small group size, consisting of just 20 rats of each sex per group (Mellert & Hildebrand, 1994c).

In a chronic toxicity and carcinogenicity dietary study, mepiquat chloride was fed to rats for 24 months at 0, 290, 2316 or 5790 ppm (equal to 0, 13, 106 and 268 mg/kg bw per day for males, 0, 18, 146 and 371 mg/kg bw per day for females). The NOAEL for chronic toxicity was 2316 ppm (equal to 106 mg/kg bw per day), based on decreased body weight at 5790 ppm (equal to 268 mg/kg bw per day). The NOAEL for carcinogenicity was 5790 ppm (equal to 268 mg/kg bw per day), the highest dose tested. (Mellert & Hildebrand, 1994d)

In a combined chronic toxicity and carcinogenicity study, rats were fed mepiquat chloride in the diet for 104 weeks at 0, 100, 300, 1000, 3000 or 9000 ppm (equal to 0, 6. 18, 62, 186 and 684 mg/kg bw for males, 0, 7, 21, 72, 212 and 670 mg/kg bw per day for females). The NOAEL for chronic toxicity was 3000 ppm (equal to 186 mg/kg bw per day) based on depressed body weight compared to controls at 9000 ppm (equal to 670 mg/kg bw per day). The NOAEL for carcinogenicity was 9000 ppm (equal to 670 mg/kg bw per day), the highest dose tested (Leuschner et al., 1979b).

Overall, the NOAEL for chronic toxicity in rats was 3000 ppm (equal to 186 mg/kg bw per day) based on effects on body weight at 5790 ppm (equal to 268 mg/kg bw per day). The overall NOAEL for carcinogenicity was 9000 ppm (equal to 670 mg/kg bw per day), the highest dose tested.

The Meeting concluded that mepiquat chloride is not carcinogenic in mice or rats.

Mepiquat chloride was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found.

The meeting concluded that mepiquat chloride is unlikely to be genotoxic.

In view of the lack of genotoxicity and the absence of carcinogenicity in mice and rats, the Meeting concluded that mepiquat chloride is unlikely to pose a carcinogenic risk to humans.

In a multigeneration reproductive toxicity study, mepiquat chloride was administered to rats in the diet at 0, 500, 1500 or 5000 ppm, (equivalent to 0, 52, 155 and 500 mg/kg bw per day). The NOAEL for parental and offspring toxicity was 1500 ppm (equal to 155 mg/kg bw per day) based on decreased food consumption and body weight, as well as tremors during lactation in the dams, and decreased pup body weight, at 5000 ppm (equivalent to 500 mg/kg bw per day). The NOAEL for reproductive toxicity was 5000 ppm (equal to 500 mg/kg bw per day), the highest dose tested (Hellwig, 1993).

In a developmental toxicity study, rats were administered mepiquat chloride by gavage at 0, 50, 150 or 300 mg/kg bw per day from gestation day (GD) 6 to GD 15. The NOAEL for maternal toxicity was 150 mg/kg bw per day, based on clinical signs of toxicity including tremors, piloerection, unsteady gait and hypersensitivity, as well as decreased body weight gain at 300 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 300 mg/kg bw per day, the highest dose tested (Hellwig & Hildebrand, 1992).

In a developmental toxicity study, mepiquat chloride was administered by gavage to rabbits at dose levels of 0, 50, 100 or 150 mg/kg bw per day from GD 7 to GD 19. The NOAEL for maternal toxicity was 50 mg/kg bw per day based on reduced body weight gain at 100 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 150 mg/kg bw per day, the highest dose tested (Bachmann et al., 1998).

The Meeting concluded that mepiquat chloride is not teratogenic.

In an acute neurotoxicity study, rats were dosed by gavage with mepiquat chloride as a single oral dose of 0, 58, 174 or 697 mg/kg bw. The NOAEL was 58 mg/kg bw, based on signs of toxicity in the functional observation battery and slightly decreased motor activity at 174 mg/kg bw (Mellert, Kaufmann & van Ravenzwaay, 2002a).

In a subchronic neurotoxicity study, rats were fed mepiquat chloride in the diet for 13 weeks at concentrations of 0, 943, 3770 or 7540 ppm (equal to 0, 65.6, 259 and 517 mg/kg bw per day for males, 0, 79.4, 367, and 617 mg/kg bw per day for females). The NOAEL for neurotoxicity was 7540 ppm (equal to 517 mg/kg bw per day), the highest dose tested. The NOAEL for overall toxicity was 943 ppm, (equal to 66 mg/kg bw per day), based on decreased body weight gain at 3770 ppm (equal to 259 mg/kg bw per day) (Mellert, Kaufmann & van Ravenzwaay, 2002b; amendment in 2002c).

The potential for developmental neurotoxicity was investigated with mepiquat chloride being administered to pregnant female rats at 0, 15, 30 or 60 mg/kg bw per day from GD 6 to post partum day (PPD) 10. Mepiquat chloride was then administered to pups at the same dose levels from PPDs 11 to 21. The NOAEL for development neurotoxicity was 60 mg/kg bw per day, the highest dose tested. The NOAEL for systemic toxicity in pups was 30 mg/kg bw per day, based on lethality at 60 mg/kg bw per day, primarily during the first three days of direct dosing (Scheider et al., 2006a).

The action of mepiquat chloride on nicotinic and muscarinic acetylcholine receptors was investigated. These studies indicate a specific, though slight, effect (ca 1% of acetylcholine effect) on nicotinic acetylcholine-activated receptors (Franke & Mellert, 1991), and a measurable but very low and nonselective affinity to muscarinic receptors (Weifenbach, 1991).

The Meeting concluded that mepiquat chloride was neurotoxic only at doses that produced systemic toxicity.

The potential for immunotoxic effects was investigated in rats at dietary doses of mepiquat chloride of 0, 972, 2981 or 9000 ppm (equal to 0,82, 258 and 744 mg/kg bw per day) for four weeks. The NOAEL for systemic toxicity was 2981 ppm (equal to 258 mg/kg bw per day) based on decreased body weight at the highest dose of 9000 ppm (equal to 744 mg/kg bw per day). The NOAEL for immunotoxicity was 9000 ppm (equal to 744 mg/kg bw per day), the highest dose tested (Stark et al., 2013).

The Meeting concluded that mepiquat chloride is not immunotoxic.

## Toxicological data on metabolites and/or degradates

### 4-Hydroxy-mepiquat chloride

In an acute oral toxicity test in rats, the $LD_{50}$ for 4-hydroxy-mepiquat chloride was greater than 464 mg/kg bw (Gamer & Hellwig, 2005).

Metabolite 4-hydroxy-mepiquat chloride was negative for mutagenicity in *Salmonella typhimurium* strains TA1535, TA100, TA1537, TA98 and *Escherichia coli* strain WP2 uvrA in the presence and absence of metabolic activation (Engelhardt & Leibold, 2005).

An in silico comparison of mepiquat chloride and 4-hydroxy-mepiquat chloride was carried out using DEREK Nexus and OECD QSAR Toolbox. All SAR alerts identified for 4-hydroxy-mepiquat chloride were also present for mepiquat chloride (Pellizzaro & Beevers, 2019).

### *N*-Methyl piperidine

Metabolite *N*-methyl piperidine is a residual impurity detected in mepiquat chloride and was negative for mutagenicity in an Ames test (Engelhardt, 1998).

The Meeting concluded that 4-hydroxy-mepiquat chloride is toxicologically relevant, and of no greater potency than the parent.

## Microbiological aspects

There was no information available in the public domain and no experimental data were submitted that addressed the possible impact of mepiquat chloride residues on the human intestinal microbiome.

## Human data

No information was provided on the health of workers involved in the manufacture or use of mepiquat chloride. No information on accidental or intentional poisoning in humans was available.

The Meeting concluded that the existing database on mepiquat chloride was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

## Toxicological evaluation

The meeting established an acceptable daily intake (ADI) for mepiquat chloride of 0–0.3 mg/kg bw based on the overall NOAEL of 32.4 mg/kg bw per day in the 90-day and 12-month dog studies, and using a safety factor of 100. This ADI is considered applicable also to 4-hydroxy-mepiquat chloride.

The meeting established an acute reference dose (ARfD) of 0.6 mg/kg bw, based on the NOAEL of 58 mg/kg bw in the rat acute neurotoxicity study. A safety factor of 100 was used. This ARfD is considered applicable also to 4-hydroxy-mepiquat chloride.

***Levels relevant to risk assessment of mepiquat chloride***

| Species | Study | Effect | NOAEL | LOAEL |
|---|---|---|---|---|
| Mouse | 104-week studies of toxicity and carcinogenicity [a, d] | Toxicity | 3000 ppm, equal to 513.5 mg/kg bw per day | 7500 ppm, equal to 1140 mg/kg bw per day |
| | | Carcinogenicity | 7500 ppm, equal to 1140 mg/kg bw per day [c] | - |
| Rat | Acute neurotoxicity study [b] | Toxicity | 58 mg/kg bw per day | 174 mg/kg bw per day [e] |
| | 90-day dietary studies [a] | Toxicity | 4632 ppm, equal to 319 mg/kg bw per day | 12 000 ppm, equal to 826 mg/kg bw per day |
| | Two-year studies of chronic toxicity and carcinogenicity [a, d] | Toxicity | 3000 ppm, equal to 186 mg/kg bw per day | 5790 ppm, equal to 268 mg/kg bw per day |
| | | Carcinogenicity | 9000 ppm, equal to 670 mg/kg bw per day [c] | - |
| | Two-generation study of reproductive toxicity [a] | Parental toxicity | 5000 ppm, equal to 500 mg/kg bw per day [c] | - |
| | | Offspring toxicity | 1500 ppm, equal to 155 mg/kg bw per day | 5000 ppm, equal to 500 mg/kg bw per day |
| | | Maternal toxicity | 1500 ppm, equal to 155 mg/kg bw per day | 5000 ppm, equal to 500 mg/kg bw per day |
| | Developmental toxicity study [b] | Embryo/fetal toxicity | 150 mg/kg bw per day | 300 mg/kg bw per day |
| | | Maternal toxicity | 300 mg/kg bw per day [c] | - |
| Rabbit | Developmental toxicity study [b] | Embryo/fetal toxicity | 50 mg/kg bw per day | 100 mg/kg bw per day |
| | | Toxicity | 150 mg/kg bw per day [c] | - |
| Dog | 13-week and 1 year studies of toxicity [a, d] | Toxicity | 1000 ppm, equal to 32.4 mg/kg bw per day | 1800 ppm, equal to 58.4 mg/kg bw per day |

[a] Dietary administration; [b] Gavage administration; [c] Highest dose tested;
[d] Two or more studies combined; [e] Lowest dose tested;

*Acceptable daily intake (ADI), applies to mepiquat chloride and 4-hydroxy-mepiquat chloride, expressed as mepiquat chloride*

0–0.3 mg/kg bw

*Acute reference dose (ARfD), applies to mepiquat chloride and 4-hydroxy-mepiquat chloride, expressed as mepiquat chloride*

0.6 mg/kg bw

### Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure.

*Critical end-points for setting guidance values for exposure to mepiquat chloride*

| | |
|---|---|
| **Absorption, distribution, excretion and metabolism in mammals** | |
| Rate and extent of oral absorption | Mepiquat chloride was rapidly and extensively absorbed after oral dosing; absorption > 80% of administered dose |
| Dermal absorption | Absorbed, followed by urinary excretion |
| Distribution | Widely distributed, with highest levels in gastrointestinal tract, urinary bladder, liver and kidney |
| Potential for accumulation | No evidence of accumulation |
| Rate and extent of excretion | Rapid and nearly complete, with around 85% of administered radioactivity excreted via urine, and up to 15% in faeces; very limited excretion (< 1%) via bile |
| Metabolism in animals | Extremely limited, with no metabolites detected in urine or faeces in in vivo studies<br>Limited quantities of 4-hydroxy-mepiquat chloride detected in an in vitro comparative metabolism study |
| Toxicologically significant compounds in animals and plants | Mepiquat chloride and 4-hydroxy-mepiquat chloride |
| **Acute toxicity** | |
| Rat, $LD_{50}$, oral | 464 mg/kg bw |
| Rat, $LD_{50}$, dermal | > 2000 mg/kg bw |
| Rat, $LC_{50}$, inhalation | > 4.89 mg/L |
| Rabbit, dermal irritation | Not irritating |
| Rabbit, ocular irritation | Not irritating |
| Mouse, dermal sensitization | Not sensitizing |
| Guinea pig, dermal sensitization | Not sensitizing |
| **Short-term studies of toxicity** | |
| Target/critical effect | Decreased body weight gain and food consumption (rat, dog)<br>Effects on erythrocytes, along with increased haemosiderin in the spleen (dog) |
| Lowest relevant oral NOAEL | 32.4 mg/kg bw per day (dog) |
| Lowest relevant dermal NOAEL | 1000 mg/kg bw per day, highest dose tested (rat) |
| Lowest relevant inhalation NOAEC | No data |
| **Long-term studies of toxicity and carcinogenicity** | |
| Target/critical effect | Decreased body weight |
| Lowest relevant NOAEL | 186 mg/kg bw per day (rat) |
| Carcinogenicity | Not carcinogenic in mice or rats [a] |
| **Genotoxicity** | Unlikely to be genotoxic |
| **Reproductive toxicity** | |
| Target/critical effect | Decreased body weight gain and food consumption |
| Lowest relevant parental NOAEL | 155 mg/kg bw per day (rat) |
| Lowest relevant offspring NOAEL | 155 mg/kg bw per day (rat) |
| Lowest relevant reproductive NOAEL | 500 mg/kg bw per day (rat) |
| **Developmental toxicity** | |
| Target/critical effect | Decreased body weight gain and food consumption, associated with delayed fetal development |
| Lowest relevant maternal NOAEL | 50 mg/kg bw per day (rabbit) |
| Lowest relevant embryo/fetal NOAEL | 150 mg/kg bw per day (rabbit) |

| | |
|---|---|
| **Neurotoxicity** | Signs of systemic toxicity were seen in a number of neurotoxicity studies, with specifically neurotoxic effects observed only in the presence of general toxicity |
| Acute neurotoxicity NOAEL | 58 mg/kg bw (systemic toxicity relevant for ARfD) |
| Subchronic neurotoxicity NOAEL | 517 mg/kg bw per day, highest dose tested (rat) |
| Developmental neurotoxicity NOAEL | 60 mg/kg bw per day, highest dose tested (rat) |
| **Other toxicological studies** | |
| Immunotoxicity NOAEL | > 744 mg/kg bw per day, the highest dose tested (rat) |
| **Studies on toxicologically relevant metabolites** | |
| 4-Hydroxy-mepiquat chloride | Acute oral $LD_{50}$: > 464 mg/kg bw (rat)<br>Not genotoxic (Ames)<br>In silico comparison with mepiquat chloride indicated comparable alerts |
| **Microbiological aspects** | No data submitted |
| **Human data** | No clinical cases or poisoning incidents have been recorded |

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

## Summary

| | Value | Study | Safety factor |
|---|---|---|---|
| ADI | 0–0.3 mg/kg bw [a] | Overall NOAEL in 90-day and 12-month studies (dog) | 100 |
| ARfD | 0.6 mg/kg bw [a] | Acute neurotoxicity study, based on signs of systemic toxicity (rat) | 100 |

[a] Applies to mepiquat chloride and 4-hydroxy-mepiquat chloride, expressed as mepiquat chloride

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# Prochloraz

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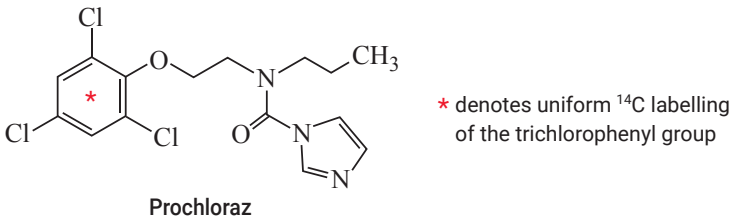
## Explanation

Prochloraz is the International Organization for Standardization (ISO)-approved common name for *N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy)ethyl]-*1H*-imidazole-1-carboxamide (IUPAC), which has the Chemical Abstracts Service number 67747-09-5. Prochloraz is a broad-spectrum fungicide which acts by inhibiting ergosterol biosynthesis.

Prochloraz was previously evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1983 and 2001, when an acceptable daily intake (ADI) of 0–0.01 mg/kg body weight (bw) and an acute reference dose (ARfD) of 0.1 mg/kg bw were established. Prochloraz was reviewed by the present Meeting as part of the periodic review programme of the Codex Committee on Pesticide Residues (CCPR).

All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with the relevant national or international test guidelines unless otherwise specified. A literature search was conducted and the toxicological information obtained in this search was submitted for the current assessment. Some studies were performed by the Laboratory of Pharmacology and Toxicology in Hamburg. The Meeting is aware of the current debate about allegations of fraud that have been made and investigated concerning this laboratory. As no formal conclusion about these allegations has been reached as yet, the Meeting has decided to include the results from such studies. The Meeting noted that these studies have no impact on the overall evaluation of prochloraz.

***Figure 1. Chemical structure of prochloraz***




Prochloraz

# Evaluation for acceptable intake

## 1 Biochemical aspects

### 1.1 Absorption, distribution and excretion

#### (a) Oral route

***Mouse***

Six male and six female CD-1 mice received [*phenyl-U*-$^{14}$C]prochloraz (radiochemical purity, greater than 99%) by gavage at a single dose of 100 mg/kg bw. Urine and faeces were collected 24, 48, 72 and 96 hours after dosing. After 96 hours all animals were killed and various tissues were removed and prepared for analysis by liquid scintillation counting (LSC). The study was conducted before GLP regulations were issued in the testing facility, but the study protocol and reporting of the results were considered acceptable.

Excretion was virtually complete within 72 hours. The overall recovery of radiolabelled material was 111% in males and 104% in females, with 63% excreted in urine (both sexes) and 47% and 41% excreted in the faeces of males and females, respectively. There were no significant differences between the sexes in the rate or route of excretion or in the concentrations of residues in tissues. The concentrations were highest in liver (4–7 mg equiv./kg tissue) and lowest in muscle, genitalia, eyes, spleen and fat (0.5–1.0 mg equiv./kg tissue). All other tissues contained mean concentrations between 1.0 and 2.5 mg equiv./kg tissue (Needham, 1982a).

***Rat***

In a study conducted in compliance with the principles of GLP (with QA certification), five male and five female Charles River rats were given [*phenyl-U*-$^{14}$C]prochloraz (radiochemical purity, greater than 98.9%) as a single oral dose of 5 mg/kg bw. Urine, faeces and expired carbon dioxide were collected. After four days all rats were killed by exsanguination after ether anaesthesia. Samples of various tissues were analyzed by LSC.

Excretion of radiolabelled material in the urine and faeces was rapid and complete, with 83%–87% of radioactivity excreted within 24 hours and 98%–99% excreted within 96 hours. A sex-related difference in the route of excretion was apparent, with faecal excretion accounting for 59% and 70% of the dose in males and females respectively, and urinary excretion accounting for 36% and 26% of the dose in males and females respectively. Tissue residue concentrations were very low, often below the limit of detection. Only liver samples contained concentrations consistently greater than 0.1 mg/kg tissue (0.20 ± 0.04 mg/kg) (Challis & Creedy, 1988).

In a toxicokinetic study, five male and five female Charles River rats were given [*phenyl-U*-$^{14}$C]prochloraz (radiochemical purity, 97%) as a single oral dose of 100 mg/kg bw. Urine,

faeces and expired carbon dioxide were collected. After four days, all rats were killed and samples of various tissues were analysed by LSC.

Excretion of radiolabelled material in the urine and faeces was rapid and complete (recovery, 104% in males and 98% in females), with 63% and 64% excreted within 24 hours in males and females respectively. A sex difference in the route of excretion was apparent, with faecal excretion accounting for 33% and 50% of the administered dose (AD) in males and females respectively, and urinary excretion accounting for 73% and 41% of AD in males and females respectively. The pattern of excretion with time also differed between the sexes: peak faecal excretion was at 48 hours in females but 24 hours in males. There was no significant excretion of radiolabelled material into expired air. The tissue residue concentrations also showed sex differences, some concentrations in female rats being up to twice those in males. The highest concentrations were found in liver (2.8 ± 0.22 mg equiv./kg tissue in males and 5.1 ± 0.78 mg equiv./kg tissue in females) and kidneys (1.5 ± 0.18 mg equiv./kg tissue in males and 2.1 ± 0.18 mg equiv./kg tissue in females). The concentrations of residues in blood and plasma of both sexes and in lungs and adrenals of females were greater than 1 mg equiv./kg tissue. In all other tissues, the residue concentrations were below 1.0 equiv./kg tissue (Dawson, 1989).

In a study of the elimination of prochloraz, five male Sprague Dawley rats were given [*phenyl-U*-$^{14}$C]prochloraz (radiochemical purity, greater than 98%) as a single oral dose of 50 or 250 mg/kg bw. Urine and faeces were collected daily for four days, and radiolabel was measured. For analysis of urinary metabolites, the rats were given $^{14}$C-labelled prochloraz at 250 mg/kg bw, urine was collected for 48 hours, and radiolabel in excreta was assayed by liquid scintillation spectrometry. The study was reported in detail in a publication and was considered to provide useful additional information.

At both doses, virtually all of the ingested [$^{14}$C]prochloraz was excreted in the urine or faeces within 96 hours, the bulk of excretion occurring between 24 and 48 hours after dosing. Urinary elimination accounted for 61% and 68% of the respective initial doses. Prochloraz was completely metabolized, no unchanged compound being detectable in the urine. The main biotransformation products in urine were 2,4,6-trichlorophenoxyacetic acid and its corresponding alcohol, the latter as the glucuronic acid conjugate. Aromatic hydroxylation also occurred, with small amounts of hydroxy-2,4,6-trichlorophenoxyethanol and hydroxy-2,4,6-trichlorophenoxyacetic acid excreted in the urine. Otherwise, 2,4,6-trichlorophenol and unconjugated 2-(2,4,6-trichlorophenoxy)ethanol were identified as minor urinary metabolites (Laignelet, Rivière & Lhuguenot, 1992).

In a study conducted in compliance with the principles of GLP (with quality assurance certification), the absorption of radiolabelled prochloraz after a single oral dose to Crl:CD(SD)BR rats was determined by characterizing its route and rate of excretion in urine, bile and faeces. Four male and four female rats with cannulated bile ducts were given [*phenyl-U*-$^{14}$C]prochloraz (radiochemical purity, 98.3%) at a nominal dose of 5 mg/kg bw. The animals were maintained in metabolism cages, and urine, faeces and bile collected for up to 48 hours. After this the animals were killed, and the radioactive content of the urine, faeces, bile, the gastrointestinal (GI) tract and its contents, and residual carcass was determined by LSC.

The overall mean recovery of radiolabel over 48 hours was 95% ± 4.5% in males and 91% ± 5.2% in females. No sex differences in the disposition of prochloraz were apparent. Biliary excretion was the predominant route of elimination and accounted for an overall mean of 48% (range 18%–75%) of the dose in both sexes. Renal elimination (urine plus cage washings) accounted for 21% (range 15%–34%) in males and 23% (range 11%–41%) in females. Excretion in faeces (primarily within 24 hours) represented 22% in males and 14% in females, while only very low levels of radioactivity remained in the GI tract (males 0.16%, females 1.1%). The residual carcass retained a very small fraction of the dose (mean, 2.6% in males and 3.0% in females). Thus, there were no apparent sex differences. Prochloraz was reasonably well absorbed with a mean combined total of 74% of the AD (range, 60%–96%) being detected in bile, urine, cage washings and carcass. Biliary excretion was the major route of elimination (D'Souza, 1995).

In a study conducted in compliance with the principles of GLP (with quality assurance certification), the clearance of radiolabelled residues from tissues in CRL:CD(SD)BR Sprague Dawley rats after a single low or high oral dose of [*phenyl-U*-$^{14}$C]prochloraz (radiochemical purity, greater than 99%) were compared. Groups of 18 males and 18 females were given a gavage dose of either 5 or 100 mg/kg bw. Those at the low dose were necropsied in groups of three males and three females at

2, 10, 20, 40, 56 and 72 hours after treatment, and those at the high dose were necropsied in groups of three males and three females at 10, 20, 40, 56, 72 and 96 hours after treatment. These times were set on the basis of the findings of preliminary studies in which the maximum concentration ($C_{max}$) in the blood were determined at the low and high doses. At necropsy, blood, plasma, liver, kidney, spleen, heart, lung, brain, muscle, gonads, eyes, adrenals, bone, renal fat, GI tract and residual carcass were collected, and residual radiolabel quantified by LSC.

At both the low and high doses, radiolabelled residues of prochloraz were cleared rapidly from the tissues, with a marked decrease in concentration by the end of the study. After the low dose, the highest tissue concentrations were seen in the GI tract (24 ± 1.0 mg equiv./kg and 28 ± 1.7 mg equiv./kg in males and females, respectively), and liver (8.5 ± 2.3 mg equiv./kg and 7.3 ± 0.9 mg equiv./kg in males and females, respectively) two hours after dosing. By 72 hours after dosing, the residues in all tissues except the liver (0.49 mg equiv./kg), kidney (0.22 mg equiv./kg) and GI tract (0.22 mg equiv./kg) of males and females and the plasma (0.13 mg equiv./kg) of males had fallen to less than 0.1 mg equiv./kg tissue and were below the level of quantification (LOQ) in some tissues. After the high dose, the highest concentrations were seen in the GI tract (230 ± 73 mg equiv./kg and 340 ± 37 mg equiv./kg), liver (65 ± 19 mg equiv./kg and 90 ± 12 mg equiv./kg) and kidney (81 ± 4.6 mg equiv./kg and 74 ± 10 mg equiv./kg) in males and females respectively, 10 hours after dosing, with high concentrations also seen in the blood (59 mg equiv./kg and 38 mg equiv./kg) and plasma (93 mg equiv./kg and 57 mg equiv./kg) in males and females respectively. By 96 hours after dosing, the tissue concentrations were still above the limit of quantification but had decreased markedly, by at least one order of magnitude.

The tissue distribution ratios indicated maximum distribution of radiolabel 2–10 hours after the low dose and 10– 20 hours after the high dose. The highest values were seen in the GI tract, carcass, blood, plasma, muscle, liver and renal fat. For the low dose study, the $C_{max}$ was calculated to occur at two hours post-dosing for the males and 0.25 hours for the females. Since little variation was observed in the female rat in the period 0.25–3 hours post dose, the time to maximum concentration ($T_{max}$) was set at two hours for future low-dose studies. Similarly, a $T_{max}$ of 10 hours was set for future high-dose studies. The calculated terminal half-lives in blood, plasma, liver and kidney were similar in all these tissues in both sexes at both the high and the low dose. In blood, the terminal half-life ($t_{½t}$) was 11 hours in males and 14 hours in females at the low dose, and 12 hours in males and 17 hours in females at the high dose. There was no significant sex difference in the half-lives in blood, plasma and kidney at either dose. For the liver at the high dose, the $t_{½t}$ was 17 hours in males and 18 hours in females at the low dose and 22 hours in males and 24 hours in females (Reynolds, 1995, 1996).

In another study, the route and rate of excretion, and tissue distribution of chemical residues in both male and female rats was examined following the administration of a high, single, oral, gavage dose at 113.5 mg/kg bw *phenyl*-$^{14}$C-labelled prochloraz–copper complex (radiochemical purity 99%), an amount equivalent to 100 mg/kg bw of prochloraz, to five fasting rats of each sex. Rats were placed individually in all-glass metabolism cages and had free access to feed and water. Faeces and urine were collected for four days and carbon dioxide was trapped for the first 24 hours. After the four days, animals were killed and the following organs removed for analysis: liver, kidney, spleen, heart, lung, brain, muscle, genitals, thyroid, eyes, adrenals, bone, renal fat, pituitary, blood, plasma and the remaining carcass. The amounts of radioactivity in the various samples were determined by radioassay using LSC.

Excretion was found to be rapid and virtually complete within 48 hours. Over the 96 hour period of the study 96.5% of the administered label was excreted by the males and 95.0% excreted by the females. The excretion of prochloraz in this study exhibited a marked sex difference. At 96 hours after dosing in the males 50.8% and 41.2% of the total dose was excreted in urine and faeces respectively, while in females at this same time 39.6% and 51.9% of the total dose were excreted in urine and faeces respectively.

The findings demonstrate that excretion occurs entirely in the urine and faeces in rats with no significant excretion into expired air. Total tissue residue levels at 96 hours were low (about 1% of AD). In most tissues, residue levels were below 1 mg prochloraz equivalents/kg 96 hours post-dosing. The highest residue levels were detected in the liver (3.36 and 4.75 mg/kg tissue in males and females respectively) and kidneys (1.59 and 1.90 mg/kg tissue in males and females respectively). Residues found in the tissues of female rats were generally higher (with some being up to 1.7 times higher) than the levels found in the corresponding tissues of males (Needham & Hemmings, 1989).

In a study investigating the kinetics of prochloraz, groups of male and female Sprague Dawley rats were gavaged once with 10 or 100 mg/kg bw of [*phenyl-U*-$^{14}$C]prochloraz (purity greater than 97.1%). Seven different tests were carried out. In the preliminary test (Test 1), expired air, urine, faeces, GI tract and carcass were analyzed for radioactive residues 48 hours after a single administration (low and high dose). Subsequent tests were as follows: Test 2 (low dose, single exposure), Test 3 (high dose, single exposure) and Test 4 (low dose, repeated exposure to unlabelled prochloraz for 14 consecutive days followed by a single administration of $^{14}$C-labelled prochloraz at the same dose rate). In all these tests urine, faeces and organs were collected 168 hours after the last dosage. Whole blood was collected from animals in group 2. All samples were analyzed for radioactive residues. The pharmacokinetic profile of total radioactivity in whole blood was investigated 168 hours after single oral administration of the low or high dose (Tests 5 and 6), with blood collected at 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 48, 96, 120 and 168 hours after administration of prochloraz. In Test 7, during the 24 hours after administration of a low dose of prochloraz to bile duct-cannulated rats, bile, urine, faeces, blood, organs and carcass were collected.

Test 1 showed that elimination via carbon dioxide in the expired air was negligible.

In Test 2, after a single oral dose of $^{14}$C-labelled prochloraz (10 mg/kg bw), there was rapid absorption and excretion of radioactive material. The mean recoveries in urine at 168 hours after dosing were 45.0% for males and 29.2% for females. Recoveries in faeces were 40.9% for males and 59.5% for females. The highest concentration of radioactive material was found in the liver of males and in the Harderian gland in females (154 ± 43.2 ng equiv./g and 153 ± 36.9 ng equiv./g respectively). All other tissues had mean concentrations of radioactivity below 120 ng equiv./g tissue, except the liver of females (131 ± 59.5 ng equiv./g).

In Test 3, after a single high oral dose of $^{14}$C-labelled prochloraz (100 mg/kg bw), there occurred also rapid absorption and excretion of radioactivity. At 168 hours after dosing recoveries of radioactivity in urine were 54.0% in males and 42.5% in females. Recoveries in faeces were 37.2% in males and 48.8% in females. There was little radioactivity in the tissues at 168 hours; 0.5% ± 0.1% in males, and 0.4% ± 0.1% in females.

In test 4, repeated administration of prochloraz at a low dose (10 mg/kg bw) had no appreciable influence on the rates of absorption or elimination nor on the distribution of the parent compound in the tissues of treated animals, based on tissue radioactivity levels. At 168 hours after dosing recoveries of radioactivity in urine were 49.6% in males and 34.0% in females. Recoveries in faeces were 31.2% in males and 45.3% in females. Little radioactivity remained in the tissues at 168 hours; 0.5% ± 0.1% in males and 0.3% ± 0.1% in female. It was noted that residue levels in the livers of males were higher in the repeat dose study (253 ± 42.5 ng equiv./g) than in the single dose study (154 ± 43.2 ng equiv./g).

Tests 5 and 6 showed that the time course of $^{14}$C concentration in whole blood peaked at six hours after administration of the low dose to males and females, while the $C_{max}$ was reached 12 hours after administration of the high dose to males and females. The concentration declined rapidly during the following 36 hours and thereafter declined more gradually. The peak concentration of radioactive material in blood was higher in males than in females (10.6 and 3.3 µg equiv./mL in males and females respectively following a low dose, and 85.5 and 31.1 µg equiv./mL in males and females respectively following a high dose).

Test 7, conducted with bile duct-cannulated rats, showed that prochloraz was extensively absorbed and predominantly excreted in bile. After 24 hours between 73.7% (females) and 74.0% (males) of AD was recovered in urine, bile and the organs and tissues of the body. Only 9.7% (females) to 14.3% (males) of AD was detected in the faeces. In urine 14.8% and 6.4% of the total AD was recovered in males and females respectively. Biliary excretion accounted for 47%–52 % of AD.

The quantitative analysis of metabolites present in urine, faeces and bile collected from animals administered [*phenyl-U*-$^{14}$C]prochloraz showed that extensive metabolism had occurred. Only one major metabolite was evident in urine, (2,4,6-trichlorophenoxy)-acetic acid, amounting to approximately 50% of the radioactivity in urine from males and approximately 30% in urine from females. The higher production of this hydrophilic metabolite in males was regarded as explaining why more radioactivity is detected in urine of males than females (Crawley, 1994).

In another study, the excretion, tissue kinetics and metabolism of prochloraz was investigated in the rat. During the study, six test groups of three male and three female Sprague Dawley rats (6–7 weeks old, body weight range 165–203 g) received either a single or multiple oral dose of [$^{14}$C]-phenyl- labelled prochloraz (purity 99.5% for batch 1, 98.6% for batch 2) dissolved in corn oil at a dose level of 5 mg/kg bw per day. Group A received a single dose with urine and faecal samples taken 24 hour post dose for investigation of excretion, metabolite identification and tissue kinetics. Groups B, C and D received daily administrations for 3, 6 and 10 days respectively for investigation of tissue kinetics. Group E received daily administration for 14 days with urine and faecal samples taken 24 hour post dose for investigation of metabolite identification and tissue kinetics. Following dose administration, the rats used for the excretion phases were individually housed in all-glass metabolism cages. Group F received daily administrations for 14 days and were sacrificed seven days after final dose administration for investigation of tissue kinetics following a seven day recovery period. Animals in Groups A to E were sacrificed 24 hours after the final dose of prochloraz. The following organs were removed for residue analysis: adrenals, whole blood, bone, brain, eyes, GI tract, heart, kidney, liver, lung, muscle, ovaries/testes, plasma, renal fat, spleen and residual carcass. Urine samples and faecal extracts from groups A (single dose, 24 hour study) and E (multiple dose, 14 day study) were analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS). Results from the metabolite analysis are presented under section 1.2 Biotransformation.

Study findings revealed that prochloraz was rapidly eliminated from the body. The major route of excretion was via the faeces, which accounted for 56.20% and 64.99% of AD in male and female rats respectively, in the 24 hours after dose administration.

Tissue distribution of radiolabelled prochloraz residues was similar in both sexes, and levels rose slightly after multiple dosing periods. Total tissue residues following a single dose of 5 mg/kg bw and expressed as a percentage of AD accounted for approximately 16% and 12% in male and female animals respectively. Once dosing ceased, residues were cleared from tissues within seven days with no indication of accumulation. Tissue residue levels were highest in the kidney, liver, GI tract and plasma. In general, tissue residue levels increased to a plateau with multiple dosing (except for the GI tract which increased throughout the dosing period), followed by a rapid decline as typified by residues in the liver (Gedik & Kidd, 2003).

In a biokinetics study, the absorption, distribution and elimination of [*phenyl-U*-$^{14}$C]prochloraz (radiochemical purity 97.2%) in four male and four female rats (around eight weeks old) was investigated after repeated oral administrations of prochloraz at dose levels of 100 mg/kg bw/day (14 daily oral doses of unlabelled prochloraz, purity 96.8%) followed by a single oral dose of labelled prochloraz. Radioactivity was recovered from urine, faeces and tissues. The mean total recoveries of radioactivity were greater than 96% for both sexes.

After 168 hours the total amount of radioactivity excreted in urine was found to be 71.9% and 56.0% administered radioactivity (AR) for males and females respectively. Via faeces for male rats 24.1% and for females 39.4% of the AR was excreted within 168 hours. The timecourse for levels of radioactivity found in urine and faeces indicated rapid excretion. Within 48 hours of the last oral administration 65.2% and 49.1% of the AR were found in the urine, while 21.6% and 34.2% of the AR was excreted via faeces by males and females, respectively. After five days, daily urinary excretion was below 1% of AR. Radioactive material was distributed in all tissues and organs throughout the body but mean concentrations of radioactivity were below 4 µg equiv./g or 0.24% of AD in all organs and tissues at 168 hours after dosing. Highest tissue concentrations were found in thyroid, liver, blood cells, plasma and kidney (Fabian & Landsiedel, 2009).

The pharmacokinetics of [*phenyl-U*-$^{3}$H]prochloraz (radiochemical purity 99%) were studied in rats by measuring radioactivity in plasma and tissues after a single oral dose of 100 mg/kg bw (to 16 male and 16 female rats) and repeated oral doses of 25 mg/kg bw (to 24 male and 24 female rats). Radioactivity was measured for 96 hours after the single dose and at specified intervals for 20 days after the repeated doses. The study complied with the general principles of GLP (statement of authenticity from the project manager).

After the single dose, the plasma concentrations were highest at 24 hours (males 166 µg equiv./mL, females, 82 µg equiv./mL) and declined steadily to consistently low concentrations by 96 hours (males

13 µg equiv./mL, females 11 µg equiv./mL). The tissue concentrations showed a similar pattern, being consistently high up to 24 hours after dosing and then declining fairly rapidly to low concentrations at 96 hours. In general the plasma concentrations in males were appreciably higher than those in females, but the tissue concentrations were similar in the two sexes, with the exception of the kidney, where the concentrations in females were lower than those in males. Apart from the GI tract, the highest concentrations of radiolabel were found in liver and kidneys and the lowest in brain, eyes, male gonads and muscle.

After repeated exposure, the plasma concentrations were once more higher in males than in females. The tissue concentrations showed no obvious sex differences, with the exception of the kidney, in which the concentrations in females were lower. In males, the plasma concentration of radiolabel rose irregularly to a peak (72 µg equiv./mL) at 15 days, but then had declined at day 20. In females, the peak plasma concentration (35 µg equiv./mL) was observed after seven days and was maintained at about this level for the remainder of the treatment. Four days after the last dose, the plasma concentrations in both sexes had fallen considerably (males to11 µg equiv./mL, females to 14 µg equiv./mL) and were similar to measurements four days after the single dose. The tissue concentrations in both sexes rose significantly for the first seven days of dosing and rose only slightly for the remainder of treatment. When selected tissues were analyzed for radiolabel during repeated dosing, liver and kidney showed the highest concentrations and muscle and fat the lowest. Thus, the phenoxy moiety of prochloraz was rapidly eliminated from the body, leaving no significant concentrations of residues in tissues, and at a comparable rate after repeated and single oral doses. Repeated doses gave rise to residues, the concentration of which reached a plateau after 7–14 days (Boardman, 1979).

***Dog***

Three dogs of each sex were given [*phenyl-U*-$^{14}$C]prochloraz (radiochemical purity greater than 99%) as a single oral dose of 18 mg/kg bw in a gelatine capsule. Blood samples were collected 1, 2, 4, 6, 8, 12, 24, 48, 72 and 96 hours after dosing. Urine and faeces were collected daily. The dogs were killed 96 hours after dosing by exsanguination after barbiturate anaesthesia. An extensive range of tissues were removed and prepared for analysis by LSC. The study was conducted before GLP regulations were issued in the testing facility, but the protocol and results were well reported.

Within the first 24 hours, 60% ± 10% of AD was excreted. After 96 hours about 96% ± 4% was excreted, and faecal excretion was the major route, accounting for 64% ± 3% of the total dose. There were no significant differences in the pattern or rate of excretion between the two sexes. The high concentrations found in bile (19 ± 14 mg equiv./kg in males and 41 ± 36 mg equiv./kg in females) indicate that biliary excretion was a significant route of elimination for this compound. The plasma concentrations 96 hours after dosing (15 ± 6 µg equiv./mL) were significantly greater than those in other tissues. The highest concentrations in tissues were found in liver (7.6 ± 1.7 mg equiv./kg) and kidney (5.6 ± 2.3 mg equiv./kg). Most tissues contained less than 3 mg equiv./kg, and the lowest concentrations were found in bone (0.5 ± 0.4 mg equiv./kg), cerebellum (0.7 ± 0.3 mg equiv./kg) and cerebrospinal fluid (0.3 ± 0.6 µg equiv./mL). The plot for plasma concentration against time showed a rapid initial absorption phase, followed by a slow final elimination phase with a $t_{½t}$ of approximately 72 hours. The peak plasma concentrations (29–31 µg equiv./mL) occurred 8–24 hours after dosing (Needham & Campbell, 1982).

## 1.2 Biotransformation

*Study 1*

In a nonguideline study, one of the major urinary metabolites of prochloraz, identified as 2,4,6-trichlorophenoxyacetic acid (BTS 9608) was measured following a single oral dose of three prochloraz equivalents. Three rats of each sex were dosed with prochloraz at 90.1 mg/kg bw, prochloraz–manganese complex (BTS 46828) at 98.2 mg/kg bw, or a major plant metabolite of prochloraz (BTS 44596) at 88.7 mg/kg bw. Urine and faeces were collected at 24-hour intervals until the rats were sacrificed 96 hours after dose administration, and the level of 2,4,6-trichlorophenoxypropionic acid in the samples was determined by gas chromatography.

Metabolite BTS 9608 (2,4,6-trichlorophenoxyacetic acid) proved to be a major urinary

metabolite of all three compounds tested in both male and female rats. It accounted for 19%–53% of AD on a mass basis of the urinary excretion and 9.5%–26.9% of the total dose. A sex difference in excretion of the amount of BTS 9608 was observed. Approximately twice as much metabolite was excreted by males (36%–53%) in their urine as by females (19%–29%). Prochloraz and its manganese co-ordination complex (BTS 46828) gave rise to similar quantities of BTS 9608 in both sexes. With BTS 44596 however, compared to females, the excretion of BTS 9608 from male rats was relatively low; 36% in males compared to 49%–53% in females. Female rats did not show the same test article-related effect as males; the quantity of the metabolite BTS 9608 in their urine was similar for all compounds tested (Campbell & Needham, 1981).

The biotransformation of $^{14}$C-labelled prochloraz (radiochemical purity greater than 99%), uniformly labelled on the phenyl or imidazole ring, was studied in male and female rats given an oral dose of 100 mg/kg bw as a suspension in gum acacia or dissolved in corn oil. A similar dosing regimen was followed in separate experiments with mice and dogs, except that only 18 mg/kg bw was administered to dogs. Urine was collected at 24-hour intervals. In another experiment, a female goat was given a single oral dose of 1.5 mg/kg bw of prochloraz, and one urine sample was collected during the first 24 hours after dosing. The study was conducted before GLP regulations were issued in the testing facility, but the study protocol and reporting of the results were considered acceptable.

Prochloraz was extensively metabolized in rats, with no unchanged compound detectable in the urine. The main metabolites found were 2,4,6-trichlorophenoxyacetic acid (Metabolite 3) and 2-(2,4,6-trichlorophenoxy)ethanol glucuronide conjugate (Metabolite 7), which accounted for up to 80% of the urinary radiolabel. Five other metabolites were detected:

- 2-(2,4,6-trichlorophenoxy)ethanol (Metabolite 2),
- 2-(3-hydroxy-2,4,6-trichlorophenoxy)ethanol (Metabolite 8),
- *N*-2-(3-hydroxy-2,4,6-trichlorophenoxy)ethyl-*N′*-propylurea (Metabolite 6),
- *N*-2-(4-hydroxy-2,6-dichlorophenoxy)ethyl-*N′*-propylurea (Metabolite 5), and
- *N*-2-(2,4,6-trichlorophenoxy)ethyl-urea (Metabolite 4).

The metabolism proceeded via cleavage of the imidazole ring, giving rise to two-carbon fragments which were incorporated into the general metabolic pool, followed by hydroxylation of the phenyl ring and/or side-chain hydrolysis to form more polar compounds. The urinary metabolic profile of prochloraz showed no significant qualitative differences between males and female, or between rats, mice, dogs and the female goat. The metabolism did differ quantitatively according to sex, female rats excreting more of the most polar metabolites than males, but no new metabolites were seen. The major plant metabolites, BTS 44596, BTS 44595, and BTS 45186 were not detected in the urine of dosed rats (Needham, 1982b).

*Study 2*

In another study, five male and five female rats were given [*phenyl-U*-$^{14}$C]prochloraz (radiochemical purity greater than 98.9%) as a dose of 5 mg/kg bw, and two rats of each sex were given 100 mg/kg bw, all by gavage. Urine and faeces were collected daily. Rats at 100 mg/kg bw were killed by cervical dislocation after 24 hours, while those at 5 mg/kg bw were killed four days after dosing. The samples were analyzed by LSC, thin-layer chromatography and high-performance liquid chromatography (HPLC).

At both doses, prochloraz and its metabolites were rapidly and completely excreted in the urine and faeces, faecal excretion dominating at the low dose and urinary excretion dominating at the higher dose (Table 1).

*Table 1. Excretion of orally dosed prochloraz in rats in the period 0–24 hours*

| Dose | Sex | Percentage excreted | | |
|---|---|---|---|---|
| | | Urine | Faeces | Total |
| 5 mg/kg bw | Male | 30 ± 4.4 | 53 ± 6.1 | 83 ± 4.6 |
| | Female | 23 ± 6.9 | 64 ± 8.6 | 87 ± 1.7 |
| 100 mg/kg bw | Male | 33 ± 10 | 37 ± 15 | 70 ± 5.0 |
| | Female | 20 ± 7.0 | 29 ± 10 | 49 ± 17 |

Sources: Challis & Creedy (1989); Needham (1997)

The main metabolic pathway at both doses involved opening of the imidazole ring and initial loss of small fragments, which along with a considerable quantity of unchanged prochloraz, were the main compounds found in faeces. Further metabolism of Metabolite 1 yielded Metabolite 4, which was excreted mainly in faeces or further metabolized to Metabolite 2 and then Metabolite 3. The latter two compounds were excreted mainly in the urine in free or conjugated form. Metabolite 3 (2,4,6-trichlorophenoxyacetic acid) was the main constituent in urine. A small amount of this acid was further metabolized to the trichlorophenol, which was also excreted in urine. A minor metabolic pathway involved aromatic hydroxylation to give Metabolites 5 and 8, which were excreted in small amounts in urine and faeces. Few differences in the metabolic profiles were seen according to dose, but quantitative differences by sex were apparent with respect to the urinary metabolites in animals at the low dose (Challis & Creedy, 1989; Needham, 1997).

*Study 3*

Male and female rats were given [*phenyl-U*-$^{14}$C]prochloraz (radiochemical purity greater than 98%) as a single dose of 100 mg/kg bw by gavage. Urine, faeces and expired carbon dioxide were collected. Four days after dosing the rats were killed and tissues removed for analysis by LSC, thin-layer chromatography and HPLC or gas–liquid chromatography.

Prochloraz underwent extensive metabolism, the primary route being opening of the imidazole ring followed by hydrolysis of the alkyl chain. The main products were Metabolites 3 and 2, which were present mainly as glucuronide conjugates. Aromatic hydroxylation occurred to produce several minor metabolites. No unchanged prochloraz was detected in the urine. The concentrations of tissue residues 96 hours after dosing were generally less than 1 mg equiv./kg tissue. The highest concentrations were found in the liver (2.8–5.1 mg equiv./kg) and kidney (1.5–2.1 mg equiv./kg). The concentrations of residues in female rats were generally slightly higher than those in males. The metabolites were excreted quantitatively within 96 hours, more than 50% of the AR being found in the excreta in the period 0–24 hours. Urinary excretion accounted for 65% of AD in males and 41% in females (Needham & Challis, 1991).

*Study 4*

The excretion, tissue kinetics and metabolism of prochloraz was investigated in the rat. During the study, six test groups of three male and three female Sprague Dawley rats (6–7 weeks old, body weight range 165–203 g) received either a single or multiple oral dose of [*phenyl-U*-$^{14}$C]prochloraz (purity 99.5% for batch 1, 98.6% for batch 2) dissolved in corn oil at a dose level of 5 mg/kg bw per day. Group A received a single dose, urine and faecal samples being subsequently taken 24 hour post dose for investigation of excretion, metabolite identification and tissue kinetics. Groups B, C and D received daily administrations for three, six and 10 days, respectively, for investigation of tissue kinetics. Group E received daily administration for 14 days, urine and faecal samples being subsequently taken 24 hour post dose for metabolite identification and investigation of tissue kinetics. Following dose administration, the rats used for the excretion phases were individually housed in all-glass metabolism cages. Group F received daily administration for 14 days and were sacrificed seven days after final dose administration for investigation of tissue kinetics following a seven day recovery period. Animals in Groups A to E were sacrificed 24 hours after the final dose of prochloraz. The following organs were removed for residue analysis: adrenals, whole blood, bone, brain, eyes, GI tract, heart, kidney, liver, lung, muscle, ovaries/testes, plasma, renal fat, spleen and residual carcass. Urine samples and faecal extracts from groups A (single dose, 24 hour study) and E (multiple dose, 14 day study) were analyzed by LC-MS/MS. Results

from the toxicokinetic investigations are presented in section 1.1 Absorption, distribution and excretion.

The structure of metabolites excreted in urine and faeces was investigated from samples taken from Group A and Group E (sampled 24 hours after dose). Only those metabolites detected in urine and faeces from Group A were taken into account, because quantification was only possible in animals that received a single oral dose. Though not all structures could be unambiguously identified, two processes were found to drive the degradation of the molecule: oxidative hydroxylation (sometimes accompanied by substitution with chlorine) in the phenyl ring, and stepwise degradation of the imidazole ring. There are indications that oxidative ring hydroxylation occurs very rapidly in the rat, so that the main plant metabolites BTS 44595 and BTS 44596 were not observed in this study, though analogous structures with a hydroxylated phenyl ring were detected. The main metabolite in faeces was unchanged prochloraz at 41%–48% of AD, while in urine no prochloraz could be detected. In urine 2,4,6-trichlorophenol, the main metabolite formed in plants, was detected only in small amounts, but its sulfate was found in the urine of male and female rats at 2.05% and 1.20% of AD, demonstrating that this major plant metabolite also occurs in animals, but probably only as an intermediate. In total 61% of AD was identified in males and in the females 68% could be identified. For quantitation as a percentage of dose, a calculation was performed from data given in the study.

Few other metabolites were found in the samples from the multiple dose Group E. These metabolites were not quantifiable in terms of percentage of dose. Among the metabolites detected after multiple dosing were BTS 44770, BTS 3037 and other compounds with a degraded imidazole ring. No structure that contradicted the proposed pathway or could not be accommodated by it, was found in this study (Gedik & Kidd, 2003).

*Study 5*

The metabolic fate of $^{14}$C-labelled prochloraz was investigated in a follow-up study to the aforementioned biokinetics study of Fabian & Landsiedel (2009). Material from the biokinetics study was used for investigation of metabolite patterns in urine and faeces. Urine and extracts of faeces were analyzed by HPLC. Individual metabolites were detected down to a level of about 0.4% of AD or even less. Metabolites were identified by mass spectrometry in isolated fractions of urine and faeces samples, but also in the original pooled urine sample from female rats.

In urine the main metabolite identified in both genders was 2,4,6-trichlorophenoxyacetic acid, amounting to about 41% of the dose for male rats and 19% for female rats. The glucuronic acid conjugate of 2,4,6-trichlorophenoxyethanol was identified as another main metabolite in urine, which eluted with a derivative hydroxylated at the propyl side chain. Both metabolites together amounted to about 12% of the dose in male rats and contributed ca 7% of the dose in female, whereas 2,4,6-trichlorophenoxyethanol was the major compound.

A further 24 metabolites were identified in urine at levels between less than 0.4% of AD to 5.8% of AD. Numerous metabolites were generated by opening of the imidazole ring and further modification of the intermediates by phenyl ring hydroxylation, chlorine substitution by a hydroxyl group and conjugation with glucuronic acid. Modification included *N*-formylated and urea derivatives. Further metabolites were additionally hydroxylated at the propyl side chain, or contained a five- or six-membered cyclic carbamate. Several urea derivatives without a *N*-propyl group were identified, one of which had the imidazole moiety substituted by *N*-acetyl-lysine. In other metabolites, the imidazole moiety was first substituted by 3-thioglyceric acid or mercapturate. Other metabolites were formed by oxidative ring opening and further degradation.

The complete loss of the imidazole and carbamide moiety generated secondary and primary amines. Additionally, small amounts of the glucuronic acid conjugate of 2,4,6-thrichlorophenol were detected. Three metabolites were identified containing the intact imidazole ring and were only modified at the phenyl ring by hydroxylation or chlorine substitution.

In faeces extracts a total of 20 metabolites were identified at concentrations in range of about 1% to 6.9% of the dose each. Several of these metabolites were also detected in urine or were identified to be related compounds to the metabolites already described for urine, however, some differences were observed. The main metabolites of urine were not detected in faeces, while the intermediates 1-propyl-1-[2-(2,4,6-trichlorophenoxy)ethyl]urea and 2,4,6-trichlorophenoxyethylamine were only identified in faeces. The two most prominent metabolites in faeces were present at about 7% of AD in female rats

and about 2%–3% of AD in male rats. They contained the intact imidazole ring and were only modified by hydroxylation of the phenyl ring. Parent compound was detected at amounts less than 1.7% of AD.

From these studies it was concluded that prochloraz was metabolized by oxidative opening of the imidazole ring, and initial loss of small fragments to generate 3-formyl-1-propyl-1-[2-(2,4,6-trichlorophenoxy)ethyl]urea and 1-propyl-1-[2-(2,4,6- trichlorophenoxy)ethyl]urea. Several metabolites were generated by modification of the phenyl ring by hydroxylation, chlorine substitution by a hydroxyl group and subsequent conjugation. Additionally, hydroxylation at the propyl side chain, substitution of the imidazole moiety by glutathione followed by degradation of the tripeptide, and substitution by amino acids yielded further metabolites. Cleavage of the propyl group or loss of the complete imidazole and carboxamide moiety generated secondary and primary amines. Further metabolism leads to 2,4,6-trichlorophenoxyethanol and 2,4,6-trichlorophenoxyacetic acid, the major metabolite component of urine (Hafemann et al., 2008).

*Study 6*

The final study was designed to compare the metabolite pattern in rats using the 0–24 hour urine and faecal samples taken from two previous kinetic studies involving, prochloraz–copper complex (Needham & Hemmings, 1989) and, prochloraz (Dawson, 1989). Analysis of the samples was carried out using HPLC on an ODS C18 column with solvent systems containing methanol, acetonitrile, water and acetic acid (or heptane-sulfonic acid in the case of faecal samples). Urine samples were subjected to acid (heating in hydrochloric acid, but note that this destroys BTS 45186) and enzyme hydrolysis (glusulase obtained from snail digestive juice with glucuronidase, sulfatase and other hydrolytic activity) to cleave conjugate groups from the prochloraz residues. Faecal samples were homogenized in water, freeze-dried and extracted with methanol prior to chromatographic analysis. Identification was performed by chromatographic comparison with synthetic reference compounds.

The same metabolic pattern was detected in urine and faecal samples from the two studies that employed prochloraz–copper complex (SN 125671) and prochloraz alone. There were no significant differences in the metabolism of prochloraz and its copper complex.

The main metabolite detected in urine from both treatment groups was BTS 9608 (trichlorophenoxyacetate, free and conjugated), which accounted for up to approximately 40% of the radioactivity present in urine after dosing with the copper complex and approximately 38% of the radioactivity from prochloraz-treated rats.

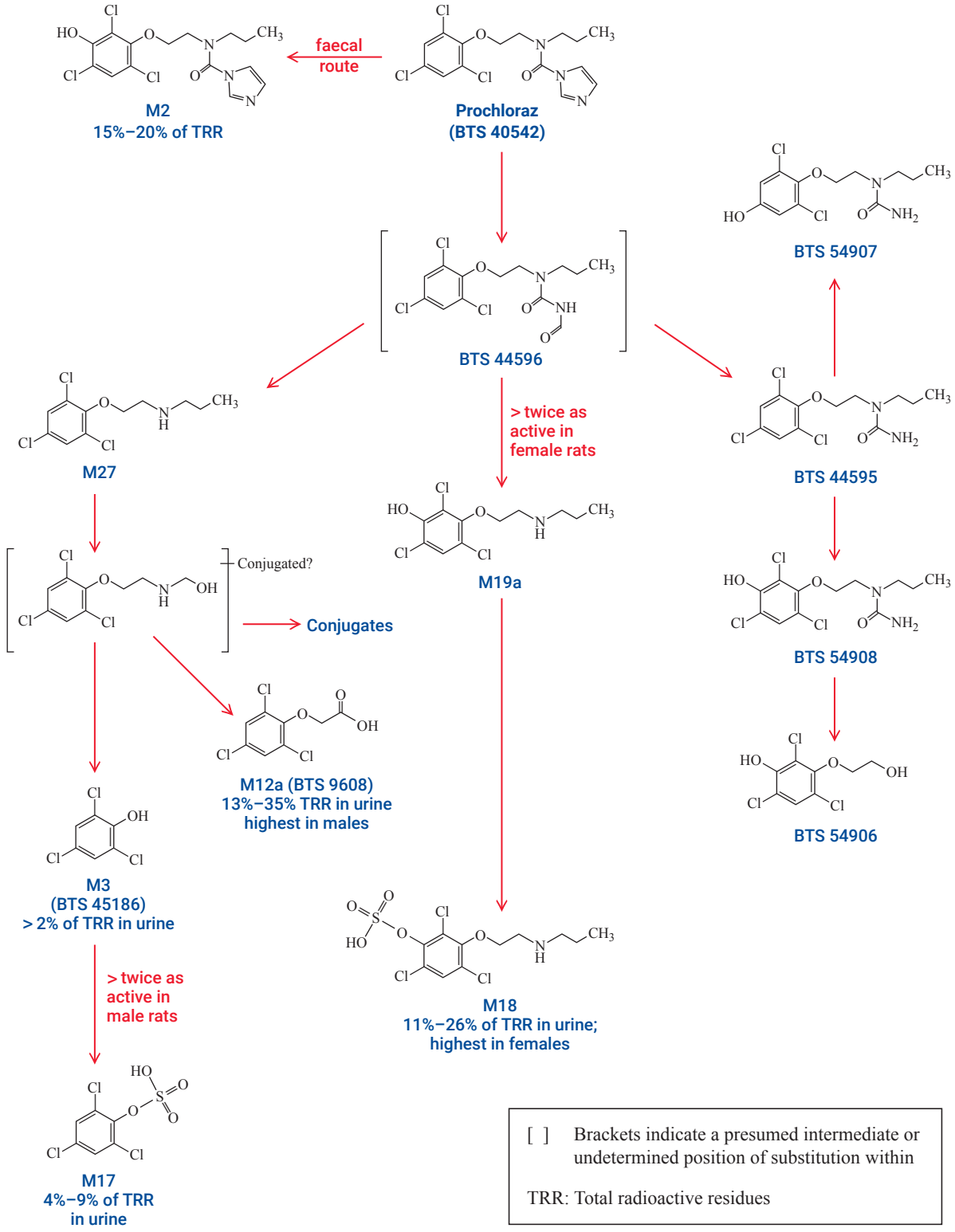
The overall pattern of metabolites produced by prochloraz was similar to that produced by the copper complex. There were some quantitative differences in the amount of free BTS 3037 (trichlorophenoxyethanol) and BTS 45186 (trichlorophenol) excreted in the urine between male rats dosed with prochloraz or the copper the complex. After enzymatic incubation, increased amounts of BTS 45186 were detected, accounting for about 6% of the radioactivity present in urine after dosing with the copper complex (SN 125671) and about 9% in the case of the prochloraz-dosed rats.

The major metabolites present in faeces were BTS 3037 and unchanged prochloraz in most of the animals, regardless of the parent compound administered. In faeces the other major metabolites were BTS 45186, BTS 9608 and BTS 44595 and/or BTS 44596. The presence of the prochloraz parent molecule also demonstrates that the copper co-ordination complex (SN 125671) readily dissociates prior to absorption in the GI tract, and that a significant amount passes through the GI tract unabsorbed. Slightly less prochloraz was absorbed by the gut when the copper complex was administered, particularly in female animals (Needham, 1989).

***Dog***

The stability of prochloraz and its plant metabolites M2 and M1 in the gastric juice and plasma of dogs was investigated in vitro. While both prochloraz and M1 were completely stable in gastric juice, M2 underwent slow hydrolysis to form M1 (less than 10% in two hours). After incubation in plasma, less than 2% of the prochloraz present underwent hydrolysis (to yield M2), and about 20% of M2 was converted to M1. No 2,4,6-trichlorophenol was detected as a hydrolysis product in any of these experiments. The initial step in the metabolism of prochloraz is therefore likely to occur in the liver (Needham, 1980).

*Figure 2. Proposed metabolic pathway for prochloraz in rats.*



### 1.3 Effects on enzymes and other biochemical parameters

Prochloraz was found to induce hepatic mixed-function oxidases in a series of investigations in rats and mice.

Prochloraz was administered to three groups of mice at doses of 0, 10 or 100 mg/kg bw, by gavage twice daily for four days. A separate group of mice received phenobarbital at 80 mg/kg bw by intraperitoneal injection once daily for four days. Twenty-four hours after the last dose all animals were killed, and a postmitochondrial supernatant, containing microsomal enzymes, was isolated from each liver, and the content of protein and of cytochromes P450 and b5 was determined. At 100 mg/kg bw, prochloraz was a potent enzyme inducer, liver weight and microsomal protein and cytochrome contents all being significantly increased. At 10 mg/kg bw, only the cytochrome P450 and microsomal protein contents were increased. As the peak of the cytochrome P450 difference spectrum was 450 nm, prochloraz is not a polycyclic aromatic hydrocarbon-type inducer, which would have a peak at 448 nm, and was therefore similar to phenobarbital (Challis & Campbell, 1983).

The effect of prochloraz (purity 95.7%) on the hepatic mixed-function oxidase system was studied in five male and five female CD-1 mice. The mice received 0, 80, 325 or 1300 ppm prochloraz in the diet for 14, 14, 6 and 2 weeks respectively. A positive control group received four daily intraperitoneal doses of 80 mg/kg bw phenobarbitone five days prior to the study. After termination, livers were removed and weighed. Subsequently, microsomal fractions were prepared and analyzed for protein, cytochrome P450 and b5 concentrations.

At at concentrations 325 and 1300 ppm prochloraz induced cytochrome P450 activity, 2–3- and 3.6–6.3-fold, respectively, also inducing cytochrome B5 activity and microsomal protein concentration at these doses. Liver weight was increased (statistically significant) at these concentrations. At 80 ppm, effects were less consistent and smaller. However, at all measurement points a change in at least one parameter was statistically significant. There was no significant sex difference at any dose level (Needham, 1983a).

The effect of prochloraz (purity greater than 98%) on liver was studied in male COBS rats. Six rats in each of three groups received prochloraz at dose levels of 0, 10 or 100 mg/kg, by oral gavage twice daily for four days. A positive control group of six male rats received 1 g/L phenobarbitone in the drinking water for seven days. At termination, all animals were killed and livers removed. Livers were weighed and supernatant, containing microsomal enzymes, prepared from each liver. Subsequently, aniline hydroxylase activity, *p*-nitroanisole demethylase activity, cytochrome P450 and cytochrome b5 concentrations were measured. Liver weight was increased (statistically significant) in rats receiving 100 mg/kg bw prochloraz. Furthermore, microsomal protein concentration, aniline hydroxylase activity and *p*-nitroanisole demethylase activity were increased with statistical significance at 100 mg/kg bw. The concentration of cytochrome P450 showed a statistically significant increase at both 10 and 100 mg/kg bw. The concentration of cytochrome B2 did not change after prochloraz exposure. The effects of prochloraz were comparable to those of phenobarbital (Needham, 1983b).

Similar induction of mixed-function oxidases was seen when male rats were fed diets containing prochloraz at a concentration of 2500 ppm for seven days, whereas only a small increase was found at 100 ppm (Rivière, 1983).

The profile of the hepatic mixed-function oxidase system in male rats was examined after treatment with prochloraz or one of three major metabolites of prochloraz: M1, 2,4,6-trichlorophenoxy-ethanol and 2,4,6-trichlorophenoxyacetic acid. All animals were dosed twice daily for four days with a solution or suspension of a molar equivalent of prochloraz or its metabolite in corn oil. A first group of rats were given prochloraz or one of the first two metabolites at a rate equivalent to 100 mg of prochloraz per kg bw, or 2,4,6-trichlorophenoxyacetic acid at a rate equivalent to 50 mg of prochloraz per kg bw, as the higher dose had been shown to have adverse effects. In a second group of rats all four compounds were tested, each at a rate equivalent to 50 mg prochloraz per kg bw. Control animals received corn oil only, and another group received phenobarbital in drinking-water at 0.1% (w/v) for 14 days. A further group received naphthoflavone intraperitoneally at 80 mg/kg bw per day for four days, and hepatic microsomes were prepared from rats that had been induced with clofibrate by an intraperitoneal dose of 400 mg/kg bw per day for three days before the study. At the end of dosing, all animals were killed and a postmitochondrial supernatant, containing microsomal enzymes, was isolated from each liver and

analyzed for protein and cytochromes P450 and b5 content, and for the activities of 7-ethoxyresorufin-*O*-deethylase), 7-pentoxyresorufin-*O*-dealkylase, 7-ethoxycoumarin-*O*-deethylase, aldrin epoxidase and lauric acid 12-hydrolase.

The profile of induction caused by prochloraz reflected the contribution of the individual metabolites. The activity of lauric acid hydroxylase was slightly increased, but aldrin epoxidase was induced by seven-fold and 7-pentoxyresorufin-*O*-dealkylase by 14-fold. Metabolite M1 was a phenobarbital-type inducer, increasing the activity of aldrin epoxidase by 120% and that of 7-pentoxyresorufin-*O*-dealkylase by eight-fold. Metabolites 2-(2,4,6-trichlorophenoxy)ethanol and 2,4,6-trichlorophenoxyacetic acid were both inducers of the clofibrate type, increasing the activity of lauric acid 12-hydrolase. The induction profile of prochloraz was mixed, but the predominant characteristics were those of a phenobarbital-type inducer (Needham, Creedy & Dawson, 1992).

## 2. Toxicological studies

### 2.1 Acute toxicity

#### (a) Lethal doses

The results of studies of acute toxicity with prochloraz are summarized in Table 2.

***Table 2. Summary of acute toxicity studies with prochloraz***

| Species | Strain | Sex | Route | Vehicle | Purity (%) | $LD_{50}/LC_{50}$ | Reference |
|---|---|---|---|---|---|---|---|
| Mouse | CD-1 | M | Oral | 10% acacia solution | Not reported | 2400 mg/kg bw | Shaw & Carter (1976)[a] |
| Mouse | CD-1 | M | Oral | 10% acacia solution | Not reported | 800–1600 mg/kg bw (1600–2400 mg/kg bw for copper complex) | Stobart, Lancaster & Smithson, 1979a[b] |
| Rat | Boots Wistar | M, F | Oral | 10% acacia solution | Not reported | 1600–2400 mg/kg bw (M) | Shaw, Lancaster & Smithson, (1979a)[c] |
| Rat | CFY | M, F | Oral | 10% acacia solution | Not reported | 2400 mg/kg bw (M/F) | Shaw, Lancaster & Smithson, (1979a)[c] |
| Rat | Wistar | M | Oral | 10% acacia solution | Not reported | 1600–3200 mg/kg bw | Shaw, Lancaster & Smithson, (1979b)[d] |
| Rat | CD-1 | M, F | Oral | Sesame oil | 97.2 | 300–1000 mg/kg bw | Chevalier (2005)[e,†] |
| Rat | Sprague Dawley CD | F | Oral | Arachis oil | 96.7 | 1023 mg/kg bw | Dreher (2002a)[f] |
| Rat | Sprague Dawley CD | F | Oral | Arachis oil | 96.7 | > 2000 mg/kg bw (F) | Dreher (2002b)[g] |
| Rat | Sprague Dawley | M, F | Oral | Maize oil | 94.6 | 1204 mg/kg bw (male) | Privman, Pirak & Nysk, (1989)[h] |
| Rat | Sprague Dawley | M, F | Oral | Not stated | Prochloraz-Zn complex (not stated) | > 4000 mg/kg bw | Dreher (1990)[i] |
| Dog | beagle | M, F | Oral | Gelatin capsule | Not reported | Sample size too small for $LD_{50}$ evaluation | Morgan et al. (1978)[j] |
| Baboon | - | F | Oral | Gelatin capsule | Not reported | Sample size too small for $LD_{50}$ evaluation | Morgan et al. (1977)[k] |

| Species | Strain | Sex | Route | Vehicle | Purity (%) | $LD_{50}/LC_{50}$ | Reference |
|---|---|---|---|---|---|---|---|
| Rat | Sprague Dawley | M, F | Dermal | None | 96.3 | > 2100 mg/kg bw | Hounsell & Ogle (1987)[l] |
| Rat | Wistar | M, F | Dermal | None | 98 | > 2000 mg/kg bw | Bioassay Labor für biologische Analytik, 2012a[m] |
| Rat | Wistar | M | Dermal | Purified water | Prochloraz-Cu complex (not stated) | > 5000 mg/kg bw | Stobart, Lancaster & Smithson, 1979b[n] |
| Rat | CD | M, F | Dermal | Sesame oil | Prochloraz-Zn complex 98.6 | > 2000 mg/kg bw | Chevalier, 2004a[o,#] |
| Rabbit | Albino | M, F | Dermal | None | 94.6 | > 2000 mg/kg bw | Kenan & Nyska, 1989[p] |
| Rabbit | New Zealand White | M, F | Dermal | None | 98.2 | > 3 ml/kg bw | Kynoch et al., 1979[q] |
| Rat | Wistar | M, F | Inhalation | Air | 96.3 | > 2.16 mg/L | Jackson & Hardy, 1987[r] |
| Rat | Wistar | M, F | Inhalation | Air | 94.6 | > 2.41 mg/L | Hardy et al., 1989[s] |
| Rat | CD | M | Intraperi-toneal | Corn oil | 96.4 | 400–800 mg/kg bw | Smithson & Lancaster, 1980[t] |

M: Male; F: Female; bw: Body weight; $LC_{50}$: Median lethal concentration; $LD_{50}$: Median lethal dose;

[#] The study was performed by the Laboratory of Pharmacology and Toxicology in Hamburg. The Meeting is aware of the current debate about allegations of fraud at this Laboratory. As no formal conclusion about these allegations has been reached yet the Meeting decided to include results from the study. It has no impact on the overall evaluation of this substance.

[a] No guideline reported. groups of 10 male CD-1 mice were given 400 to 2400 mg/kg bw in a single dose. Mortality was recorded up to day 7 after treatment. After termination alanine aminotransferase (ALT) estimations were conducted (method not reported).

[b] No guideline reported. Groups of 10 male CD-1 mice received prochloraz or prochloraz–copper complex at a single oral dose of 400, 800, 1600 or 2400 mg/kg bw. The mice were observed for 14 days after treatment. Clinical signs were observed in all treatment groups consisting of ataxia, slow respiration rate, coolness to touch, closed eyes and diarrhoea. At 800 mg/kg bw and above convulsions and increased lacrimation and salivation were observed.

[c] Study copied from the 2001 JMPR evaluation. Study report not included in the current dossier.

[d] No guideline reported. Groups of five male Boots Wistar rats received prochloraz or its copper complex as a single oral dose at 800, 1600 or 3200 mg/kg bw. Clinical signs were observed at all dose levels consisting of slow respiration rate, piloerection, ataxia, increased salivation, cool to touch, tremors, hunched posture, closed eyes and increased lacrimation.

[e] Study conducted in accordance with OECD TG 423. Clinical signs were observed at 1000 mg/kg bw consisting of reduced motility, ataxia, reduced muscle tone and dyspnoea.

[f] Study performed according to OECD TG 425. Signs of systemic toxicity noted in one animal treated with 2000 mg/kg bw were hunched posture, decreased respiratory rate, laboured respiration, loss of righting reflex and increased lachrymation. Animals at 2000 mg/kg bw that died or where killed in extremis showed abnormally red lungs, dark or pale liver, dark kidneys and slight haemorrhage of the gastric mucosa at necropsy.

[g] Study performed according to OECD TG 425. Signs of systemic toxicity included hunched posture, diarrhoea and diuresis at 2000 mg/kg bw.

[h] Study performed according to OECD TG 401. Clinical signs were observed at 966 mg/kg bw and above, consisting of hunched posture, ataxia, diarrhoea, salivation, tremors, bradypnoea, piloerection, decreased motor activity, opacity and urogenital staining.

[i] Study performed according to OECD TG 425. Two animals treated with 2000 mg/kg bw were found dead and one animal treated with 2000 mg/kg bw was killed in extremis one day after dosing. No deaths were noted at a dose level of 510 or 550 mg/kg bw. Signs of systemic toxicity noted in one animal treated with 2000 mg/kg bw were hunched posture, decreased

respiratory rate, laboured respiration, loss of righting reflex and increased lachrymation. No signs of systemic toxicity were noted in animals treated at 510 or 550 mg/kg bw.

[j] No guideline reported. Groups of two dogs (one M/one F) were given a single dose of 0, 10, 100, or 250 mg/kg bw prochloraz. Clinical signs, body weight and food consumption were recorded. Haematological and biochemical evaluation and urinalysis was conducted. After termination organ weights were measured and histopathological evaluation was conducted. At 100 mg/kg bw and above clinical signs were observed including increased salivation, licking and diarrhoea. At 250 mg/kg bw a decrease in body weight as well as increased alkaline phosphatase activity was observed. Relative liver weight was also increased at the mid and high dose although no dose response was observed. Due to the low number of animals no NOAEL could be set from this study.

[k] No guideline reported. One female baboon was given a single oral dose of 50 then 250 mg/kg bw of prochloraz on days 1 and 17. Second female served as control. Haematological, biochemical and urinary analysis were conducted. Sections of the liver, kidney and bone marrow smears were examined histologically. Clinical signs were observed after dosing at 250 mg/kg bw. A slightly decreased serum potassium level was observed, but no other adverse effects. Due to the few animals involved, no conclusion could be drawn as to a NOAEL.

[l] Study conducted in accordance with OECD TG 402. No clinical signs nor local effects were observed.

[m] Study performed according to OECD TG 402. No clinical signs nor local effects were observed.

[n] No guideline reported. Groups of five rats were given a single dermal application of 5000 mg/kg bw and observed for 15 days. Clinical signs included sedation for one hour on the day of dosing and excitability in one rat from days 3–8. Mild erythema was observed in most rats.

[o] Study performed according to OECD TG 402. No clinical signs nor local effects were observed.

[p] Study performed according to OECD TG 402. No clinical signs were observed. Slight exfoliation of the skin was noted on days 9–15 in most animals.

[q] No guideline reported. Groups of two male and two female rabbits were treated with a single dose of 3 mL/kg bw. Increased respiration, piloerection, hunched posture and poor general condition was observed in treated animals. Well defined dermal reactions appeared on day 2 which were reversible by day 12.

[r] Study conducted in accordance with OECD TG 403. The highest attainable dose of 2.16 mg/L was tested. Clinical signs included partial closing of the eyes, lacrimation, abnormal respiratory rate and/or movement, and hunched posture.

[s] No guideline reported but generally agreed with OECD TG 403. The highest attainable dose was 2.41 mg/L. The clinical signs observed were abnormal breathing and soiled fur.

[t] No guideline reported. Groups of five male CD rats received a single intraperitoneal dose of 50, 100, 200, 400, 800 or 1600 mg/kg bw. Rats were observed for 15 days. Clinical signs were observed at 100 mg/kg and above, including coma, prostration, lethargy, sedation, inactivity, ataxia, piloerection, partially closed eyes, irregular respiration rate, limpness to touch, hunched posture and walking on tiptoe.

### (b) Dermal irritation

The results of studies of dermal irritation with prochloraz are summarized in Table 3.

***Table 3. Summary of dermal irritation studies with prochloraz***

| Species | Strain | Sex | Purity | Result | Reference |
|---|---|---|---|---|---|
| Rabbit | New Zealand White | M/F | 98.0% | Slightly irritating | Bioassay Labor für biologische Analytik, 2012b |
| Rabbit | New Zealand White | M/F | 96.3% | Non-irritating | Cuthbert & D'Arcy-Burt, 1984a |
| Rabbit | Albino | M/F | 94.6% | Slightly irritating | Kenan, 1989a[a] |
| Rabbit | New Zealand White | F | Prochloraz–copper chloride complex; 99.4% | Non-irritating | Jackson & Wood, 1990[a] |
| Rabbit | New Zealand White | M | Prochloraz–Zinc complex; 99.5% | Non-irritating | Tos, 1995[a] |

M: Male; F: Female;

[a] Study performed in accordance with OECD TG 404

The dermal irritation potential of technical-grade prochloraz (purity, 96.3%) was tested in three male and three female New Zealand White rabbits to which 0.5 mL of test material was applied under a gauze patch and covered with occlusive tape for four hours. At the end of this period the patch was removed, and skin reactions were assessed 48 and 72 hours later. No irritation was seen at treated sites at any time during the study. No clinical signs were seen. A quality assurance statement was included (Cuthbert &

D'Arcy Burt, 1984a).

In a dermal irritation study, 500 mg of undiluted technical grade prochloraz (purity: 94.6%, batch P-3) was applied under a semi-occlusive dressing to the clipped intact skin of three male and three female albino rabbits for four hours. Observations were performed according to Draize at 5, 24, 48, 72 hours and eight days after patch removal. Two rabbits showed slight erythema 48 hours after administration of prochloraz. At the 72-hour examination, one rabbit showed slight erythema and two rabbits showed well defined erythema. All signs had resolved between 72 hours and eight days after administration (Kenan, 1989a).

In a dermal irritation study, the intact skin of three female New Zealand White rabbits was exposed for four hours, under occlusion, to 500 mg prochloraz–copper chloride complex (purity 99.4%, batch CR 19417/1) moistened with 1% (w/v) methyl cellulose in distilled water. Dermal irritation was scored according to Draize 0.5–1, 24, 48 and 72 hours after patch removal. No skin irritation was observed at any time point (Jackson & Wood, 1990).

In a dermal irritation study, the intact skin of two male and one female Crl:KBL New Zealand White rabbits was exposed for four hours under semi-occlusion to 0.5 mL prochloraz (purity 98.0%, batch COD-000718). Dermal irritation was scored 1, 24, 48 and 72 hours and seven days after patch removal. Directly after removal, very slight erythema was observed in all three animals. In two animals, the observed erythema persisted until 72 hours after removal. With the third animal, very slight erythema was observed 1 and 24 hours after removal, followed by well-defined erythema after 48 and 72 hours after removal. All observed effects were reversible within seven days. No oedema was observed (Bioassay Labor für biologische Analytik, 2012b).

In a dermal irritation study, the intact skin of three New Zealand White rabbits was exposed for four hours under semi-occlusion to 0.5 g prochloraz (purity 97.0%, batch 931108). Dermal irritation was scored 1, 24, 48 and 72 hours after patch removal. None of the treated animals showed any clinical sign or behavioural alteration. At the application sites, no signs of dermal irritancy were noted in any animal at any time (Tos, 1995).

### (c) Ocular irritation

The results of studies of eye irritation with prochloraz are summarized in Table 4.

***Table 4. Summary of eye irritation studies with prochloraz***

| Species | Strain | Sex | Purity | Result | Reference |
|---|---|---|---|---|---|
| Rabbit | New Zealand White | F | 98.0% | Minimal irritation | Bioassay Labor für biologische Analytik, 2012c |
| Rabbit | New Zealand White | M/F | 96.3% | Minimal irritation | Cuthbert & D'Arcy-Burt, 1984b |
| Rabbit | Albino | M/F | 94.6% | Minimal irritation | Kenan, 1989b |
| Rabbit | New Zealand White | F | Prochloraz–Cu complex; 99.4% | Moderately irritating | Davies, 1990 |
| Rabbit | Himalayan | M | Prochloraz–Zn complex 98.6% | Irritating | Leuschner, 2004[#] |

M: Male; F: Female;

[#] The study was performed by the Laboratory of Pharmacology and Toxicology in Hamburg. The Meeting is aware of the current debate about allegations of fraud conducted by this Laboratory. As no formal conclusion about these allegations has been reached yet the Meeting decided to include the results from the study.

[a] Study performed in accordance with OECD TG 405.

The ocular irritation potential of technical grade prochloraz (purity 96.3%) was tested in three male and three female New Zealand White rabbits, to which 0.1 mL of test material was applied onto the lower-right eyelid. The lids were then gently held together for 1–2 s. The right eye remained

untreated to serve as a control. The eyes were examined for irritation under standard illumination, and any ocular reactions recorded 1, 24, 48 and 72 hours after instillation. No corneal or iridal response was seen. Slight conjunctival responses (redness, score 1) were seen only one hour after instillation. The eyes were normal after 24 hours. No clinical signs were seen. A quality assurance statement was included (Cuthbert & D'Arcy Burt,1984b).

In a test for acute eye irritation 100 mg of technical grade prochloraz (purity 94.6%, batch P-3) was applied to the eye of six rabbits. Ocular damage or irritation was scored according to Draize 1, 24, 48 and 72 hours following treatment. A slight redness was seen in the eyes of four rabbits and diffuse, crimson redness was seen in one rabbit one hour after administration of the test material. Some of these rabbits showed slight chemosis. All signs related to treatment resolved within 24 hours. No corneal lesions were observed using the fluorescein dye test (Kenan, 1989b).

In a primary eye irritation study 100 mg of undiluted prochloraz–Zn complex (purity 98.6%, batch 70868018) was placed into the conjunctival sac of the right eye of three male Himalayan rabbits (2.5–2.6 kg); the left eye served as control. Ocular damage or irritation was assessed at 1, 24, 48, 72 hours and daily between four and 14 days following instillation, according to the guidelines. In addition, eyes were examined with fluorescein 24 hours, 7 and 14 days after administration. Corneal opacity (grades 1 to 3) was observed in all animals for up to 13 days after instillation of prochloraz–Zn. The fluorescein tests performed after 24 hours revealed corneal staining amounting to a quarter of the surface in two animals and half of the surface in one animal. At seven days after treatment, corneal staining amounted to a quarter of the surface in two animals and to half of the surface in one animal. The fluorescein test on day 14 revealed no pathological findings. Irritation of the iris (grade 1) was observed in all animals for up to 11 days. Conjunctival redness (grade 1) was observed in all animals for up to nine days. Conjunctival chemosis (grade 1) was observed in one animal at 24 and 48 hours after instillation. In addition, white deposits in the conjunctival sac were observed for up to four days after treatment (Leuschner, 2004).

In an acute eye irritation study, 0.1 mL of technical prochloraz–copper chloride complex (purity 99.4%, batch CR 19417/1) was instilled into the conjunctival sac of one eye of each of three female New Zealand White rabbits. As result of malfunctioning of a syringe, one of the rabbits was instilled with only 0.05 mL of the test article. The untreated eyes served as controls. Ocular irritation was scored based on conjuctival redness and chemosis, corneal opacity and the condition of the iris at one hour and 1, 2, 3, 4, 7 and 14 days after application. Swelling inside the lower eyelid was observed in all three animals from one or two days after application and persisted until day 3 in the rabbit that had received 0.05 mL, but day 7 in the other rabbits. No other treatment-related clinical signs were observed in the animal receiving 0.05 mL. Slight changes of the iris were observed in one animal only at day 2 after application, and in the another from day 1 until day 3. Slight conjuctival redness was observed in two animals; in one animal from 1 hour until day 1 after application, and in the other from day 1 to day 2 after application. Conjuctival chemosis was observed in two animals. In one animal, slight conjuctival chemosis was observed after days 1 and 2. In the other animal obvious conjuctival chemosis was observed at day 1 and this was still present but slight at day 2. All observed effects were reversible within four days of application, except from swelling of the eyelid which was reversed within 14 days. No treatment-related clinical signs were observed in the cornea. It was concluded that technical prochloraz–copper chloride complex was moderately irritating to the rabbit eye, eliciting transient reversible conjunctival irritation, and slight transient reversible changes in the iris (Davies, 1990).

In an acute eye irritation study, 0.1 mL of prochloraz (purity 98.0%, batch COD-000718) was instilled into the right conjunctival sac of each of three female New Zealand White rabbits (Crl:KBL). The untreated left eye served as a control. Ocular irritation was scored based on conjunctivae redness, chemosis and discharge, corneal opacity and the condition of the iris at 1, 24, 48 and 72 hours after application. At 24 hours after application eyes were examined for corneal lesions. Slight conjuctival redness was observed in all three animals one hour after application and persisted in two animals until 24 hours after application. Slight conjuctival chemosis was only observed in one animal at one hour after application and this was reversible within 24 hours. Obvious conjuctival discharge was observed in all animals at one hour after application, but was reversible within 24 hours. In one animal scleral vessels were injected at 1 and 24 hours after application. All observed effects were reversible by 48 hours after application. No treatment-related clinical signs were observed in either the cornea or iris (Bioassay Labor für biologische Analytik, 2012c).

### (d) Dermal sensitization

The results of studies of dermal sensitization with prochloraz are summarized in Table 5.

***Table 5. Summary of sensitization studies with prochloraz***

| Species | Strain | Sex | Method | Purity | Result | Reference |
|---|---|---|---|---|---|---|
| Guinea pig | Crl:HA | F | Buehler | 98.0% | Not sensitizing | Frey-Tox, 2012 |
| Guinea pig | Dunkin Hartley LAC | F | Maximization | > 96.2% | Not sensitizing | Shaw, 1979 [b] |
| Guinea pig | Not stated | M/F | Buehler | 94.6% | Not sensitizing | Marom & Crown, 1989 [c] |
| Guinea pig | Albino | M | Maximization | Prochloraz–copper; complex; 97.6% | Not sensitizing | Dreher, 2002c [a] |
| Guinea pig | Dunkin Hartley | M | Maximization | Prochloraz–zinc complex; 98.6% | Not sensitizing | Chevalier, 2004b [a,#] |

M: Male; F: Female;

[#] The study was performed by the Laboratory of Pharmacology and Toxicology in Hamburg. The Meeting is aware of the current debate about allegations of fraud conducted by this Laboratory. As no formal conclusion about these allegations has been reached yet the Meeting decided to include the results from the study.

[a] Study conducted in accordance with OECD TG 406.

[b] No guideline reported, but study in line with OECD TG 406.

[c] No guideline reported. Compared to OECD TG 406 the number of tested animals was too low; five in the control group, 10 in the treatment group.

The skin sensitization potential of prochloraz (purity unspecified) was assessed in 20 female Guinea pigs by the method of Magnusson & Kligman. None of the animals showed any dermal reaction either 24 or 48 hours after challenge. Therefore, there was no evidence that prochloraz acts as a delayed dermal sensitizer in Guinea pigs. The study was conducted before GLP regulations were issued in the testing facility, but the study protocol and reporting of the results were considered acceptable (Shaw, 1979).

The skin sensitization potential of prochloraz technical (purity 94.6%) was investigated in fifteen (10 test and five control) albino Guinea pigs according to the Buehler method. The Guinea pigs received three dermal induction doses (days 1, 8 and 15), semi-occlusive applications of 0.5 mL undiluted prochloraz, and a challenge application on day 29. The exposure period was six hours. The application areas were scored at 24 hours after the induction periods, and at 27 and 48 hours following the challenge. At the induction phase, no responses were observed at the first and second examinations. Slight erythema was observed in two animals after the third application. After the challenge, one animal of the test group exhibited severe erythema with oedema, while a second animal displayed slight erythema 27 hours after dosing. At 48 hours, one animal in the control group displayed slight erythema, while no responses were observed in the test substance group (Marom & Crown, 1989).

In a dermal sensitization study using the Guinea pig (Magnusson & Kligman maximization method), prochloraz (purity 97.6%, batch AABA00315) was tested on ten male, albino Dunkin Hartley Guinea pigs. The vehicle control group consisted of five animals. As positive controls, 2-mercaptobenzothiazole and α-hexylcinnamaldehyde were used. In a preliminary study the concentrations of prochloraz for the intradermal induction, topical induction and topical challenge were determined as 5%, 50% and 10% and 25% (w/w) respectively of prochloraz in arachis oil (BP), . In the test phase, skin reactions to the challenge were observed 24 and 48 hours after removal of the challenge patch. In the control group no skin reaction was observed at the 10% site, except for desquamation in one animal at 48 hours. In the test animals, discrete or patchy erythema was observed in four cases and oedema in one case after 24 hours. Small superficial scattered scabs were observed in three test animals. All observed effects were reversible within 48 hours, except for the desquamation observed in five test and one control animal. At the 25% site discrete or patchy erythema was observed in nine test animals and slight oedema in two test animals 24 hours after removal. One control animal showed moderate and

confluent erythema and slight oedema after 24 hours. Small, superficial, scattered scabs were observed in five test animals and three controls 24 hours after removal. All observed effect were reversible within 48 hours, except for one animal showing small, superficial, scattered scabs and desquamation observed in six test animals and three controls 48 hours after removal. The sensitization rate was calculated as 0%. The positive control groups, using 2-mercaptobenzothiazole and α-hexylcinnamaldehyde, produced 20%–100% positive results (Dreher, 2002c).

In a dermal sensitization study using the Buehler test, prochloraz (purity 98.0% , batch COD-000718) was tested on 20 female SPF albino Guinea pigs (Crl:HA). The vehicle control group consisted of 10 animals. As a positive control α-hexylcinnamaldehyde was used. In a preliminary study the concentrations of prochloraz for the induction and challenge were determined as 90% and 50% (w/w) respectively, dissolved in olive oil. In the test phase, skin reactions to the challenge were observed 24 hours and 48 hours after removal of the challenge patch. In the control group no skin irritation was observed following challenge by vehicle or test substance. In the test animals no skin reaction was observed following challenge by vehicle, while discrete or patchy erythema was observed following challenge by prochloraz in two animals at 24 hours. At 48 hours no skin irritation was observed in the test animals. The sensitization rate was calculated as 10%. The α-hexylcinnamaldehyde group gave 75% positive results (Frey-Tox, 2012).

The skin sensitization potential of prochloraz–Zn complex (purity 98.6%) was investigated according to OECD TG 406, using the method of Magnusson & Kligman. Fifteen (10 test and five control) male Dunkin Hartley Guinea pigs were tested according to the following dosing regimen: (a) induction by intradermal injection at 10% (w/w) prochloraz–zinc in sesame oil (day 0); (b) topical application of 0.5 mL sodium lauryl sulfate in order to induce a local irritation (day 6); (c) induction by topical application of 50 % prochloraz–zinc suspended in sesame oil (day 7), semi-occlusive for 48 hours; then (d) two weeks after the topical induction, topical application of 50 % prochloraz–zinc suspended in sesame oil, semi-occlusive dressing for 24 hours. Skin sites were evaluated at 48 and 72 hours after challenge application. There were no effects on body weight gain of the animals treated with prochloraz–zinc and behaviour remained unchanged. Intradermal induction did not cause skin irritation. Application of sodium lauryl sulfate followed by topical application of a 50% suspension of prochloraz–zinc in sesame oil induced local irritation (grade 1). After challenge with 2 mL of a 50% suspension of prochloraz–zinc in sesame oil per animal, no skin irritation was observed in any animal. The positive control, benzocaine, induced a sensitizing reaction in all animals in form of discrete or patchy erythema (grade 1). On the basis of these results, prochloraz–zinc was regarded as non-sensitizing (Chevalier, 2004b).

## 2.2 Short-term studies of toxicity

### (a) Oral administration

***Mouse***

In a non-GLP, 21-day gavage, range-finding toxicity study, prochloraz (purity 95.29% , batch 312/A) dissolved in 10% aqueous acacia) was administered to groups of five male and five female CFLP mice at 0, 96, 240 or 600 mg/kg bw per day. The animals were checked daily for mortality and clinical signs of toxicity. Body weights were measured weekly. Prior to termination, blood samples were taken for measurement of alanine transaminase (ALT) activity. All the mice were macroscopically examined. The livers of all mice, duodena of all mice showing macroscopic abnormalities in the liver, and all organs with macroscopic abnormalities were examined microscopically.

At the high dose one male and one female died due to dosing accidents. Two high-dose males died after the second dose. The animals that died showed lethargy, half-closing of eyes and lacrimation ten minutes after dosing, subsequently followed by comatose state, reduced respiratory rate and death overnight. The cause of death of one male on day 18 could not be established. The remaining high-dose animals showed poor condition, sedation or inactivity, piloerection and coolness to touch. In addition, the cage litter was saturated with urine. Body weight gain in surviving males given 600 mg/kg bw was reduced by up to 40% during the first two weeks of the study, but recovered during the last week of treatment. Final body weights in high-dose males were only slightly lower (3%) than for controls.

Body weight gain and clinical signs in the other male test groups were unaffected by treatment. In females body weight gain was increased compared to controls at the mid dose (by up to 15%) and high dose (by up to 11%), although all treated groups tended to slightly lose weight from day 7 to day 14. Plasma ALT activity was elevated up to 3-fold in the surviving female mice given 600 mg/kg bw. The ALT levels for control males were not available and there was insufficient sample for an accurate estimation in the surviving high-dose male.

Macroscopic examination showed hyperkeratinization of the forestomach in three males and all the females at 600 mg/kg bw per day, in one male at 240 mg/kg bw per day and in one control female. Although there was a high incidence of minor pathological changes livers, there was no alteration in their severity or in the numbers of mice affected in relation to treatment with prochloraz. No further treatment-related microscopic changes were observed. No findings were attributed to treatment at a dose level of 96 mg/kg bw per day in either sex (Lancaster & Shaw, 1980b).

Groups of 15 male and 15 female CD-1 mice were given diets containing technical grade prochloraz (purity 98.2%) at concentrations providing a dose of 6, 25, 100 or 400 mg/kg bw per day for 13 weeks. A control group comprised 24 mice of each sex. Further groups of 15 male and 15 female controls, and mice treated at 400 mg/kg bw per day were kept for four weeks after the end of dosing, and, in addition groups of nine males and nine females were treated for six weeks. Solutions of the test material were prepared in corn oil and added to the diet. Overt signs of toxicity were recorded daily, and body weight and food consumption recorded three times weekly throughout the study. Haematological and blood biochemical analyses were conducted at 6 and 13 weeks and after four weeks on a control diet. All mice were killed at the end of dosing or the recovery period and were dissected and examined macroscopically, and the main organs weighed. A comprehensive microscopic examination of tissues from 10 male and 10 female controls and all mice at 400 mg/kg bw per day killed at 13 weeks was carried out, and the livers of all remaining animals killed at 13 weeks and all mice killed after four weeks on control diet were examined.

No treatment-related deaths were observed. At 400 mg/kg bw per day, the incidence of piloerection was increased. Weight gain was slightly reduced compared to controls in both sexes at 400 mg/kg bw per day (by 30% in males and by16% in females), and in males at 100 mg/kg bw per day (by 23%). Feed consumption was higher in both sexes at the highest dose throughout dosing and during the first week of the recovery period, but thereafter was similar to that of controls.

At week 6, the haemoglobin (Hb) concentration, packed cell volume PCV) and erythrocyte count were increased in both sexes at 400 mg/kg bw per day. Mean corpuscular Hb was also marginally increased in high-dose males, and their leukocyte counts increased in females due to lymphocytosis. These parameters were no longer affected by treatment at week 13 or after four weeks of recovery. The total leukocyte count was increased in females at the highest dose at weeks 6 and 13. Plasma ALT activity was increased at week 6 in some mice of each sex at 400 mg/kg bw per day and in some males at 100 mg/kg bw per day; at week 13, it was increased in the majority of females at 400 mg/kg bw per day and in some mice of each sex at 100 mg/kg bw per day; after four weeks on a control diet the ALT levels of some males at 400 mg/kg bw per day were still affected. At weeks 6 and 13 the plasma albumin concentration and, consequently the albumin:globulin (A:G) ratio was slightly reduced in both sexes at 400 mg/kg bw per day and in males at 100 mg/kg bw per day. At week 13, the plasma glucose concentration was increased and the blood urea nitrogen (BUN) concentration slightly decreased in both sexes at 400 mg/kg bw per day.

At autopsy at week 6 the weights of the liver were increased in both sexes at 100 and 400 mg/kg bw per day and were slightly increased in females at 25 mg/kg bw per day. The ovaries of some females at the highest dose, and in most at 100 mg/kg bw per day, were small, and the prostate and seminal vesicles of males at 400 mg/kg bw per day were small. The weight of the spleen was increased in both sexes at 100 mg/kg bw per day and in a few females at 25 mg/kg bw per day, but was low in most females at 6 mg/kg bw per day (this decrease was considered not to be related to treatment). At week 13, the weight of the liver was increased in both sexes at 25, 100 or 400 mg/kg bw per day. The ovaries were small in females and the prostate and seminal vesicles small in males at 400 mg/kg bw per day (Table 5). After four weeks of recovery liver weight was still increased in both sexes at the highest dose, but to a much lesser extent than at weeks 6 or 13. The livers of some mice at 400 mg/kg bw per day appeared to be enlarged, and the lobular pattern was prominent.

Histopathological examination showed treatment-related changes only in the liver. Minimal liver changes, with loss of glycogen and periportal fat droplets, were seen in both sexes at the highest dose and in females at 100 mg/kg bw per day after 13 weeks. Some males at the two higher doses also showed centrilobular hepatocyte enlargement. In mice killed after four weeks of recovery none of these changes were present.

The NOAEL was 25 mg/kg bw per day on the basis of the reduced body weight gain and effects on the liver at 100 mg/kg bw per day. The hepatic effects were reversible (Gale, 1980; Keene, 1988a; Gale, 1980; Mallyon, 1988a).

***Table 6. Organ weight findings in week 13 of the 90-day feeding study in mice***

| | Dose (mg/kg bw per day) | | | | |
|---|---|---|---|---|---|
| | **0** | **6** | **25** | **100** | **400** |
| **Liver** | | | | | |
| Absolute weight (g) | | | | | |
| males | 1.91 ± 0.26 | 1.90 ± 0.26 | **2.13** ± 0.24** | **2.37** ± 0.22** | **2.80** ± 0.32** |
| females | 1.61 ± 0.21 | 1.56 ± 0.24 | 1.72 ± 0.27 | **1.92** ± 0.17** | **2.17** ± 0.36** |
| Relative weight (%) | | | | | |
| males | 5.18 ± 0.68 | 5.07 ± 0.38 | **5.64** ± 0.39* | **6.51** ± 0.30** | **8.03** ± 0.68** |
| females | 5.44 ± 0.52 | 5.17 ± 0.57 | 5.71 ± 0.85 | **6.52** ± 0.50** | **7.87** ± 0.70** |
| **Ovary (females)** | | | | | |
| Absolute weight (g) | 0.046 ± 0.014 | 0.040 ± 0.011 | 0.041 ± 0.007 | 0.047 ± 0.022 | **0.034** ± 0.011** |
| Relative weight (%) | 0.157 ± 0.048 | 0.137 ± 0.044 | 0.138 ± 0.026 | 0.156 ± 0.64 | 0.126 ± 0.46 |
| **Prostate and seminal vesicles (males)** | | | | | |
| Absolute weight (g) | 0.47 ± 0.09 | 0.52 ± 0.16 | 0.51 ± 0.09 | 0.46 ± 0.04 | 0.40 ± 0.05 |
| Relative weight (%) | 1.28 ± 0.21 | 1.37 ± 0.32 | 1.36 ± 0.29 | 1.28 ± 0.14 | **1.14** ± 0.14* |

Statistical difference from control mean at : * $p < 0.05$; ** $p < 0.01$; *Source*: Gale, 1980

***Rat***

*Study 1*

In a range finding study, technical grade prochloraz (purity 92.7%, batch 331/394) was emulsified in aqueous acacia and given by gavage to groups of five male and five female Boots Wistar rats at doses of 25, 100 or 400 mg/kg bw per day for 30 days. A control group of 10 males and 10 females received the vehicle alone. Overt signs of toxicity were recorded daily and body weight and food consumption three times weekly throughout the study. Haematological, blood biochemical and urinary analyses were conducted during week 4. All rats were killed at the end of the dosing period and serum samples collected for estimation of enzyme activity and electrolyte content. The rats were then dissected and examined macroscopically, and the main organs were weighed. A comprehensive histological examination of tissues from half the controls and all the rats at 400 mg/kg bw per day was completed. In addition, the liver, lymph nodes, spleen and bone marrow smears from the remaining controls and rats given 25 or 100 mg/kg bw per day were examined. The study was conducted before GLP regulations were issued in the testing facility, but the study protocol and reporting of the results were considered acceptable. The study was considered to provide useful additional information.

Increased salivation was seen in both sexes given 100 or 400 mg/kg bw per day, and slight loss of condition was noted in females at 400 mg/kg bw per day. Slight weight loss (1.6 g in males, 1.0 g in females) and decreased food intake were seen at the start of treatment in both sexes at 400 mg/kg bw per day, and feed consumption was also marginally reduced in both sexes given 100 mg/kg bw per day. The erythrocyte count was decreased in some rats of each sex at 400 mg/kg bw per day and in some females at 100 mg/kg bw per day. Haemoglobin concentration was also slightly decreased in one rat of each sex at 400 mg/kg bw per day. The mean corpuscular volume was high in some animals at 400 mg/kg bw per day. Leukocyte count was increased in females at 400 mg/kg bw per day due to neutrophilia and lymphocytosis. Urine flow was increased, and the specific gravity before water was withheld, was

decreased in some rats of each sex at 400 mg/kg bw per day and in one male at 100 mg/kg bw per day. Urinary aspartate aminotransferase (AST) activity was slightly increased in females, and the urinary pH was reduced in a few rats receiving 400 mg/kg bw per day. The weight of the liver was increased in both sexes at 100 and 400 mg/kg bw per day, the females tending to be more severely affected. The weight of the adrenals was increased, and the prostate and seminal vesicles were small in males at 100 and 400 mg/kg bw per day. The only finding possibly related to treatment observed at a microscopic level was slight haematopoiesis in the spleen in most females at 25 mg/kg bw per day and in both sexes at 100 and 400 mg/kg bw per day, although this change was also seen in one male and three female controls. Reported incidences off slight haematopoies were 1, 1, 4 and 4 in the control, low-, mid- and high-dose males and 3, 4, 2 and 3 in the control, low-, mid- and high-dose females.

The authors concluded that, because the dose of 100 mg/kg bw per day was tolerated by both sexes, it would be appropriate as the highest dose for a 90-day study. The increased salivation may have been related to the method of administration, as similar effects were not seen after dietary administration (Lancaster & Shaw, 1980a).

*Study 2*

In a study conducted before GLP regulations were issued in the testing facility but which complied with the general principles of GLP and the final report of which was reviewed for QA, groups of 20 male and 20 female Boots Wistar rats were given technical grade prochloraz (purity 97.5%, batch 9/DM 2723) by gavage at doses of 0, 6, 25 or 100 mg/kg bw per day in 10% aqueous acacia suspension for 13 weeks. The control group of 20 males and 20 females received the vehicle alone. Further groups of 20 male and 20 female controls, and rats at 100 mg/kg bw per day were kept for four weeks after the end of the dosing period. In addition, groups of 10 male and 10 female controls, and rats at 6, 25 or 100 mg/kg bw per day were examined after six weeks. Overt signs of toxicity, body weight and food consumption were recorded daily throughout the study; haematological, blood biochemical and urinary analyses were conducted at weeks 6 and 13 and after the four-weeks recovery period. All rats were killed at the end of their dosing or recovery periods, and serum samples collected for estimation of enzyme activity and electrolyte content. The rats were dissected and examined macroscopically, and the main organs were weighed. A comprehensive histological examination of tissues from half the controls and all rats at 100 mg/kg bw per day and those killed at 13 weeks was carried out. In addition, the livers from all remaining animals were examined. The investigation was extended to quantify the size of liver cells by counting the number of nuclei in 10 periportal and 10 centrilobular fields of the left lateral lobe of the liver from all rats killed at weeks 6 and 13 and those killed after the four-weeks recovery period.

All prochloraz-treated rats and one of the controls had respiratory disease and/or a middle ear infection. Rats of each sex at 25 and 100 mg/kg bw per day and a few at 6 mg/kg bw per day showed increased salivation. The incidence of diarrhoea was increased during the first four weeks of dosing in males at 100 mg/kg bw per day and possibly during the first week of dosing in males at 25 mg/kg bw per day. In females, a few instances of diarrhoea were recorded at each dose. Body weight gain was increased in females at 25 and 100 mg/kg bw per day during dosing. During the first five days of the recovery period females at 100 mg/kg bw per day lost a small amount of weight relative to controls, the overall weight gain difference being statistically significant. Food consumption was unaffected by treatment except for a slight increase during the recovery period in males at 100 mg/kg bw per day.

At week 6, Hb concentrations were slightly decreased in males at all doses, although only statistically significant at the low and high dose, and in females at 100 mg/kg bw per day. At week 13 concentration of Hb was slightly decreased (but statistically significant) in females at 25 and 100 mg/kg bw per day. Leukocyte counts were increased, due to lymphocytosis, in all treated males. At week 13, mean corpuscular volumes were slightly decreased in all treated males and in females at 100 mg/kg bw per day, and Hb concentrations were slightly decreased in females at 25 and 100 mg/kg bw per day and in males at 100 mg/kg bw per day after the four-week recovery period. Serum bilirubin concentrations were slightly decreased in all treated rats at week 6, in males at 25 and 100 mg/kg bw per day and in females at 6 and 100 mg/kg bw per day at week 13, and in a few treated rats of each sex after the four-week recovery period. Serum potassium concentrations were slightly increased (by 11%–12%) at week 6 in males at 25 and 100 mg/kg bw per day and in three females at 100 mg/kg bw per day. At week 6, urinary AST activity was slightly increased in a few rats of each sex at each dose. At week 13 the specific gravity of urine, before water was withheld, and sodium excretion were slightly decreased in both sexes

at 100 mg/kg bw per day, urinary AST was increased in males at 100 mg/kg bw per day, urinary pH was reduced in some females at 25 and 100 mg/kg bw per day and the urinary protein content was reduced in all treated females and in males at 100 mg/kg bw per day. After the four-week recovery period potassium excretion and urinary pH were slightly reduced in females given 100 mg/kg bw per day.

Autopsy at week 6, revealed that liver weights were increased in both sexes at 100 mg/kg bw per day and in females given 6 or 25 mg/kg bw per day, and kidney weights were increased in males at 100 mg/kg bw per day and in both sexes at 25 mg/kg bw per day. The prostate and seminal vesicles were smaller than usual in males and the ovaries were larger in females at 100 mg/kg bw per day. Thyroid weight was increased in all treated females. At week 13 the weight of the liver was increased in males at 6 mg/kg bw per day and in both sexes at 25 and 100 mg/kg bw per day; kidney weight was increased in both sexes at 100 mg/kg bw per day, the spleen weight was slightly decreased in all treated males, ovary weight was increased in all treated females, and thyroid weights were increased in females at 6 and 100 mg/kg bw per day. After the four-weeks recovery period the weights of liver, ovaries and thyroids of the females were slightly greater than those of the controls.

At weeks 6 and 13 and after the four-week recovery period the centrilobular liver cells were larger than the periportal cells in control rats in both sexes, reflecting the normal morphology of the liver. This difference was also seen in all treated groups, irrespective of changes in liver weight or cell size. At week 6, liver cell size was increased in females at 6 and 25 mg/kg bw per day and in both sexes at 100 mg/kg bw per day. At week 13 the liver cell size had increased in both sexes given 6, 25 or 100 mg/kg bw per day. After four weeks on the control diet the liver cell size was still increased in males but in females had returned to normal or was even decreased in comparison with the controls. The changes in liver cell size generally correlated closely with increases in liver weight. The only instance in which such a correlation was not apparent was after four weeks of recovery, when in treated males the liver cell size was still slightly increased, even though there was no change in liver weight, and conversely in treated females where the liver cell size was decreased although liver weight was slightly increased.

Although various treatment-related effects were seen in all groups the effects were generally small. The most obvious effects were increased liver weight and hepatocyte size. In the absence of changes in serum chemistry parameters or histopathological changes these liver effects were not considered adverse. The Meeting noted that the effects were generally small and often lacked a dose–response relationship, and that all prochloraz-treated rats, but only one control rat had respiratory disease and/or a middle ear infection. This complicated the interpretation of the findings. The Meeting concluded that the study was not suitable for the identification of a no-observed-adverse-effect level (NOAEL) or a lowest-observed-adverse-effect level (LOAEL) for risk assessment purposes (Jackson, 1988; Lancaster & Shaw, 1979; Markham, 1988a; Shaw et al., 1979b).

*Study 3*

In a 90-day oral toxicity study, 20 male and 20 female Fischer F344 rats received technical grade prochloraz (purity 93.4%, batch P3) in basal diet for 13 weeks at concentrations of 0, 50, 500 or 1500 ppm (equal to 0, 4.4, 43.3 and 137.2 mg/kg bw per day for males, 0, 4.7, 46.6 and 144.9 mg/kg bw per day for females) . All groups were observed regularly for mortality (daily), clinical signs (weekly), water consumption (weeks 1, 4, 8 and 12), food consumption (weekly) and growth (weekly). Ophthalmological examinations were performed on 10 rats per sex and per dose before the start of the treatment and in the control and high-dose groups upon study termination. Blood and urine samples were taken from 10 rats per sex and per dose after 12 weeks of treatment for haematology, plasma clinical chemistry and urinalysis. Animals were sacrificed after 13 weeks of treatment. All rats were subjected to detailed gross pathology and organ weight analysis. Histopathological examinations were performed on kidneys, liver and lungs from all animals. All other required tissues were examined from high-dose and control animals.

No treatment-related, preterminal deaths or clinical signs were observed. Ophthalmological examinations at termination revealed no difference between the high-dose and control groups. High-dose males showed significantly lower body weights from week 2 onwards, by up to10%, when compared to controls. Concurrently, the feed consumption was significantly reduced by 8% to 10% during weeks 3 and 4. Among the females, the mean body weight was similar to that of controls except in week 3 when the mean body weight of the high-dose group was significantly lower (by 5.6%) than the controls. Water intake was reduced in both sexes at 1500 ppm.

Small decreases in PCV values and mean corpuscular volume (MCV) were noted in mid- and high-dose males (by 3% and 4%). Mean corpuscular haemoglobin concentrations (MCHCs) were slightly increased in high-dose males (by 2%). Changes noted in the leukocyte pattern were inconsistent and not considered related to treatment. Changes in enzyme activity compared with controls were noted. Declines in the activity of ALT were observed in mid-dose females (by 35%) and high-dose animals of both sexes (by 14%–35%). Declines in AST occurred in mid- and high-dose females (by −26% and −19%). In addition, alkaline phosphatase (ALP) levels were decreased in mid- and high-dose males (by 15% at both doses). Activity of GGTP was increased in high-dose males and females. Other increases in GGTP activity in the remaining groups were very small and were within the normal range for rodents of this age and strain. Total protein and albumin concentrations were increased in high-dose males compared with controls. A few other values (for example phosphorus in males at 50 ppm) which were significantly different from the controls were considered to be of no toxicological relevance since differences were negligible and not related to dose. Urinary volume was decreased in high-dose males.

No treatment-related macroscopic findings were observed upon necropsy. The fasting body weights of high-dose males were significantly lower than controls. Increased weights of thyroids, kidneys and livers from mid- and high-dose males, as well as increased liver weights in high-dose females, were considered to be treatment-related based on covariant analysis using final body weights (see Table 7). Decreased absolute brain, heart, spleen and adrenals weights compared to controls in high-dose males were attributed to the lower fasting body weights. Covariant analysis indicated that the testes of high-dose males were significantly heavier than in controls, which was primarily attributed to the body weight changes as no effect on absolute weight was observed. Spleen weights were significantly increased in low-dose females but were considered to be of no toxicological relevance since the difference from the control value was negligible and not related to dose.

***Table 7. Effects of prochloraz on organ weights in 90-day study in rats***

| | | Dietary dose level | | | |
|---|---|---|---|---|---|
| | | **0 ppm** | **50 ppm** | **500 ppm** | **1500 ppm** |
| | | **Males** | | | |
| Liver weight: | absolute | 12.28 ± 1.08 | 12.40 ± 1.14 | 13.00 ± 1.43 | 12.94 ± 2.04 |
| | relative | 11.95 ± 0.21 | 11.94 ± 0.21 | 12.65 ± 0.21* | 14.08 ± 0.23*** |
| Kidney weight: | absolute | 1.98 ± 0.16 | 1.99 ± 0.14 | 2.06 ± 0.20 | 1.97 ± 0.24 |
| | relative | 1.94 ± 0.02 | 1.92 ± 0.02 | 2.01 ± 0.02* | 2.13 ± 0.03*** |
| Thyroid weight: | absolute | 12 ± 2 | 13 ± 2 | 15 ± 3* | 15 ± 2* |
| | relative | 12.21 ± 0.55 | 13.12 ± 0.55 | 14.40 ± 0.55** | 14.98 ± 0.62** |
| | | **Females** | | | |
| Liver weight: | absolute | 6.88 ± 0.66 | 6.61 ± 0.58 | 6.98 ± 0.67 | 7.72 ± 0.68*** |
| | relative | 6.80 ± 0.12 | 6.55 ± 0.13 | 7.01 ± 0.14 | 7.84 ± 0.12*** |
| Kidney weight: | absolute | 1.39 ± 0.11 | 1.38 ± 0.11 | 1.35 ± 0.06 | 1.31 ± 0.08 |
| | relative | 1.38 ±0.02 | 1.37 ± 0.02 | 1.35 ± 0.02 | 1.33 ± 0.02 |
| Thyroid weight: | absolute | 14 ± 3 | 13 ± 2 | 12 ± 2 | 13 ± 4 |
| | relative | 13.87 ± 0.71 | 13.07 ± 0.74 | 12.37 ± 0.78 | 13.43 ± 0.71 |

Statistical difference from control mean at: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ *Source:* Marom et al., 1990

Histopathology revealed treatment-related hepatic changes in mid- and high-dose groups of both sexes (Table 8). These changes included a reduction in the incidence of hepatocytic centrilobular anoxic vacuolation and a reduction in the quantity of hepatocytic cytoplasmic glycogen reserves ("hepatocytic pallor"). These changes were more prominent among males. Changes confined to the centrilobular hepatocytes included an increase in the hepatocytic cellular size (hypertrophy), associated with an increase in eosinophilic cytoplasmic fine granular material, and cytoplasmic peripheral displacement of basophilic granularity. Perilobular or midzonal hepatocytic fine and large fatty vacuolation was observed in high-dose males only.

*Table 8. Incidences of conspicuous histological findings in rat livers*

| | Incidence of conspicuous histological findings | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Sex** | **Male** | | | | **Female** | | | |
| **Dose (ppm)** | **0** | **50** | **500** | **1500** | **0** | **50** | **500** | **1500** |
| **Number of animals examined** | **20** | **20** | **20** | **20** | **20** | **18** | **16** | **20** |
| Anoxic vacuolation: | | | | | | | | |
| centrilobular, slight | 8 | 10 | 7 | 4 | 1 | 2 | 0 | 0 |
| centrilobular, moderate | 12 | 10 | 2 | 1 | - | - | - | - |
| Hepatocytic pallor: | | | | | | | | |
| perilobular, slight | 1 | 1 | 9 | 3 | - | - | - | - |
| centrilobular, slight | - | - | - | - | 3 | 5 | 0 | 0 |
| perilobular, moderate | 2 | 6 | 2 | 3 | - | - | - | - |
| diffuse, slight | 10 | 8 | 1 | 1 | - | - | - | - |
| diffuse, moderate | 3 | 2 | 0 | 0 | - | - | - | - |
| no hepatocytic pallor | 4 | 3 | 8 | 13 | 17 | 13 | 16 | 20 |
| Hepatocytic hypertrophy: | | | | | | | | |
| centrilobular, slight | 0 | 0 | 2 | 6 | 0 | 0 | 0 | 8 |
| centrilobular, moderate | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 |
| Increase in eosinophilic cytoplasmic fine granular material: | | | | | | | | |
| centrilobular, slight | 0 | 0 | 18 | 6 | 0 | 0 | 13 | 8 |
| centrilobular, moderate | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 12 |
| Cytoplasmic peripheral displacement of basophilic granularity: | | | | | | | | |
| centrilobular | 0 | 0 | 18 | 18 | 0 | 0 | 13 | 20 |
| Hepatocytic fine and large fatty vacuolation: | | | | | | | | |
| perilobular, slight | 0 | 0 | 0 | 1 | - | - | - | - |
| midzonal, slight | 0 | 0 | 0 | 7 | - | - | - | - |

*Source:* Marom et al. (1990)

The NOAEL was 50 ppm (equal to 4.4 mg/kg bw per day) on the basis of the effects on organ weights and the observed histopathological changes observed in livers at 500 ppm (equal to 43.3 mg/kg bw per day) (Marom et al., 1990).

### *Dog*

*Study 1*

In a dose range-finding study, groups of two male and two female beagle dogs were given technical grade prochloraz (purity 96.6%) suspended in a 5% aqueous acacia solution and administered by gastric intubation for 14 days at a dose of either 10 mg/kg bw per day in a volume of 0.3 mL/kg bw, or 40 mg/kg bw per day in a volume of 1.2 mL/kg bw. A group of two males and two females receiving 5% acacia solution at the higher dose volume served as controls. Body weight, food consumption and faecal appearance as well as occult blood content were recorded daily for one week before dosing and then throughout the study. Overt signs of toxicity were recorded throughout dosing. Comprehensive haematological and blood biochemical analyses were conducted once before dosing and on day 14; additional blood samples were collected on days 3 and 8 for estimation of bilirubin concentration and the activity of certain serum enzymes. The dogs were killed the day after the final dose, dissected and examined macroscopically. The main organs were weighed, and sections of selected organs and tissues examined histologically. The study was conducted before GLP regulations were issued in the testing facility, but the protocol and results were well reported. The study is considered to provide useful additional information although the number of animals was too small to draw any firm conclusions as to a NOAEL.

Dogs given 40 mg/kg bw per day usually vomited within one hour of dosing and had diarrhoea within two hours. Because of the emesis, the daily dose was divided and administered in two equal volumes morning and afternoon to each dog from day 6; thereafter, the incidence of vomiting decreased and, as for the diarrhoea, usually occurred only after the morning dose. In addition, increased salivation was seen sporadically in some dogs at both doses. Both males at 40 mg/kg bw per day were intermittently anorexic and lost weight during the study.

On day 14, in animals at 40 mg/kg bw per day, low values were recorded for erythrocyte count, Hb concentration and erythrocyte volume fraction in one female, for Hb concentration in the other female and for erythrocyte count in one male. Serum ALP activity increased progressively (to 384% of the control value) throughout the dosing period in both males and one female at the higher dose, and two of these animals also had very slight increases in serum leucine aminopeptidase activity.

At autopsy on day 15, the livers of all dogs at 40 mg/kg bw per day appeared large and bulbous, and the absolute and relative weights in these dogs and the relative weight in one female at 10 mg/kg bw per day were increased compared to controls. The prostates of both males at 40 mg/kg bw per day and both testes of one of one dog appeared small and flaccid; the testes had spermatid giant cells. In addition, copious amounts of mucus were seen in the stomachs of one male at the higher dose and both males at the lower dose, and a cream–grey layer was observed on the mucosal surface of the ileum in one male at each dose. Additionally, one female at the higher dose had microscopic evidence of gastritis. Microscopic examination revealed no other change that was considered to be directly related to treatment.

The study authors concluded that a single oral dose of 40 mg/kg bw per day would be unacceptable in a long-term study and a single dose of 20 mg/kg bw per day would be well tolerated. The lower dose was therefore chosen as the highest dose for the 90-day study that followed. Due to the small number of animals no conclusion could be drawn as to a NOAEL (Morgan et al. 1979).

*Study 2*

In a study conducted before GLP regulations were issued in the testing facility but which complied with the general principles of GLP and for which the final report was reviewed for QA, groups of four male and four female beagle dogs were given technical-grade prochloraz (purity, 97.9%). The test article was suspended in a 10% aqueous acacia solution at doses of 0, 1, 2.5, 7 or 20 mg/kg bw per day and administered by gastric intubation for 13 weeks; a group of four dogs of each sex receiving the vehicle as controls. Additional groups of four male and four female controls and dogs at 20 mg/kg bw per day were retained for four weeks while receiving no treatment once dosing had ended. Overt signs of toxicity, body weights, food consumption and faecal appearance were recorded daily throughout the study. At weeks 6 and 13 a clinical examination, including ophthalmoscopy and electrocardiography, and haematological, blood biochemical, faecal and urinary analyses were conducted. Parameters that had appeared affected at week 13 were measured again at the end of the four-week recovery period. On each occasion, parameters with values outside normal limits were measured again one week later, and serum ALP and leucine aminopeptidase (LAP) activities were estimated in all dogs at week 9. All dogs were killed at the end of the dosing or recovery period, dissected and examined macroscopically, the main organs were weighed and a comprehensive histological examination was carried out.

The only overt signs of toxicity were isolated bouts of emesis and increased salivation in some dogs from all groups, including controls, although effects were more prevalent at the highest dose. In addition, all dogs at 20 mg/kg bw per day and one at 7 mg/kg bw per day occasionally produced yellow mucoid and/or liquid faeces. During the recovery period no overt signs of toxicity were observed. Body weight gain was comparable in control and treated groups. Reduced food consumption was recorded for two dogs at 20 mg/kg bw per day. Clinical examination at week 13 revealed that four dogs at 20 mg/kg bw per day had apparently small and/or flaccid testes; in two of these dogs that were allowed to recover, the change was still apparent at week 17. No treatment-related effects were detected by ophthalmoscopy or electrocardiography. There was no treatment-related effect on haematological parameters. Serum ALP activity was high during dosing in most dogs at 20 mg/kg bw per day and in some at 7 mg/kg bw per day; in those dogs retained for recovery, the activity returned to within normal limits. However, the increases in ALP levels were considered not toxicologically relevant as it is now established that isolated ALP changes in dogs are not adverse (Yokoyama et al., 2019, 2021). Serum

LAP activity was very slightly increased at week 6 at 20 mg/kg bw per day (males and females) and at 7 mg/kg bw per day (males only). The activity had returned within normal limits by week 9 or 13. There was no effect of treatment on urinary parameters. At necropsy, all dogs at 20 mg/kg bw per day and three at 7 mg/kg bw per day had large, heavy livers; this effect was still apparent in only one treated dog at the end of the recovery period. Mean absolute and relative prostate weights were reduced significantly in dogs at 7 and 20 mg/kg bw per day after 13 weeks of treatment (see Table 9). After the four-week recovery period, prostate weights were slightly reduced, but this was not statistically significant. In this study the increases in prostate weights were not accompanied by histopathological changes, but in a one-year and a two-year study in dogs prostatic atrophy was observed at similar doses. In two females at the highest dose that were killed at week 14 and one killed at week 18, a clear brown exudate from freshly sectioned mammary glands was observed. The only histological finding of note was apparent immaturity of the testes with no spermatozoa present in some dogs at 20 mg/kg bw per day. Preterminal clinical examination revealed small and/or flaccid testes in four of the eight dogs. Histological changes that were indistinguishable from immaturity were observed in one of two dogs killed after 13 weeks, and comparable findings were made in another dog which had shown no associated clinical signs. At the end of the recovery period the testes of all dogs appeared normal, indicating that the change was reversible. Counts of liver nuclei revealed no statistically significant differences in liver cell size between controls and animals at the highest dose.

The NOAEL was 2.5 mg/kg bw per day on the basis of reduced prostate weights at 7 mg/kg bw per day. These effects were reversible (Jackson, 1989; Keene, 1988b; Lancaster, Morgan & Stobart, 1979; Markham, 1988b).

***Table 9. Effect of prochloraz on terminal organ weights in 13 week study in dogs***

| | Dose (mg/kg bw/day) | | | | | | |
|---|---|---|---|---|---|---|---|
| **Parameter** | **0** | **1.0** | **2.5** | **7.0** | **20** | **0 (R)** | **20 (R)** |
| **Liver weight (males)** | | | | | | | |
| Absolute (g) | 404.0 | 381.8 | 421.3 | 462.9 | **564.8*** | 416.0 | 464.4 |
| Relative (%) | 3.07 | 2.74 | 2.977 | 3.409 | **4.296*** | 3.180 | 3.609 |
| **Liver weight (females)** | | | | | | | |
| Absolute (g) | 306.8 | 312.9 | 326.3 | **367.4*** | **474.1*** | 339.1 | 354.2 |
| Relative (%) | 3.14 | 2.80 | 3.12 | 3.34 | **4.45*** | 3.07 | 3.51 |
| **Prostate weight (males)** | | | | | | | |
| Absolute (g) | 12.6 | 8.6 | 9.8 | **7.7*** | **3.8*** | 10.2 | 5.7 |
| Relative (%) | 0.095 | 0.061 | 0.070 | **0.058*** | **0.028*** | 0.077 | 0.044 |
| **Testes weight (males)** | | | | | | | |
| Absolute (g) | 28.7 | 27.4 | 31.9 | 29.8 | 23.1 | 31.9 | 28.3 |
| Relative (%) | 0.216 | 0.197 | 0.225 | 0.221 | 0.176 | 0.244 | 0.220 |
| **Thyroid weight (females)** | | | | | | | |
| Absolute (g) | 1.19 | 1.34 | 1.36 | 1.31 | 1.16 | 1.06 | **1.51*** |
| Relative (%) | 0.013 | 0.012 | 0.013 | 0.012 | 0.011 | 0.010 | 0.015* |

Statistically significantly different from control: * $p \leq 0.05$; (R) recovery group;

*Study 2*

In a one year toxicity study, beagle dogs (four of each sex per group; 5–6 months old) received prochloraz (purity 97.4%, batch XY-1) in gelatin capsules at 0, 1, 5 or 25 mg/kg bw per day for 52 weeks. All groups were observed regularly for mortality, toxicological symptoms, food consumption and growth. Ophthalmological examinations were performed at study initiation and after 12, 24 and 50 weeks of treatment. Laboratory investigations for haematology and plasma clinical chemistry were performed at study initiation and after 13, 24 and 50 weeks. Urine analysis was performed at study initiation and after 11, 23 and 49 weeks. All surviving animals were sacrificed after the treatment period and were subjected to detailed gross pathology, organ weight analysis and histopathology.

No treatment-related effects on survival, clinical signs or ophthalmology findings were observed during the study. In high-dose females, food consumption, and consequently body weight gain, was depressed during the first six weeks of treatment. Overall, body weight gain in high-dose females was slightly lower than in controls.

No treatment-related haematological changes were observed. Statistically significant differences from controls in some parameters (for example, total white blood cell count, mean corpuscular Hb MCHC, nitrogen, leucocytes and monocytes) represented single events, no trend in time or no dose–response relationship. A treatment-related increase (up to 8.5-fold) in plasma ALP was observed in all animals of the high-dose group throughout the study period. One mid-dose female also showed an increase in ALP. In the absence of adverse effects on other liver parameters increased ALP levels were considered not toxicologically relevant as it is now established that isolated ALP changes in dogs are not adverse (Yokoyama et al., 2019, 2021). All other findings which attained statistical significance when compared with the controls, for example AST, ornithine carbamoyltransferase (OCT), LAP, urea, total protein, represented single events. With these parameters there were no trends in time or dose–response relationship, or they reflected trends that were apparent before treatment commenced; they were therefore considered to represent normal biological variation. No treatment-related effects on urinalysis were observed.

Compound-related gross lesions were confined to three males receiving 25 mg/kg bw per day which displayed smaller prostate glands. No other treatment-related gross lesions were observed. No treatment-related effects were observed on fasting body weights. Statistically significant increases in relative liver weights occurred in high-dose males (50%) and high-dose females (22%) and increased absolute liver weights in high- (54% increase) and mid-dose males (27% increase). In addition, significantly decreased prostate weights were observed in mid-dose males when compared to controls (absolute decrease by 27%; decrease relative to bw by 32%) and high-dose males (absolute decrease by 82%; relative decrease by 83%). High-dose females showed significantly reduced absolute and relative thymus weights. All other findings (for example, absolute weight of brain, adrenals, ovaries, uterus) were minor or lacked a dose–response relationship.

Histopathology revealed fatty vacuolation of the zona fasciculata in the adrenals of mid- (one male, one female) and high-dose (three males, two females) animals, as well as one low-dose female. Increased glycogen content in the liver was evident in mid-dose animals (two males, one female) and three high-dose males. Prostatic atrophy was seen in three mid-dose males and four high-dose males. Other findings were of the type and incidences commonly seen in dogs of this age.

The main target organs of toxicity appeared to be the prostate and the adrenal. The NOAEL was 1 mg/kg bw per day based on the effects on prostate weights and histopathological changes in prostate (atrophy) and adrenals (fatty vacuolation of the zona fasciculata) observed in at 5 mg/kg bw per day (East, 1994).

*Study 3*

In a two-year toxicity study, groups of five male and five female beagle dogs received diets containing technical grade prochloraz (purity 95.2%–97.1%) for 104 weeks at 0, 30, 135 or 600 ppm (increased to 1000 ppm from week 57) This was equal to 0, 0.94, 4.5, 18/29 mg/kg bw per day for males, 0, 0.90, 4.1, 18/28 mg/kg bw per day for females, . Control animals received a diet containing just the vehicle, corn oil. An additional group of two males and two females given 600 ppm prochloraz were treated for 13 weeks, and formed the interim kill group. All animals were observed daily for clinical signs; body weight and food consumption were recorded weekly. Ophthalmoscopy, electrocardiography and laboratory investigations were carried out before treatment and during weeks 13, 26, 50–51, 78 and 104. Samples were taken for haematology, blood chemistry and urine analysis. At termination all dogs were killed by exsanguination under pentobarbital anaesthesia, and a full post mortem was carried out. Bone marrow was obtained by sternal puncture and examined. Various organs were weighed, and a large number of tissues were selected and (except those from interim kill animals) examined microscopically.

Four dogs were killed for humane reasons during the treatment period, but none of the deaths nor the findings post mortem were considered to be treatment-related. There were no overt signs of toxicity and no effects of treatment on body weight, food consumption, ophthalmoscopy or electrocardiography were seen. Mean serum ALP activity in males and females receiving 600 ppm was higher than that of

controls during weeks 13, 26 and 50. After the increase in week 57 of the top dose from 600 ppm to 1000 ppm a marked increase in ALP activity was seen (on average 6–7-fold) which persisted for the remainder of the dosing period. Activity of ALP was also higher (about 2-fold) in males at 135 ppm from week 13, but the difference was not as marked as at the higher dose. Slight increases in cholesterol level were observed at the highest dietary concentration. Slight but inconsistent increases in platelet count and blood glucose level were also seen in these animals, but the effects could not be definitely related to treatment. Treatment had no effect on urinary end-points or faecal occult blood content.

Macroscopic examination post mortem revealed no changes that were considered to be related to treatment. At the interim kill, the liver of one animal was heavier than usual, and the liver of one other animal slightly exceeded the normal upper limit when expressed as a percentage of body weight. The prostate of one dog was lighter than expected. At termination the mean liver weight of males and females at 600/1000 ppm was significantly higher than the corresponding control mean (increased by 42% in males and 30% in females), and the mean prostate weight of animals at these concentrations was significantly lower than the control mean (a decrease by 63%). Treatment-related histopathological changes observed after 104 weeks were minimal swelling and rarefaction of centrilobular hepatocytes with associated low-grade hepatitis in all dogs at the highest dietary concentration, minimal swelling and rarefaction of occasional centrilobular hepatocytes and polymorphic leukocyte infiltration in central areas in one dog at the intermediate concentration, and minimal prostatic atrophy or incomplete acinar development with associated stromal, trabecular or capsular fibrosis in four male dogs at the highest dietary concentration. No evidence of neoplasia was found.

The NOAEL was 135 ppm (equal to 4.1 mg/kg bw per day) on the basis of decreased prostate weight, increased liver weight and ALP, and histopathological changes in the prostate and liver at 600/1000 ppm (equal to 28 mg/kg bw per day) (Chesterman et al., 1981; Malarkey, 1993; Mallyon, 1988b; Woodhouse, Almond & Ball, 1979).

### (b) Dermal application

In a 28-day dermal toxicity study, prochloraz (purity 97.1%, batch 2050017) was applied under occlusion to the clipped skin of groups of 22 male and 22 female Sprague Dawley (Crl:CD IGS BR) rats at 0, 100, 300 or 1000 mg/kg bw per day, for five days per week, six hours per day. The animals were checked daily for mortality and clinical signs of toxicity. A detailed physical examination was performed weekly. Body weights and food consumption were measured prior to treatment, weekly throughout the study and on the day of scheduled necropsy. Water consumption was monitored daily by visual inspection. In week 4 blood was sampled for haematological and clinical biochemistry examinations. All the rats were macroscopically examined, and weights of brain, thyroids (including parathyroids), adrenals, thymus, spleen, heart, liver, kidneys, testis/ovaries, epididymides, prostate, seminal vesicles were determined. An extensive range of organs and tissues was preserved. Several organs and tissues (adrenals, heart, liver, ovaries, skin, seminal vesicles, spleen, thyroids (including parathyroids)) from controls and animals exposed to the highest dose were examined histopathologically. Also, kidneys from males and macroscopically abnormal tissue from all animals were likewise examined.

There were no treatment-related effects on any of the parameters tested, except for hyaline droplet nephrosis in some male animals receiving 300 and 1000 mg/kg per day. The study author reported that hyaline droplet nephrosis was considered to be unique to male rats and constitutes a well established syndrome. Therefore this effect was considered to be unique to male rats and not applicable to humans. The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Palmer, 2001).

### (c) Exposure by inhalation

No data relevant to this topic were submitted.

## 2.3 Long-term studies of toxicity and carcinogenicity

***Mouse***

*Study 1*

In a chronic dietary carcinogenicity study, prochloraz (purity 96.58%, batch 17) was administered to groups of male CD-1 mice at 0, 78, 325 or 1300 ppm, for 106 weeks to males and for 121 weeks to females. Dietary doses were equal to 0, 7.5, 33 and 134 mg/kg bw per day for males, 0, 8.8, 36 and 149 mg/kg bw per day for females. Control groups consisted of 104 animals per sex, treatment groups consisted of 52 animals per sex and per dose. The mice were checked daily for mortality and clinical signs. A detailed clinical examination including palpation for masses was carried out weekly. Body weights and food consumption were recorded weekly. Blood was sampled from 10 animals per sex and per group for haematological examinations at weeks 52 and at termination. Animals found dead or euthanized before or at the end of the treatment period underwent necropsy. Organs (brain, heart, liver, kidneys, spleen and testes) were weighed. A wide range of tissues was examined microscopically from all animals that were killed (or died) prematurely or were killed at the end of the treatment period. Three coronal sections through the head were examined from each of 10 males and 10 females from each group killed at termination.

There was no effect of treatment on mortality. The percentage survival at termination was 52%, 50%, 25% and 48% in males at 0, 78, 325 and 1300 ppm respectively, 30%, 27%, 40% and 29% in females at 0, 78, 325 and 1300 ppm respectively. Distended abdomen was observed in some mice of both sexes at 1300 ppm and in some males at 325 ppm. Necropsy of these mice revealed that they had multiple liver tumours. During the course of the study body weights were slightly lower than controls in high-dose males and females, but this difference never reached 10%. Food consumption was significantly increased in males at 325 ppm (by up to 12%) and 1300 ppm (by up to 7%). Haematology at week 52 showed no treatment-related effects. At week 121 (termination) compared with controls, slightly lower Hb concentrations (by 8%) were found in treated female mice at 1300 ppm, and lower total leukocyte counts due to lower lymphocyte counts were found in some females in all treated groups, in particular at 1300 ppm. At 1300 ppm some treated female mice showed lower total white blood cell counts, mainly due to the presence of fewer lymphocytes.

Dose-dependent increases in liver weights were seen in males and females at 325 ppm (by 26% and 20% respectively) and 1300 ppm (by 81% and 60% respectively). Most of these livers presented with tumours. A dose-related increase in the incidence of macroscopic liver masses was observed in males and females that had died during the study or were killed at termination (Table 10). No other treatment-related macroscopic lesions were observed. Microscopic examination revealed increases in the number of benign and/or malignant tumours in the livers of mice of both sexes at 325 ppm and 1300 ppm (see Table 10). At these doses there was also an increase in number of animals with multiple tumours. Liver cell tumours contributed to the deaths of more males (20/52) and females (14/52) at 1300 ppm, than amongst controls (9/104 males and 1/104 females), and more males at 325 ppm (13/52) compared with these same controls. No treatment-related effect on the incidence of other tumours was found. Also no treatment-related other non-neoplastic changes were seen.

***Table 10. Summary of liver tumour occurrence; from terminal scheduled and unscheduled deaths***

| | Dietary dose level | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 ppm | | 78 ppm | | 325 ppm | | 1300 ppm | |
| **Decedents (D) terminal kill (T)** | **D** | **T** | **D** | **T** | **D** | **T** | **D** | **T** |
| | | | | **Males** | | | | |
| **Number of mice with masses in liver** | | | | | | | | |
| Mice examined | 50 | 54 | 26 | 26 | 39 | 13 | 27 | 25 |
| Number with masses | 15 | 20 | 8 | 12 | 23 | 8 | 24 | 20 |
| Total | 35/104 | | 20/52 | | 31/52 | | 44/52 | |

| | | Dietary dose level | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 ppm | | 78 ppm | | 325 ppm | | 1300 ppm | |
| **Decedents (D) terminal kill (T)** | | **D** | **T** | **D** | **T** | **D** | **T** | **D** | **T** |
| **Number of mice with tumours in liver** | | | | | | | | | |
| Mice examined | | 50 | 54 | 26 | 26 | 38 | 14 | 27 | 25 |
| Number with benign tumours | | 3 | 18 | 6* | 9 | 6 | 5 | 6* | 14* |
| Total | | 21/104 | | 15/52 | | 11/52 | | 20/52** | |
| | | **Number of mice with malignant tumours** | | | | | | | |
| Tumours per mouse: | 1 | 9 | 4 | 2 | 1 | 7 | 1 | 7 | – |
| | 2 | 2 | 1 | – | – | 5 | – | 4 | 2 |
| | 3 | – | – | 1 | 1 | 1 | – | – | – |
| | ≥ 4 | – | – | – | – | 1 | 2 | 6 | 5 |
| | Total | 11 | 5 | 3 | 3 | 14 | 3 | 17*** | 7* |
| Total D + T | | 16/104 | | 6/52 | | 17/52** | | 24/52*** | |
| | | **Number of mice with any tumour** | | | | | | | |
| Tumours per mouse: | 1 | 12 | 22 | 6 | 9 | 12 | 5 | 9 | 4 |
| | 2 | 2 | 1 | 2 | – | 5 | 1 | 5 | 4 |
| | 3 | – | – | 1 | 2 | 2 | – | 2 | 4 |
| | ≥ 4 | – | – | – | – | 1 | 2 | 7 | 9*** |
| | Total | 14 | 23 | 9 | 12 | 20 | 8 | 23 | 21 |
| Total D + T | | 37/104 | | 21/52 | | 28/52* | | 44/52*** | |
| | | **Females** | | | | | | | |
| **Number of mice with masses in liver** | | | | | | | | | |
| Mice examined | | 73 | 31 | 38 | 14 | 31 | 21 | 37 | 15 |
| Number with masses | | 3 | 3 | 6 | 3 | 5 | 7 | 22 | 13 |
| Total | | 6/104 | | 9/52 | | 12/52 | | 35/52 | |
| **Number of mice with tumours in liver** | | | | | | | | | |
| Mice examined | | 73 | 31 | 38 | 14 | 31 | 21 | 37 | 15 |
| Number with benign tumours | | 1 | 3 | 4* | 2 | 3 | 7* | 18*** | 12*** |
| Total | | 4/104 | | 6/52 | | 10/52** | | 30/52*** | |
| | | **Number of mice with malignant tumours** | | | | | | | |
| Tumours per mouse: | 1 | 1 | – | – | – | – | 1 | – | 1 |
| | 2 | – | – | – | – | – | – | – | 1 |
| | 3 | – | – | – | – | – | – | 1 | – |
| | ≥ 4 | – | – | – | – | – | – | 6 | – |
| | Total | 1 | – | – | – | – | 1 | 7 | 2 |
| Total D + T | | 1/104 | | 0/52 | | 1/52 | | 9/52*** | |
| | | **Number of mice with any tumour** | | | | | | | |
| Tumours per mouse: | 1 | 2 | 3 | 3 | 1 | 2 | 2 | 8 | 5 |
| | 2 | – | – | 1 | 1 | 1 | 4 | 2 | 5 |
| | 3 | – | – | – | – | – | – | 2 | – |
| | ≥ 4 | – | – | – | – | – | 2 | 13 | 4 |
| | Total | 2 | 3 | 4 | 2 | 3 | 8 | 25 | 14 |
| Total D + T | | 5/104 | | 6/52 | | 11/52** | | 39/52*** | |

Statistical difference from control mean at: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ Source: Colley, 1983

The NOAEL was 78 ppm (equal to 7.5 mg/kg bw per day) based on increased liver weights and liver tumours (not further specified) observed in both sexes at 325 ppm (equal to 33 mg/kg bw per day) (Colley et al., 1983).

*Study 2*

In a 78-week carcinogenicity study, prochloraz (purity 97.4%, batch XY-1) was administered in the diet to groups of CD-1 mice. Fifty male and 50 female mice received prochloraz for 78 weeks at dietary concentrations of 0, 40, 220 or 1200 ppm (equal to 0.0, 4.7, 26.3 and 151.2 mg/ kg bw per day for males, 0.0, 6.0, 31.5 and 182.5 mg/kg bw per day for females). The mice were checked daily for mortality and clinical signs and a detailed examination, including palpable masses was conducted weekly. Water consumption was recorded daily and food consumption was recorded weekly. Body weight was recorded weekly during the first 14 weeks and every two weeks thereafter. Blood was sampled for haematological examinations at weeks 50 and 67. Animals found dead or euthanized before or at the end of the treatment period underwent necropsy. Adrenals, brain, heart, liver, kidneys, spleen, ovaries, uterus with cervix, testes and lungs with mainstem bronchi were weighed from all animals. A wide range of tissues was examined microscopically from all animals killed or that died prematurely, and all control and high-dose animals killed at the end of the treatment period. In addition, livers from all animals at 40 and 220 ppm and lungs (with mainstem bronchi) from males at 40 and 220 ppm and any abnormalities in tissues from the 40 and 220 ppm groups were examined histopathologically.

There were no effects of treatment on mortality, clinical signs, food consumption, incidence or distribution of masses, ophthalmology or haematology. Final body weight was reduced by 14% (statistically significant) in males at 1200 ppm compared with controls. In females final body weights were not affected by prochloraz treatment when compared to controls.

Necropsy of males showed a dose-related increase in incidences of liver masses. The increase in liver masses was statistically significant in males at 220 ppm (15 mice) and 1200 ppm (24 mice) compared to controls (four mice), and in high-dose females (15 mice) compared to controls (two mice). In the liver of males at 1200 ppm statistically significant increases in the incidences of enlargement or swelling and of single or multiple pale areas were seen. Also more swollen and larger livers were seen in males at 1200 ppm. In females a statistically significant increase in liver masses was observed only in the highest dose group at 1200 ppm. In males a dose-dependent decrease was observed in the amount of large seminal vesicles. More masses were observed in the lungs of males at 1200 ppm, and in all female prochloraz receiving groups However this difference was not statistically significant.

Liver weights were higher (statistically significant) compared to controls in both males (by 71%) and females (by 82%) at 1200 ppm. Also the liver weights of animals at 220 ppm were slightly higher compared to controls (by 9% and 7% for males and females respectively), but this difference was not significant. In females at 1200 ppm, a slightly lower, but not statistically significant, mean uterus weight was observed compared to controls. In the same group a slightly lower ovarian weight was also observed. However, this was the result of abnormally high ovarian weights in the controls which correlated with macropathological abnormalities.

After 78 weeks, histopathological examination showed a dose-related increase in the incidence of hepatocellular adenomas in the livers of males (see Table 11). This increase in hepatocellular adenomas was significant in males at 220 ppm and in males and females at 1200 ppm compared to the controls, and values were outside the range of the historical control data (0%–19.2%). Additionally, the incidence of hepatocellular carcinoma in males and females at 1200 ppm was increased compared to controls. This increase was only statistically significant in males. In the lung, the incidence of pulmonary carcinoma was significantly increased in males at 1200 ppm compared to controls. However the study author stated that these tumours occur spontaneously at a high frequency similar to that of the incidence observed in the present study. In males at 1200 ppm, single incidences of malignant thymoma and follicular cell adenoma were observed. In females at 1200 ppm single incidences of exocrine cell carcinoma in the pancreas and adenoma in the uterus observed found. In males at 1200 ppm statistically significant non-neoplastic findings including centriacinar hepatocytic fatty vacuolation and basophilic foci of hepatocellular alteration in the liver were observed (see Table 11). In females at 1200 ppm an increase was seen in periacinar hepatocytic hypertrophy and basophilic foci of hepatocellular alteration, but these were not statistically significant. In the lungs of males at 1200 ppm, a statistically significant increase in the incidence of alveolar macrophages associated with neoplasia was observed. In males at 1200 ppm an increased incidence of perivascular lymphocytic infiltration in the lung was also seen.

*Table 11a. Incidence of neoplasms in mice (including inter-current deaths)*

| | Dose level (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | 0 | 40 | 220 | 1200 | 0 | 40 | 220 | 1200 |
| **Liver** | | | | | | | | |
| Number examined | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| Hepatocellular carcinoma | 1 | 2 | 2 | 7* | 1 | 0 | 0 | 4 |
| Hepatocellular adenoma | 4 | 7 | 10* | 19*** | 1 | 0 | 1 | 16*** |
| **Lungs** | | | | | | | | |
| Number of examined | 50 | 50 | 50 | 50 | 50 | 27 | 24 | 50 |
| Pulmonary carcinoma | 4 | 7 | 4 | 13* | 3 | 3 | 2 | 1 |
| Pulmonary adenoma | 6 | 6 | 9 | 5 | 0 | 3 | 2 | 3 |

Statistical difference from control at: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; Source: Chapman et al., 1993a

*Table 11b. Incidence of non-neoplastic changes in mice (including intercurrent deaths)*

| | Dose level (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | 0 | 40 | 220 | 1200 | 0 | 40 | 220 | 1200 |
| **Liver** | | | | | | | | |
| Number examined | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| Centriacinar hepatocytic fatty vacuolation | 0 | 0 | 4 | 7* | 0 | 0 | 0 | 0 |
| Basophilic focus/foci of hepatocellular alteration | 0 | 1 | 2 | 6* | 0 | 0 | 0 | 2 |
| **Lungs** | | | | | | | | |
| Number examined | 50 | 50 | 50 | 50 | 50 | 27 | 24 | 50 |
| Alveolar macrophages associated with neoplasia | 3 | 4 | 5 | 11* | 3 | 3 | 2 | 0 |
| **Adrenal** | | | | | | | | |
| Number examined | 42 | 10 | 16 | 44 | 46 | 20 | 22 | 42 |
| Amyloidosis | 7 | 3 | 3 | 1* | 4 | 7 | 9 | 7 |
| **Spleen** | | | | | | | | |
| Number examined | 50 | 24 | 27 | 50 | 50 | 31 | 27 | 50 |
| Amyloidosis | 7 | 4 | 5 | 0* | 4 | 7 | 10 | 0 |
| **Ileum** | | | | | | | | |
| Number examined | 46 | 13 | 17 | 50 | 49 | 18 | 17 | 49 |
| Amyloidosis | 3 | 2 | 5 | 2 | 3 | 3 | 4 | 14** |

Statistical difference from control at: * $p < 0.05$; ** $p < 0.01$; Source: Chapman et al., 1993a

The NOAEL was 40 ppm (equal to 4.7 mg/kg bw per day) based on the increased incidence of liver adenomas at 220 ppm (equal to 26.3 mg/kg bw per day) (Chapman et al., 1993a).

***Rat***

*Study 1*

In a combined toxicity and carcinogenicity study, prochloraz (purity 95.1%–97.0%, batch 00097/DT) was administered in the diet to groups of Sprague Dawley CD rats. Groups of male and female rats received prochloraz at dietary concentrations of 0 ppm (150 animals per sex), 37.5 ppm (80 animals per sex), 150 ppm (80 animals per sex) and 625 ppm (90 animals per sex) (equal to 0, 1.3, 5.1, and 21.5 mg/kg bw per day for males, 0, 1.6, 6.4 and 28.1 mg/kg bw per day for females). The experiment lasted until 20% survival was reached in any group of a sex. At 13 weeks, 10 males and 10 females from the control and 625 ppm dose group were killed. At 52 weeks 20 males and 20 females from every group were killed. At week 111 treatment ended for males while at week 115 treatment ended for females.

The animals were checked twice a day for mortality. Observations for clinical signs were conducted daily during the first four weeks and weekly thereafter. From week 26, the rats were checked weekly for palpable masses. The dimensions of palpable masses were measured every two weeks. Body weights and food consumption were recorded one week before the study and weekly thereafter. Water consumption was monitored daily by visual examination. Ophthalmology was performed pretreatment and during weeks 6, 13, 26, 51, 78 and 104 on 50 males and 50 females receiving 625 ppm and the same the same number of controls. Blood was sampled for haematological and clinical biochemistry examinations at weeks 6, 13, 26, 52, 77/78 and 104 from 10 males and 10 females from the controls, the 625 ppm group and any interim kill controls.

During the experiment additional investigations were undertaken. For haematology this included; repeated sampling to examine thrombotest time in weeks 7 and 8 from 10 males from all regular dose groups; sampling from 10 not previously examined control and intermediate-dose males and females for examination of red cell parameters and platelet count in week 13; sampling of the same animals from the intermediate dose groups tested in week 13 during the measurements of week 26; sampling of from 10 males and 10 females from the intermediate group and interim kill group during the regular examinations from week 52 to the end of the study. For biochemistry additional investigations included; sampling of 10 male and 10 female controls not previously examined and the same number from intermediate dose groups to investigate several parameters; sampling of 10 males and 10 females from the intermediate group and interim kill group during the regular examinations from week 52 to the end of the study; repeated sampling from 10 males and 10 females from all groups in week 52 to examine several parameters. Urine analysis was performed during weeks 7, 13, 26, 52, 78 and 104 on 10 males and 10 females receiving 625 ppm and the same number of controls. Urine analysis was extended to 10 males and 10 females from the 37.5 ppm, 150 ppm and the interim kill groups in week 52, and 10 males and 10 females from the 37.5 ppm and 150 ppm groups in weeks 78 and 104. Animals killed at week 13 underwent macroscopic examination and their livers were weighed and inspected microscopically. Animals killed at week 52 underwent ophthalmology, macroscopic examination and organ weight analysis. Microscopic examination of the liver was only conducted in animals from the control and 625 ppm groups.

Other animals found dead or euthanized before or at the end of the treatment period underwent the same examinations as the animals killed at the end of the treatment period. The animals were macroscopically examined and the organs (adrenals, brain, heart, kidneys, liver, ovaries, pituitary, prostate with seminal vesicles, spleen, testes and thyroids) were weighed. A wide range of tissues was examined microscopically from all animals killed at the end of the treatment period.

There was no apparent effect of treatment on mortality, clinical signs, incidence or distribution of masses, water consumption or ophthalmology. A sialodacryoadenitis infection in a few rats during week 20, 21, 66 and 67 affected the body weight and food consumption during those weeks. The survival rate of males receiving prochloraz was similar to the controls, whereas females receiving 37.5, 150 and 625 ppm prochloraz achieved higher survival rates than the 23% for controls (35%, 37% and 48% respectively). Final body weight was significantly reduced ($p < 0.05$) compared to controls at the end of the treatment period in males (by 10.6%) and females (by 10.5%) receiving 625 ppm. Females receiving 37.5 ppm showed a statistically significant ($p < 0.01$) increase in body weight gain compared to controls (by 18.5%). Rats receiving 150 ppm prochloraz showed a slightly, but not significantly, lower body weight gain compared to controls; males by 4.4%, females by 5.6%. Food consumption was significantly reduced ($p < 0.01$) in males and females receiving 625 ppm. Males and females receiving 150 ppm showed a significantly ($p < 0.05$) lower food consumption until week 52. At the end of the treatment period, the food consumption of both of these150 ppm groups was only slightly lower, and not to a statistically significant extent.

The haematological investigation showed statistically significant differences in red cell parameters, like haemoglobin, mean corpuscular volume and red blood cells of rats receiving 625 ppm compared to the controls. However the differences were inconsistent and individual values were mostly within the expected range. At week 78, females receiving 625 ppm showed slightly lower neutrophil and lymphocytes counts compared to controls. At week 78 and 104, neutrophil counts were statistically significant lower in females receiving 625 ppm compared to controls.

Some changes in urinary, blood chemical and haematological parameters were detected in treated rats, but these were minimal and inconsistent and of doubtful toxicological significance.

Macroscopic examination revealed no effects of treatment in the animals killed at 13 weeks. At week 52, several animals receiving 150 or 625 ppm showed enlarged or swollen livers. At the end of the treatment period only males receiving 625 ppm (18/61) showed livers more enlarged or swollen than in the controls (24/121). In females, nodules in adipose tissue were not observed in control animals, but at least two cases were observed in every treatment group. At week 13 a statistically significant ($p < 0.001$) higher relative liver weight compared to the controls was seen in females receiving 625 ppm prochloraz. Also three males receiving 625 ppm showed increased liver weights, but this was not statistically significant. At week 52 relative liver weight was higher (statistically significant) in both males and females receiving 625 ppm compared to controls and males receiving 37.5 ppm also showed a statistically significant higher liver weight. Pituitary weights were lower in females receiving 625 ppm compared to controls, an effect that was statistically significant. At the end of the treatment period, females receiving 625 ppm showed significantly ($p < 0.05$) higher liver weights. Also some females receiving 37.5 ppm displayed higher absolute liver weights (statistically significant), although this was most likely the result of higher overall body weights.

Microscopic examination showed centrilobular hepatocyte enlargement of the liver in one male and two females receiving 625 ppm prochloraz at week 13. At week 52, centrilobular hepatocyte enlargement of the liver was observed in 13 females receiving 625 ppm. Periportal glycogen loss combined with centrilobular fat deposition was observed in animals from both sexes and especially when receiving 150 ppm. In animals receiving 625 ppm this change was accompanied by clumping of the cell organelles in some hepatocytes. At the end of the treatment period, the incidence of hyperplastic changes in the liver was slightly higher in male and female animals receiving 625 ppm (15/61 and 21/63 respectively) than in male and female controls (23/121 and 34/120 respectively). However, this was not statistically significant. In one male receiving 625 ppm a single malignant tumour was found in the liver. It was considered to be an incidental finding, when the data was compared to five concurrent studies using the same strain of rats where adenocarcinoma incidences were 2/50, 1/60, 1/55, 0/100 and 0/50 in the males and 1/60, 1/50, 1/50, 0/100 and 0/55 in the females. Adenocarcinoma of the prostate was found in two males receiving 625 ppm and hyperplastic lesions in the prostate were seen in two males receiving 625 ppm and one control male. When the data was compared to the five concurrent studies above, no effect due to prochloraz was concluded. No other histopathological changes seen were related to the exposure to prochloraz. The conclusion that the incidence of liver tumours was unaffected by treatment was confirmed by an independent pathologist who re-examined the livers.

The NOAEL was 37.5 ppm (equal to 1.3 mg/kg bw per day) based on macroscopic and histopathological signs of liver toxicity (periportal glycogen loss and centrilobular fat deposition) at 150 ppm (equal to 5.1 mg/kg bw per day) (Colley et al., 1982).

*Study 2*

Groups of 80 male and 80 female F-344 rats received prochloraz (purity 97.4%, batch XY-1) dissolved in corn oil at dietary concentrations of 0, 40, 220 or 1200 ppm (equal to 0, 1.7, 9.4 and 50.3 mg/kg bw per day for males, 0, 2.1, 11.8 and 64.1 mg/kg bw per day for females). Ten animals per sex and group assigned to the toxicity phase of the study were killed after 4, 52 and 104 weeks of treatment. All other animals assigned to the oncogenicity phase of the study were skilled after 104 weeks of treatment. All groups were observed daily for mortality, clinical signs, food consumption and body weight gain. Ophthalmological examinations were performed at test start (all toxicity-phase animals of the 52- and 104-week groups) and after 12, 24, 50, 76 and 102 weeks (control and high-dose toxicity-phase animals only), and upon study termination for some oncogenicity-phase animals (nine per group). Haematology was performed on 20 males and 20 females per toxicity-phase group after weeks 12, 24, 50, 76 and 102, and for nine oncogenicity-phase animals per group upon study termination. In addition, blood smears were prepared for each surviving oncogenicity-phase animal after 50, 76 and 102 weeks. Laboratory plasma clinical chemistry was performed on 10 males and 10 females per toxicity-phase group in the same sequence as for peripheral haematology. Urine analysis was performed on 10 males and 10 females per toxicity-phase group after weeks 11, 23, 49, 75 and 101, and for nine oncogenicity-phase animals per group upon study termination. All animals were subjected to detailed gross pathological examination and various organs were weighed. Histopathology was performed on control and high-dose

toxicity-phase animals sacrificed at 52 weeks or upon study termination, on all control and high-dose oncogenicity-phase animals upon study termination and on all animals sacrificed non-scheduled or found dead during the study. In addition, histopathological investigation of the liver was performed on all low- and mid-dose animals at 52 weeks and upon study termination.

No treatment-related effects on mortality, clinical signs, ophthalmoscopy or palpable masses were observed. Significantly reduced body weights were observed in high-dose toxicity-phase animals after one-year of treatment. In oncogenicity-phase animals, significantly reduced body weights were observed in high-dose males after one year of treatment (see Table 12). Body weights were reduced in mid- and high-dose females after one and two years of treatment. High-dose animals of both sexes showed a slightly lower daily food intake per animal. During the first 14 weeks of treatment, the overall efficiency of feed utilization was low in this high-dose group.

***Table 12. Effect of prochloraz on body weights of rats in 104-week study***

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | 0 | 40 | 220 | 1200 | 0 | 40 | 220 | 1200 |
| Toxicity phase | | | | | | | | |
| Weeks 0–52 | 343 ± 31 | 333 ± 28 | 342 ± 23 | 292 ± 26** | 143 ± 14 | 150 ± 19 | 141 ± 13 | 127 ± 12** |
| Weeks 0–104 | 286 ± 30 | 319 ± 17 | 293 ± 32 | 285 ± 37 | 190 ± 15 | 211 ± 12 | 200 ± 19 | 171 ± 15 |
| Oncogenicity phase | | | | | | | | |
| Weeks 0–52 | 361 ± 30 | 354 ± 28 | 353 ± 28 | 310 ± 29** | 146 ± 19 | 143 ± 15 | 134 ± 15** | 117 ± 12** |
| Weeks 0–104 | 317 ± 50 | 307 ± 39 | 311 ± 33 | 293 ± 32 | 218 ± 31 | 208 ± 40 | 197 ± 32** | 175 ± 24** |

Statistical difference from control at: * $p < 0.05$; ** $p < 0.01$; Source: Chapman (1993b)

Haematology investigations revealed in the high-dose animals, and occasionally in the low- and mid-dose animals, slight but statistically significant effects on Hb, erythrocyte counts (RBC), MCV and MCHC values at 12, 24 and 50 weeks, and in mid-dose females after 24 weeks of treatment when compared with the controls. However, as the changes were generally well below 10%, these effects were not considered adverse. Significantly increased total leukocyte counts (total WBC) were evident in high-dose females after 12 and 50 weeks (by up to 32%) and in mid-dose females after 50 weeks (23%). All other findings (for example MCVs in low-dose animals, PCV, mean corpuscular Hb, platelets) were slightly affected and within the normal range of variation or showed no dose–response relationship. Examination of blood smears prepared after 50, 76 and 102 weeks of treatment from oncogenicity-phase animals revealed no treatment-related findings.

Clinical chemistry investigations revealed significantly lower ALT, AST, and OCT levels in the mid- and high-dose animals, and occasionally in low-dose animals. The toxicological relevance of these findings is unclear. Slightly but significantly increased GGTP activity was evident in females receiving 1200 ppm and in high-dose males only after 12, 24, and 102 weeks. In animals of the high-dose group, α1-globulin concentration was significantly lower than in the controls (week 24, 50, 76 and 102), but no similar effects were seen at 40 or 220 ppm. All other findings (for example ALT in low-dose animals, urea, glucose, albumin, total proteins) were slight and within the normal range of variation, or showed no dose–response relationship. No treatment-related changes were observed from urinalysis; all findings (volume, pH) represented single events with no trend over time or dose–response relationship.

At the interim kill, no treatment-related macroscopic changes were observed. High-dose males killed after four weeks of treatment showed decreased absolute and relative prostate weights and increased relative liver weights (see Table 13). High-dose females killed after four weeks of treatment showed increased absolute and relative liver weights and decreased absolute and relative uterus weights. After 52 weeks of treatment significantly increased relative liver weights were observed in high-dose males and all treated females. Absolute and relative uterus weights were decreased in high-dose females. Other differences in organ weights (for example brain, kidneys, testes, spleen) were considered to reflect differences in body weight or were not considered related to treatment in the absence of similar changes at other examinations.

***Table 13. Summary of effects on absolute and relative organ weights at interim sacrifice in a 104-week rat study (means ± standard deviations shown)***

| Organ weight | Week | Dose level | | | |
|---|---|---|---|---|---|
| | | 0 ppm | 40 ppm | 220 ppm | 1200 ppm |
| | | | **Males** | | |
| Fasting body weight (g) | 4 | 232.7 ± 11.8 | 227.7 ± 17.2 | 227.8 ± 10.8 | 213.89 ± 7.6** |
| | 52 | 453.9 ± 34.2 | 455.2 ± 33.8 | 462.6 ± 32.5 | 410.4 ± 25.5* |
| Prostate absolute (g) | 4 | 0.193 ± 0.030 | 0.187 ± 0.045 | 0.192 ± 0.039 | 0.124 ± 0.041** |
| | 52 | 0.354 ± 0.106 | 0.331± 0.087 | 0.328 ± 0.165 | 0.309 ± 0.086 |
| Prostate relative (%) | 4 | 0.0825 ± 0.0101 | 0.0821 ± 0.0179 | 0.0840 ± 0.0170 | 0.0576 ± 0.0187** |
| | 52 | 0.0786 ± 0.0252 | 0.0724 ± 0.0164 | 0.0717 ± 0.0369 | 0.0759 ± 0.0228 |
| Liver relative (%) | 4 | 4.80 ± 0.44 | 4.46 ± 0.28 | 4.88 ± 0.38 | 5.22 ± 0.29* |
| | 52 | 3.55 ± 0.18 | 3.89 ± 0.23** | 3.68 ± 0.26 | 4.35 ± 0.19** |
| Brain absolute (g) | 4 | 1.74 ± 0.04 | 1.73 ± 0.03 | 1.72 ± 0.07 | 1.69 ± 0.05 |
| | 52 | 1.95 ± 0.05 | 1.95 ± 0.07 | 1.92 ± 0.05 | 1.87 ± 0.04* |
| Kidneys relative (%) | 4 | 0.831 ± 0.042 | 0.837 ± 0.025 | 0.866 ± 0.052 | 0.90 ± 0.039** |
| | 52 | 0.688 ± 0.015 | 0.708 ± 0.022 | 0.691 ± 0.021 | 0.737 ± 0.026** |
| Testes relative (%) | 4 | 1.140 ± 0.039 | 1.182 ± 0.054 | 1.179 ± 0.038 | 1.269 ± 0.067** |
| | 52 | 0.713 ± 0.068 | 0.704 ± 0.023 | 0.699 ± 0.052 | 0.763 ± 0.038 |
| Thyroids relative (%) | 4 | 0.0060 ± 0.0023 | 0.0070 ± 0.0024 | 0.0060 ± 0.0009 | 0.0073 ± 0.0027 |
| | 52 | 0.0050 ± 0.0009 | 0.0055 ± 0.0022 | 0.0049 ± 0.0008 | 0.0059 ± 0.0004* |
| | | | **Females** | | |
| Fasting body weight (g) | 4 | 148.2 ± 8.2 | 153.5 ± 11.0 | 147.4 ± 7.3 | 137.6 ± 8.1* |
| | 52 | 238.3 ± 19.1 | 240.6 ± 9.3 | 236.8 ± 12.7 | 222.7 ± 11.3* |
| Liver absolute (g) | 4 | 6.3 ± 0.8 | 6.9 ± 0.9 | 6.5 ± 0.7 | 7.2 ± 0.8* |
| | 52 | 7.4 ± 1.0 | 8.4 ± 0.9* | 8.4 ± 0.7* | 8.4 ± 0.6* |
| Liver relative (%) | 4 | 4.26 ± 0.39 | 4.48 ± 0.28 | 4.43 ± 0.35 | 5.24 ± 0.46** |
| | 52 | 3.12 ± 0.29 | 3.47 ± 0.33* | 3.54 ± 0.25** | 3.78 ± 0.17** |
| Uterus + cervix absolute (g) | 4 | 0.41 ± 0.17 | 0.38 ± 0.15 | 0.34 ± 0.13 | 0.20 ± 0.08** |
| | 52 | 0.70 ± 0.13 | 0.77 ± 0.17 | 0.94 ± 0.22* | 0.46 ± 0.12** |
| Uterus + cervix relative (g) | 4 | 0.273 ± 0.112 | 0.249 ± 0.097 | 0.230 ± 0.090 | 0.144 ± 0.057** |
| | 52 | 0.294 ± 0.057 | 0.317 ± 0.061 | 0.398 ± 0.086** | 0.208 ± 0.057* |
| Brain absolute (g) | 4 | 1.59 ± 0.05 | 1.58 ± 0.03 | 1.60 ± 0.05 | 1.56 ± 0.05 |
| | 52 | 1.77 ± 0.03 | 1.79 ± 0.06 | 1.77 ± 0.04 | 1.70 ± 0.05* |
| Brain relative (%) | 4 | 1.074 ± 0.047 | 1.031 ± 0.062 | 1.084 ± 0.030 | 1.139 ± 0.057* |
| | 52 | 0.747 ± 0.061 | 0.747 ± 0.041 | 0.7851 ± 0.045 | 0.765 ± 0.039 |
| Adrenals relative (%) | 4 | 0.0321 ± 0.0040 | 0.0278 ± 0.0031* | 0.0308 ± 0.0036 | 0.0300 ± 0.0041 |
| | 52 | 0.0196 ± 0.0016 | 0.0220 ± 0.0060 | 0.0201 ± 0.0029 | 0.0202 ± 0.0038 |
| Spleen relative (%) | 4 | 0.2477 ± 0.0199 | 0.2426 ± 0.0149 | 0.2521 ± 0.0170 | 0.2692 ± 0.0201* |
| | 52 | 0.1676 ± 0.0119 | 0.1704 ± 0.0101 | 0.1748 ± 0.0090 | 0.1723 ± 0.0161 |
| Heart absolute (g) | 4 | 0.58 ± 0.04 | 0.60 ± 0.05 | 0.58 ± 0.04 | 0.55 ± 0.06 |
| | 52 | 0.79 ± 0.05 | 0.78 ± 0.04 | 0.75 ± 0.05 | 0.70 ± 0.05** |

Statistical difference from control at: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ *Source:* Chapman (1993b)

Non-neoplastic changes at the interim kill which might be related to treatment were observed only in the liver. Incidences of hepatocytic fatty vacuolation in the centriacinar (males and females) and midzonal (males only) regions were significantly increased in high-dose animals (see Table 14). A nonsignificant increase in the incidence of periacinar hepatocytic fatty vacuolation was observed in high-dose males. An increased incidence of basophilic foci was observed in mid-dose males, but not in high-dose males. High-dose males showed a decrease in the incidence of portal tract senile changes.

*Table 14. Non-neoplastic liver changes at interim sacrifice (week 52) in 104-week rat study*

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | 0 | 40 | 220 | 1200 | 0 | 40 | 220 | 1200 |
| **Liver** | | | | | | | | |
| Number examined | 10 | 9 | 10 | 9 | 10 | 10 | 10 | 10 |
| Number of rats exhibiting finding: | | | | | | | | |
| Centriacinar hepatocytic fatty vacuolation | 0 | 0 | 0 | 7*** | 0 | 0 | 0 | 7** |
| Midzonal hepatocytic fatty vacuolation | 0 | 0 | 3 | 9*** | 0 | 0 | 0 | 3 |
| Periacinar hepatocytic fatty vacuolation | 4 | 2 | 5 | 8 | 0 | 0 | 0 | 0 |
| Basophilic focus/foci of hepatocellular alteration | 1 | 4 | 7* | 2 | 1 | 0 | 2 | 5 |
| Portal tract (senile) change including proliferation of bile ducts, hyaline degeneration and inflammation | 6 | 4 | 8 | 0* | 1 | 6 | 3 | 0 |

Statistical difference from control at: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ *Source:* Chapman (1993b)

At termination, gross pathology revealed a slightly higher incidence of masses in the liver and a significantly lower incidence of pale kidneys in high-dose males compared to controls. No other treatment-related macroscopic changes were observed. Increased relative liver weights (statistically significant at 12%) were observed in high-dose males (see Table 15). Other examples of inter-group differences in organ weights (prostate, pituitary, testes, lungs, thyroid, spleen) were attributed mainly to differences in body weight or were not considered related to treatment in the absence of similar changes at earlier examinations. Histopathology revealed treatment-related, non-neoplastic changes in the liver (see Table 16). An increased incidence of focal hepatocytic fatty vacuolation was noted in mid-dose females, progressing into increased midzonal hepatocytic fatty vacuolation in males and females at 1200 ppm. In addition, an increased incidence of clear cell foci was noted in high-dose males. There were some statistically significant differences in male secondary sex organs, including increased hypospermia of the epididymides and a reduction in prostate secretion at 1200 ppm. These findings were considered age-related and associated with the increased survival in this group. In addition, less severe nephropathy was observed in females at 1200 ppm. Reduced incidences of mineralization of the sclera (males only), decreased remodelling of the femur and decreased parathyroid hyperplasia were also found. There was also a slight reduction in thymic involution in high-dose females as well as oedema, hyperkeratisis and acanthosis in high-dose males.

*Table 15. Summary of significant effects on absolute and relative organ weights at terminal sacrifice in 104-week rat study (means ± standard deviations shown)*

| Organ weight | Group | Dietary dose | | | |
|---|---|---|---|---|---|
| | | 0 ppm | 40 ppm | 220 ppm | 1200 ppm |
| | | | Males | | |
| Fasting body weight (g) | Tox | 401.1 ± 28.3 | 432.4 ± 21.1 | 405.7 ± 28.1 | 391.9 ± 45.6 |
| | Onc | 434.0 ± 43.2 | 413.0 ± 41.4 | 426.0 ± 30.6 | 404.8 ± 31.7* |
| Liver relative (%) | Tox | 4.42 ± 0.46 | 4.12 ± 0.47 | 4.58 ± 0.52 | 4.51 ± 0.45 |
| | Onc | 4.12 ± 0.41 | 4.42 ± 1.33 | 4.38 ± 0.52 | 4.60 ± 0.47** |
| Prostate absolute (g) | Tox | 0.172 ± 0.042 | 0.201 ± 0.111 | 0.159 ± 0.051 | 0.135 ± 0.043 |
| | Onc | 0.208 ± 0.073 | 0.227 ± 0.121 | 0.192 ± 0.107 | 0.149 ± 0.070* |
| Pituitary absolute (g) | Tox | 0.016 ± 0.003 | 0.012 ± 0.003 | 0.013 ± 0.002 | 0.012 ± 0.004 |
| | Onc | 0.015 ± 0.003 | 0.014 ± 0.003 | 0.014 ± 0.004 | 0.011 ± 0.003** |
| Pituitary relative (%) | Tox | 0.0041 ± 0.0007 | 0.0028 ± 0.0006* | 0.0030 ± 0.0005 | 0.0032 ± 0.0008 |
| | Onc | 0.0035 ± 0.0007 | 0.0034 ± 0.0008 | 0.0033 ± 0.0008 | 0.0028 ± 0.0008* |

| Organ weight | Group | Dietary dose | | | |
|---|---|---|---|---|---|
| | | 0 ppm | 40 ppm | 220 ppm | 1200 ppm |
| Heart | Tox | 1.39 ± 0.17 | 1.27 ± 0.09 | 1.25 ± 0.09 | 1.21 ± 0.15 |
| absolute (g) | Onc | 1.36 ± 0.14 | 1.37 ± 0.16 | 1.33 ± 0.14 | 1.19 ± 0.12** |
| Testes | Tox | 8.01 ± 2.11 | 6.58 ± 2.35 | 5.71 ± 3.00 | 6.40 ± 2.42 |
| absolute (g) | Onc | 6.04 ± 2.01 | 5.836 ± 2.85 | 6.71 ± 2.12 | 7.69 ± 1.90* |
| Testes | Tox | 1.991 ± 0.472 | 1.521 ± 0.538 | 1.401 ± 0.718 | 1.679 ± 0.741 |
| relative (%) | Onc | 1.408 ± 0.488 | 1.409 ± 0.669 | 1.567 ± 0.462 | 1.897 ± 0.436** |
| Lungs | Tox | 0.516 ± 0.163 | 0.495 ± 0.136 | 0.461 ± 0.082 | 0.428 ± 0.034 |
| relative (%) | Onc | 0.439 ± 0.074 | 0.501 ± 0.114* | 0.482 ± 0.135 | 0.453 ± 0.066 |
| Thyroids | Tox | 0.0094 ± 0.0015 | 0.0073 ± 0.0014 | 0.0074 ± 0.0013 | 0.0103 ± 0.0049 |
| relative (%) | Onc | 0.0063 ± 0.0008 | 0.0101 ± 0.0132 | 0.0188 ± 0.0537 | 0.0086 ± 0.0053* |
| | | | **Females** | | |
| Fasting body | Tox | 284.8 ± 16.0 | 306.9 ± 16.3 | 297.1 ± 15.2 | 164.1 ± 20.5 |
| weight (g) | Onc | 309.3 ± 30.5 | 302.8 ± 37.8 | 289.5 ± 34.5** | 270.1 ± 23.9** |
| Adrenals | Tox | 0.069 ± 0.014 | 0.056 ± 0.008 | 0.067 ± 0.006 | 0.052 ± 0.007* |
| absolute (g) | Onc | 0.063 ± 0.013 | 0.064 ± 0.013 | 0.061 ± 0.014 | 0.052 ± 0.009** |
| Adrenals | Tox | 0.0241 ± 0.0042 | 0.0182 ± 0.0025** | 0.0225 ± 0.0023 | 0.0199 ± 0.0024* |
| relative (%) | Onc | 0.0206 ± 0.0046 | 0.0215 ± 0.0045 | 0.0215 ± 0.0055 | 0.0194 ± 0.0032 |
| Heart | Tox | 1.02 ± 0.09 | 0.97 ± 0.06 | 1.01 ± 0.05 | 0.86 ± 0.08** |
| absolute (g) | Onc | 0.97 ± 0.08 | 0.95 ± 0.10 | 0.95 ± 0.11 | 0.88 ± 0.08** |
| Heart | Tox | 0.359 ± 0.042 | 0.316 ± 0.019* | 0.339 ± 0.025 | 0.326 ± 0.020 |
| relative (%) | Onc | 0.316 ± 0.040 | 0.319 ± 0.042 | 0.333 ± 0.069 | 0.328 ± 0.033 |
| Spleen | Tox | 0.675 ± 0.061 | 0.901 ± 0.856 | 0.762 ± 0.421 | 0.541 ± 0.101* |
| absolute (g) | Onc | 0.681 ± 0.243 | 0.840 ± 1.039 | 0.617 ± 0.205 | 1.228 ± 3.718 |
| Thymus | Tox | 0.339 ± 0.105 | 0.455 ± 0.064* | 0.335 ± 0.059 | 0.331 ± 0.125 |
| absolute (g) | Onc | 0.371 ± 0.125 | 0.361 ± 0.125 | 0.328 ± 0.091 | 0.294 ± 0.093** |
| Kidneys | Tox | 2.62 ± 0.50 | 2.41 ± 0.19 | 2.38 ± 0.11 | 2.11 ± 0.24 |
| absolute (g) | Onc | 2.51 ± 0.30 | 2.42 ± 0.30 | 2.29 ± 0.23** | 2.11 ± 0.21** |
| Brain | Tox | 0.626 ± 0.036 | 0.580 ± 0.020 | 0.612 ± 0.024 | 0.648 ± 0.037 |
| relative (%) | Onc | 0.581 ± 0.066 | 0.585 ± 0.063 | 0.620 ± 0.073* | 0.657 ± 0.064** |
| Lungs | Tox | 0.468 ± 0.024 | 0.442 ± 0.027 | 0.514 ± 0.146 | 0.467 ± 0.065 |
| relative (%) | Onc | 0.445 ± 0.067 | 0.473 ± 0.128 | 0.468 ± 0.121 | 0.509 ± 0.193* |

Tox: Toxicity-phase group; Onc: Oncogenicity-phase group; Source: Chapman (1993b)

Statistical difference from control at: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

***Table 16. Non-neoplastic changes terminal sacrifice (week 104) in a 104-week rat study***

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | 0 | 40 | 220 | 1200 | 0 | 40 | 220 | 1200 |
| **Toxicity-phase group** | | | | | | | | |
| Liver | | | | | | | | |
| Number of animals examined | 10 | 10 | 10 | 10 | 9 | 10 | 10 | 10 |
| Number of rats exhibiting finding: | | | | | | | | |
| Midzonal hepatocytic fatty vacuolation | 0 | 1 | 1 | 6* | 0 | 0 | 2 | 3 |

| | Dietary dose (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| | 0 | 40 | 220 | 1200 | 0 | 40 | 220 | 1200 |
| **Oncogenicity-phase group** | | | | | | | | |
| **Liver** | | | | | | | | |
| Number of animals examined | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| Number of rats exhibiting finding: | | | | | | | | |
| Midzonal hepatocytic fatty vacuolation | 4 | 8 | 10 | 37*** | 9 | 4 | 6 | 24** |
| Focal hepatocytic fatty vacuolation | 2 | 3 | 3 | 5 | 5 | 8 | 16** | 4 |
| Clear cell focus/foci | 8 | 8 | 9 | 27*** | 6 | 4 | 4 | 8 |
| **Kidney** | | | | | | | | |
| Number of animals examined | 50 | 31 | 40 | 50 | 50 | 12 | 13 | 50 |
| Number of rats exhibiting finding: | | | | | | | | |
| Progressive (senile) nephropathy | 48 | 31 | 40 | 49 | 46 | 10 | 11 | 22*** |

Statistical difference from control at: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; Source: Chapman (1993b)

The NOAEL was 40 ppm (equal to 1.7 mg/kg bw per day) based on histopathological changes in the liver at 220 ppm (equal to 9.4 mg/kg bw per day). No effect of prochloraz on tumour incidence was observed. (Chapman, 1993b).

## 2.4 Genotoxicity

Prochloraz was tested for genotoxicity in a wide range of in vitro and in vivo assays. All tests gave negative results, apart from one of the two in vitro chromosome aberration studies, the in vitro sister chromatid exchange (SCE) study and one of the three in vitro mammalian gene mutation studies. All in vivo studies were negative. Overall, it was concluded that prochloraz does not show genotoxic potential (Table 17).

***Table 17. Overview of genotoxicity tests with prochloraz***

| End-point | Test system | Concentrations/ doses tested | Purity | Results | Reference |
|---|---|---|---|---|---|
| | | **In vitro** | | | |
| Reverse mutation | *S. typhimurium* TA98, TA100, TA1535 and TA1537;<br>*E.* coli WP2 *uvr*A/pKM101 | ±S9: 5–5000 µg/plate | 96.5% | Negative[a] | May, 2002a[b] |
| Reverse mutation | *S. typhimurium* TA100, TA98, TA1535, TA1537;<br>*E.coli* WP2 uvrA pKM101 | ±S9: 5–5000 µg/plate | 98.2% | Negative | May, 2007[c] |
| Reverse mutation | *S. typhimurium* TA100, TA98, TA1535, TA1537 and TA1538 | ±S9: 62.5–1000 µg/plate | ≥ 99.66% (purified prochloraz)<br>97.9–98.5% (technical Prochloraz) | Study unacceptable[d] | The Boots Co. Ltd, 1978[d] |
| Reverse mutation | *typhimurium* TA100, TA98, TA1535, TA1537 and TA1538 | ±S9: 2.5–260 µg/plate | Not stated | Negative | Pinto, 1989[e] |

| End-point | Test system | Concentrations/ doses tested | Purity | Results | Reference |
|---|---|---|---|---|---|
| Reverse mutation | *S. typhimurium* TA100, TA98 | ±S9: 12.5–100 µM, equivalent to 13.2–105.3 µg/plate | 99.2% | Negative | Alpertunga et al., 2014 [f] |
| Reverse mutation | *S. typhimurium* TA100, TA98, TA1535, TA1537<br>*E. coli* WP2 uvrA pKM101, CM891 | ±S9: 5–5000 µg/plate | 97.6% prochloraz–copper | Negative | May, 2002b [g] |
| Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538 | ±S9: 62.5, 125, 250, 500, 1000 µg/plate | 100 and 97.9% | Negative | Wilcox, 1978 [h] |
| Chromosomal aberration | Cultured human lymphocytes | −S9: 10–40 µg/mL (24 hour treatment)<br>+S9: 10–100 µg/mL (3 hour treatment) | 94.6% | Some evidence of clastogenic potential, +S9 [i] | Hodson-Walker, 1990a |
| Chromosomal aberration | CHO-K1 cells of sub-strain BH4 | −S9: 2.5–32.5 µg/mL (20 hour treatment)<br>+S9: 7–110 µg/mL (2 hour treatment) | 96.1% | Negative [j] | Allen et al., 1986a |
| Sister chromatid exchange (SCE) | Chinese hamster CHO-K1-BH4 ovary cells | −S9: 5–30 µg/mL (2 hour treatment)<br>+S9: 7–35 µg/mL (2 hour treatment) | 96.1% | Slightly increased SCE frequencies (±S9) [k] | Allen & Brooker, 1986 |
| Mammalian cell gene mutation | Mouse lymphoma L5178Y cells – TK locus | ±S9: 5–40 µg/mL (3 hour treatment)<br>−S9: 2.5–25 µg/mL (24 hour treatment) | 97.2% | Negative | Bowen, 2006 [l] |
| Mammalian cell gene mutation | Mouse lymphoma L5178Y $tk^{+/-}$ cells (sub-strain 3.7.20C) | −S9: 5–70 µg/mL (3 hour treatment)<br>+S9: 10–60 µg/mL (3 hour treatment)<br>−S9: 10–50 µg/mL (24 hour treatment) | 96.7% | Positive (+S9) in the presence of excessive levels of cytotoxicity | Clare, 2002 [m] |
| Mammalian cell gene mutation | Mouse lymphoma L5178Y $tk^{+/-}$ cells | Experiment 1 ±S9: 0.5–50 µg/mL (3 hour treatment)<br>Experiment 2 ±S9: 30–70 µg/mL (3 hour treatment) | 97.8% | Negative | McGregor, Riach & Brown, 1983 [n] |
| DNA damage and repair | Nondividing human embryonic fibroblasts | ±S9: 10–220 µg/mL (3 hour treatment) | 97.8% | Negative [o] | McGregor & Riach, 1983 |
| DNA damage (comet assay) | NRK-52E cells | −S9: 13.2–105.3 µg/plate | 99.2% | Negative [p] | Alpertunga et al., 2014 |

| End-point | Test system | Concentrations/ doses tested | Purity | Results | Reference |
|---|---|---|---|---|---|
| | | **In vivo** | | | |
| Mouse micronucleus | Mouse (CD-1) bone marrow micronucleus (gavage) | 272, 544 and 1088 mg/kg bw | 96.3% | Negative [q] | Allen, Proudlock & Pugh, 1986 |
| Mouse micronucleus | Rat (CD) bone marrow micronucleus (i.p.) | 6.25, 25 and 100 mg/kg bw | 95.7% | Negative (unreliable) [r] | Everest & Cliffe, 1980 [r] |
| Mouse micronucleus | Mouse (CD-1) bone marrow micronucleus (gavage) | 12, 60 and 300 mg/kg bw | Not stated (assumed as 94.6%) | Negative [s] | Hodson-Walker, 1990b |
| DNA damage and repair (UDS) | Han Wistar rats (male) hepatocytes (gavage) | 280 and 700 mg/kg bw | 96.7% | Negative | Beevers, 2003 [t] |
| DNA damage and repair (UDS) | Rat (CD) hepatocytes (gavage) | 40, 133 and 400 mg/kg bw | 97.9% | Negative | Foster, 1992 [u] |
| Germ cells assay (dominant lethal) | Mouse (CD-1) male (diet) | 6, 25 and 100 mg/kg bw/day | Not stated | Negative | Cozens et al., 1980 [v] |

*S. typhimurium*: *Salmonella typhimurium*; *E. coli*: *Escherichia coli*; *tk*: Thymidine kinase; CHO: Chinese hamster ovary; S9: 9000*g* supernatant fraction from rat liver homogenate

[a] Positive and negative (solvent) controls were included in all studies.

[b] Performed in accordance with OECD TG 471. The plate incorporation method was used in the first experiment followed by a pre-incubation assay. Toxicity was observed at 1500 µg/plate and above.

[c] Performed in accordance with OECD TG 471. The plate incorporation method was used in the first experiment followed by a pre-incubation assay. No toxicity was observed.

[d] Study conducted prior to GLP; no quality assurance included. Study was performed mainly in line with OECD TG 471, but did not include a test in strain TA102 or *E.coli* WP2. Furthermore, positive controls were only included for the assays with metabolic activation. Toxicity was observed at 250 µg/plate and above, resulting in only 2–3 analyzable concentrations per strain. Overall the Meeting considered the study unacceptable.

[e] Non-GLP study, QA statement included. Study was performed mainly in line with OECD TG 471, but did not include a test in strain TA102 or *E.coli* WP2. Precipitation was observed at concentrations greater than 260 µg/plate.

[f] Public literature study. Only strains TA 98 and TA100 were included.

[g] Study performed in accordance with OECD TG 471. The plate incorporation method was used in the first experiment followed by a pre-incubation assay. Toxicity was observed at 1500 µg/plate and above.

[h] Antibacterial effects of the test material precluded evaluation of mutagenicity at 1000, 500 and in some cases 250 µg/plate. The study was not submitted for the current JMPR evaluation but was assessed in the 2001 review of prochloraz.

[i] Study conducted essentially in accordance with OECD TG 473 (adopted July 1997) with the exception that the short treatment (three hours, without S9) was not performed A dose-related increase in the mean aberrant cell frequency was observed in the presence of S9 both including and excluding gaps. In the absence of S9 an increase in the mean aberrant cell frequency was observed only when gaps were excluded.

[j] Study conducted mainly in compliance with OECD TG 473 (adopted July 1997). For the short-term treatment a two hour exposure duration was used instead of the recommended 3–6 hours and this treatment was not performed in the absence of S9 metabolic activation. At each concentration 200 metaphases were scored. There was a reduction of about 50% in mitotic index at the highest doses.

[k] Study conducted essentially in compliance with OECD TG 479 (adopted October 1986). There were slight but statistically significant increases in SCE frequencies both in the presence and absence of metabolic activation at dose levels resulting in cytotoxicity (≥20 µg/mL). This test does not measure mutations and it is useful for research purposes. Nevertheless, the relevant OECD guideline has been withdrawn and the test is currently not included in the test batteries for risk assessment.

[l] Study conducted in accordance with OECD TG 476 (adopted July 1997). However, methods followed were compliant with the relevant current OECD Test Guideline. The relative total growth values were 36% and 22% at 40 µg/mL for the

three hour experiment in the absence and presence of S9 respectively. After 24 hours of exposure the relative total growth was 15% at 25 µg/mL in the absence of S9.

[m] Performed in accordance with OECD TG 476 (1997). Negative in the absence of S9 mix. Positive in the presence of S9 mix from 45 µg/mL and above in the first test; from 30 µg/mL and at 40 µg/mL in the third experiment. In all cases statistical significance was associated with high levels of cytotoxicity and significant reduction of cloning efficiency (<70%).

[n] Non-GLP study, QA statement included. The study was conducted mainly in accordance with OECD TG 476 (1984). Cytotoxicity was observed at 50 µg/mL and above. No meaningful results were obtained at 60 or 70 µg/mL owing to toxicity.

[o] Non-GLP study, QA statement included. The study was conducted mainly in accordance with OECD TG 482 which is not valid anymore (deleted on 2 April 2014). Some evidence of toxicity at 100 µg/mL and marked toxicity at 1000 µg/mL; precipitation at 1000 µg/mL.

[p] Public literature study. The study bears significant limitations in that no S9 metabolic activation was used, and long treatment (24 hours) was employed.

[q] Study conducted in accordance with OECD TG 474 valid at the time of the study. The major limitation is that only 1000 polychromatic erythrocytes per animal were scored instead of 4000 as currently required. At 48 and 72 hours there was some reduction in ratio of polychromatic:normochromatic erythrocytes (PCE:NCE) in animals at 1088 mg/kg bw, indicating bone-marrow cytotoxicity. At this dose 7/20 males and 7/19 females were found dead, indicating that the test compound was systemically available.

[r] Non-GLP study, QA statement included. The study suffers from inadequate selection of dose levels. Despite the use of the intraperitoneal injection route of administration only 100 mg/kg bw (the highest dose administered) elicited limited, and not relevant, clinical signs demonstrating systemic availability of the test compound. No PCE:NCE ratios reported and only 1000 PCEs per animal were only scored, not 4000 as currently required.

[s] Study conducted in accordance with OECD TG 474 valid at the time of performance of the study. No effect on the ratio of polychromatic/mature erythrocytes was observed. Lethargy was observed four hours after treatment at the higher dose used. Only 1000 PCEs per animal scored not 4000 as currently required..

[t] Study conducted in accordance with OECD TG 486. In a preliminary study at 630 and 900 mg/kg bw mortality was observed in all animals at 900 mg/kg bw. In the main study toxicity including mortality occurred in one animal at the high dose of 700 mg/kg bw. According to EFSA, OECD TG 486 this is no longer valid as a follow-up to positive mutagenicity tests (EFSA, 2017). Therefore, the present study serves only as supplementary information.

[u] Study conducted in accordance with OECD TG 486. In a preliminary study marked toxicity was observed at doses of 500 mg/kg bw and above. According to EFSA, OECD TG 486 is no longer a valid follow-up to positive mutagenicity tests (EFSA, 2017). Therefore, the present study serves only as supplementary information.

[v] Study conducted in accordance with OECD TG 478 (adopted April 1984). Mating performance of treated males and subsequent pregnancy rates in untreated females were unaffected by treatment. The mean numbers of implantations, viable young, embryonic deaths and post-implantation losses were unaffected by treatment. At terminal autopsy of males, no obvious treatment-related macroscopic changes were seen, and the mean weights of the testes of treated males compared favourably with that of controls. The assay is considered to be of low sensitivity, since detection of small increases in the frequency of mutations is generally limited due to the high spontaneous frequency of dominant lethal mutations.

## 2.5 Reproductive and developmental toxicity

### (a) Multigeneration studies

In a two-generation reproductive toxicity study, groups of 28 male and 28 female Charles River CD rats were exposed to prochloraz (purity 97.5%, batch DCM-I) in the diet at 0, 50, 150 or 450 ppm (equal to premating intake levels of 0, 3.8, 10.9 and 34.0 mg/kg bw per day for males, 0, 4.5, 13.7 and 43.0 mg/kg bw per day for females. Dose levels were administered throughout the experimental period up to weaning of the F3 litters (premating, mating, gestation and rearing). Both sexes were treated for 14 weeks (F0 and F1 generations) or 10 weeks (F2 generation) before pairing to produce litters. All groups were observed for mortality, clinical signs of toxicity, body weight, mating behaviour and reproductive performance. All parental animals, offspring culled at postpartum day (PPD) 4, the nonselected F1 and F2 weanlings and all pups in the F3 generation were subjected to gross necropsy. Organ weight analysis (epididymides, liver, ovaries, prostate, seminal vesicles, testes and uterus) was carried out on all parental animals (F0, F1 and F2). Histopathological examinations were performed on tissues from all parental F0 and F1 animals in the control and high-dose groups, from all adult rats that died during the study or were sacrificed unscheduled, and on tissues from any animals showing gross pathological lesions.

At 150 ppm one F0 female was euthanized in extremis on PPD 1 due to parturition difficulties. One F1 male was euthanized for humane reasons and one female in the 150 ppm group was euthanized

in extremis on PPD 4. At 450 ppm four parental females (F0) and one female in each of the F1 and F2 generations were euthanized in the perinatal period because of parturition difficulties.

At 450 ppm a slight reduction in body weight was observed in males (see Table 18). In females, body weight was reduced during the gestation and lactation periods at 450 ppm for the F0 and F2 generation, and during gestation for the F1 generation. At 150 ppm no differences were observed during the gestation period, but a trend towards lower body weights was observed during lactation, and this reached statistical significance at day 14 in the F1 generation.

***Table 18. Summary of body weights (g) of females (means ± standard deviations) in a rat multigenerational study with prochloraz***

| Dose level (ppm) | Time point | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Day of gestation (GD) | | | | Day postpartum (PPD) | | | | | |
| | 0 | 6 | 13 | 20 | 1 | 4 | 7 | 14 | 21 | 25 |
| **Generation F0** | | | | | | | | | | |
| 0 | 342±40 | 376±41 | 412±44 | 491±48 | 376±46 | 383±48 | 387±39 | 296±27 | 401±31 | 376±31 |
| 50 | 332±31 | 366±29 | 403±30 | 487±36 | 366±31 | 371±27 | 376±26 | 389±22 | 384±20 | 358±19* |
| 150 | 333±32 | 365±33 | 403±37 | 482±43 | 370±38 | 371±34 | 380±33 | 394±31 | 394±37 | 369±30 |
| 450* | 315 ±31** | 343 ±30*** | 373 ±31*** | 451 ±40** | 331 ±30*** | 331 ±32*** | 344 ±33*** | 361 ±26*** | 366 ±27*** | 347 ±27*** |
| **Generation F1** | | | | | | | | | | |
| 0 | 340±27 | 370±33 | 410±35 | 488±47 | 400±35 | 402±33 | 400±31 | 405±25 | 388±23 | 375±23 |
| 50 | 342±39 | 366±40 | 411±38 | 489±50 | 396±41 | 399±40 | 396±38 | 399±29 | 390±24 | 373±24 |
| 150 | 334±25 | 362±26 | 394±26 | 472±34 | 373±23 | 375±21 | 377±20 | 381±22* | 376±24 | 366±21 |
| 450* | 325 ±41 | 347 ±45 | 385 ±47 | 461 ±53 | 357 ±57** | 362 ±46** | 362 ±44** | 362 ±40*** | 363 ±37** | 351 ±35** |
| **Generation F2** | | | | | | | | | | |
| 0 | 335±34 | 370±34 | 407±36 | 489±35 | 384±45 | 384±49 | 388±48 | 400±40 | 391±29 | 381±31 |
| 50 | 318±28 | **349**±32* | 392±34 | 471±35 | 374±39 | 374±32 | 372±33 | 383±24 | 369±29* | 361±26* |
| 150 | 329±26 | 360±26 | 397±28 | 480±37 | 365±28 | 372±29 | 375±30 | 387±24 | 383±29 | 370±26 |
| 450* | **294** ±38*** | **320** ±39*** | **352** ±44*** | **430** ±51*** | 317 ±47*** | 321 ±46*** | 327 ±40*** | 346 ±31*** | 342 ±26*** | 330 ±29*** |

Statistical difference from control at: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; Source: Reader, 1993

Parents exposed to 150 ppm had a slightly reduced conception rate and fertility index which was observed in the F1 and F2 parental generations, however the effect was not dose-dependent. Gestation length, one of the end-points to determine endocrine disruption, was significantly increased at the high dose in all three generations (F0, F1 and F2; see Table 19). An extended gestation time was also observed in females exposed to 150 ppm in the F0, F1 and F2 generations. Although this difference was not mostly statistically significant, it was related to treatment and dose-dependent, and the increase in duration of gestation reached statistical significance at the top dose of 450 ppm. Gestation index was slightly reduced at 150 and 450 ppm. Statistically significant increases in the relative weight of male reproductive organs were seen in all three generations at 450 ppm, and this was considered to be related to reduced body weight.

*Table 19. Summary of gestation length and gestation index in a rat multigenerational study*

| Dose level | *N* | | Gestation length (days) | | | | | | Number of live litters | Gestation index (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 22 | 22.5 | 23 | 23.5 | 24 | 24.5 | | |
| **Generation F0** | | | | | | | | | | |
| 0 ppm | 23 | *n* [%] | 0 | 9 [39] | 10 [43] | 4 [17] | 0 | 0 | 23 | 100 |
| 50 ppm | 24 | *n* [%] | 2 [8] | 12 [50] | 7 [29] | 3 [13] | 0 | 0 | 24 | 100 |
| 150 ppm | 23 | *n* [%] | 0 | 6 [26] | 5 [22] | 12 [52] | 0 | 0 | 22 | 96 |
| 450 ppm* | 27 [a] | *n* [%] | 0 | 5 [20] | 8 [32] | 12 [48] | 0 | 0 | 25 | 93 |
| **Generation F1** | | | | | | | | | | |
| 0 ppm | 19 [b] | *n* [%] | 2 [11] | 6 [33] | 7 [39] | 2 [11] | [6] | 0 | 18 | 95 |
| 50 ppm | 19 | *n* [%] | 5 [26] | 9 [47] | 5 [26] | 0 | 0 | 0 | 19 | 100 |
| 150 ppm | 16 [c] | *n* [%] | 0 | 6 [40] | 7 [47] | 2 [13] | 0 | 0 | 15 | 94 |
| 450 ppm* | 20 | *n* [%] | 0 | 2 [10] | 6 [30] | 11 [55] | 0 | 1 (5) | 19 | 95 |
| **Generation F2** | | | | | | | | | | |
| 0 ppm | 26 | *n* [%] | 6 [23] | 12 [46] | 5 [19] | 2 [8] | 1 [4] | 0 | 26 | 100 |
| 50 ppm | 22 | *n* [%] | 2 [9] | 10 [45] | 8 [36] | 1 [5] | 1 [5] | 0 | 22 | 100 |
| 150 ppm | 24 | *n* [%] | 2 [8] | 11 [46] | 8 [33] | 3 [13] | 0 | 0 | 24 | 100 |
| 450 ppm* | 22 [d] | *n* [%] | 2 [10] | 4 [19] | 10 [48] | 3 [14] | 2 [10] | 0 | 21 | 95 |

Distribution of parameter significantly different from controls: * $p < 0.05$; ** $p < 0.01$; Source: Reader, 1993

*N*: Total number of pregnant rats; *n* [%]: Number of gestations of the length indicated [as percentage of *N*];

[a] Includes two pregnant females without gestation lengths, killed in extremis before onset of parturition.

[b] Percentages calculated from 18 animals. One pregnant female failed to litter – pregnancy indicated by single implantation site.

[c] Percentages calculated from 15 animals. One pregnant female failed to litter – pregnancy indicated by implantation sites.

[d] Percentages calculated from 21 animals. One pregnant female failed to litter.

In the high-dose offspring, mean pup weight was significantly decreased in all three generations (F1, F2 and F3; see Table 20).

*Table 20. Effect of prochloraz on pup weight in a rat multigenerational study*

| Dose level | Mean body weight (g ± SD) of male pups on day | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 4[a] | 7 | 14 | 21 | 25 |
| | | | Male pups | | | |
| **Generation F0 / litter F1** | | | | | | |
| 0 ppm | 6.8±0.8 | 10.4±1.7 | 17.8±2.2 | 37.8±3.1 | 65.6±5.6 | 90.8±6.5 |
| 50 ppm | 6.6±0.6 | 10.0±1.3 | 17.0±1.9 | 37.7±3.1 | 64.7±4.7 | 90.0±5.7 |
| 150 ppm | 6.8±0.5 | 9.8±1.5 | 16.7±2.0 | 37.5±3.0 | 63.9±7.6 | 88.6±8.7 |
| 450 ppm | 6.6±0.9 | 9.7±1.4 | 16.2±2.6 | 35.3±4.8 | 60.8±6.5 | 84.7±8.1** |
| **Generation F1 / litter F2** | | | | | | |
| 0 ppm | 7.0±0.9 | 11.0±2.0 | 18.6±2.7 | 39.3±4.8 | 66.3±6.2 | 91.8±7.0 |
| 50 ppm | 6.9±0.6 | 10.9±1.4 | 18.2±2.3 | 38.7±3.8 | 65.7±6.3 | 91.6±7.5 |
| 150 ppm | 7.1±0.9 | 11.0±1.9 | 18.4±2.9 | 38.5±3.6 | 65.4±5.0 | 91.1±6.0 |
| 450 ppm | 6.9±0.5 | 10.5±1.7 | 17.2±2.4 | 34.9±4.3** | 60.1±6.1** | 83.9±7.7** |
| **Generation F2 / litter F3** | | | | | | |
| 0 ppm | 6.6±0.7 | 9.9±1.8 | 16.7±3.2 | 37.2±4.6 | 63.7±7.3 | 87.8±9.4 |
| 50 ppm | 6.9±0.8 | 10.7±1.8 | 17.8±2.8 | 38.4±4.2 | 64.8±7.7 | 89.6±9.3 |
| 150 ppm | 6.6±0.6 | 9.9±1.5 | 16.9±2.3 | 37.5±3.5 | 63.3±5.7 | 88.0±6.9 |
| 450 ppm | 6.6±0.6 | 10.0±1.3 | 16.4±2.2 | 34.2±3.9 | 60.1±5.0 | 83.2±6.0 |

| Dose level | Mean body weight (g ± SD) of male pups on day | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 4[a] | 7 | 14 | 21 | 25 |
| | | | Female pups | | | |
| **Generation F0 / litter F1** | | | | | | |
| 0 ppm | 6.5 ± 0.8 | 10.2 ± 1.6 | 17.4 ± 2.3 | 37.1 ± 3.1 | 63.6 ± 5.0 | 87.2 ± 5.2 |
| 50 ppm | 6.2 ± 0.6 | 9.5 ± 1.2 | 16.5 ± 1.7 | 36.5 ± 3.0 | 61.5 ± 4.6 | 84.1 ± 5.1 |
| 150 ppm | 6.4 ± 0.5 | 9.4 ± 1.3 | 16.0 ± 1.8* | 36.4 ± 2.7 | 60.9 ± 6.4 | 83.6 ± 7.5 |
| 450 ppm | 6.1 ± 0.8 | 9.0 ± 1.4 | 15.0 ± 2.3*** | 33.5 ± 4.4*** | 57.2 ± 5.6*** | 78.8 ± 6.6*** |
| **Generation F1 / litter F2** | | | | | | |
| 0 ppm | 6.8 ± 1.2 | 10.7 ± 2.4 | 17.9 ± 3.4 | 38.8 ± 5.9 | 64.3 ± 8.4 | 86.7 ± 9.4 |
| 50 ppm | 6.4 ± 0.6 | 10.0 ± 1.3 | 17.1 ± 1.9 | 37.3 ± 3.6 | 62.5 ± 6.2 | 86.0 ± 7.8 |
| 150 ppm | 6.8 ± 0.8 | 10.6 ± 1.8 | 17.9 ± 2.6 | 37.8 ± 3.4 | 63.2 ± 4.5 | 86.9 ± 5.7 |
| 450 ppm | 6.6 ± 0.6 | 10.1 ± 1.5 | 16.4 ± 2.1 | 33.8 ± 4.2** | 57.9 ± 5.3** | 80.0 ± 7.1* |
| **Generation F2 / litter F3** | | | | | | |
| 0 ppm | 6.2 ± 0.7 | 9.3 ± 1.6 | 15.7 ± 2.9 | 35.7 ± 4.1 | 60.9 ± 6.2 | 83.0 ± 7.5 |
| 50 ppm | 6.7 ± 0.9 | 10.4 ± 1.7 | 17.1 ± 2.8 | 37.5 ± 3.7 | 62.3 ± 6.3 | 84.7 ± 7.6 |
| 150 ppm | 6.2 ± 0.6 | 9.5 ± 1.3 | 16.4 ± 1.9 | 36.5 ± 3.2 | 61.3 ± 4.9 | 83.5 ± 5.7 |
| 450 ppm | 6.3 ± 0.6 | 9.4 ± 1.3 | 15.5 ± 2.2 | 32.5 ± 3.9** | 56.6 ± 4.7* | 77.9 ± 5.1* |

Statistical difference from control at: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; Source: Reader, 1993

[a] After culling

The NOAEL for parental toxicity was 50 ppm (equal to 3.8 mg/kg bw per day) based on decreased body weight gain at 150 ppm (equal to 10.9 mg/kg bw per day).

The NOAEL for offspring toxicity was 150 ppm (equal to 10.9 mg/kg bw per day) based on reduced body weight gain at 450 ppm (equal to 34 mg/kg bw per day).

The NOAEL for reproductive toxicity was 50 ppm (equal to 4.5 mg/kg bw per day) based on the effects on gestation index, gestation length and dystocia at 150 ppm, equal to 13.7 mg/kg bw per day (Reader, 1993).

In a second multi-generation study, groups of 30 male and 30 female CrL:COBS CD (SD) rats were exposed to prochloraz (purity, 96.2%–97%, batch 17 (00097/DT)) in the diet at doses of 0, 37.5, 150 or 625 ppm. This was equal to mean achieved intakes of 0, 3.1, 13 and 57 mg/kg bw per day for F0 males, 0, 3.5, 14 and 58 mg/kg bw per day for F0 females; in the F1 generation it was equal to 0, 3.7, 16 and 70 mg/kg bw per day for males, 0, 4.5, 18 and 81 mg/kg bw per day for females. Each generation produced two litters. The parental animals were exposed from nine weeks before mating through to weaning of the second litter. After the weaning of the first litter the parental animals were then remated with different males and females, and their offspring were discarded when they were about three weeks of age, after macroscopic examination post mortem. Animals forming the second generation were mated about eight weeks after selection and were also remated with different male and female pairings. Animals were observed for clinical signs, food consumption, body weight change, pregnancy rate, mating performance and gestation period. At autopsy, selected organs from 10 male and 10 female F1a weanlings, F2a weanlings and F1a adults in each group were weighed, and tissues from the 10 male and 10 female F1a adults per group and from five male and five female F1a and F2a weanlings per group were examined histologically.

Males of the F0 generation receiving the highest dietary concentration showed a prolongation of aggressive behaviour after rehousing following both matings; this effect was not seen among F1 males. A slight prolongation was also seen in F0 males at 150 ppm (three weeks compared with two weeks for the controls), after the first mating only. Aggressive behaviour is a common finding during the first weeks of rehousing after mating. The clinical signs seen in F0 females at the highest dietary concentration in late gestation and/or the perinatal period were hunched posture, walking on tiptoes, piloerection and pallor. About one-third of the animals were affected in each mating, although not necessarily the same

individuals each time. These effects were noted less frequently in the F1 generation, the females being least affected at the second mating. Food intake before mating was not consistently related to dose but was generally lower than that of controls. Decreased mean body weight was noted in parental males and females of both generations at 625 ppm. At this dose there was evidence of extended gestation ( longer than 22 days) and parturition in some females, leading to a small number of deaths associated with dystocia in both generations, there were, however, no effects on pregnancy rates. The increased gestation length might have been related to the anti-androgenic properties of prochloraz that have been described in literature (see Developmental toxicity section below for more details). There were no obvious effects on male mating performance.

At 625 ppm the gestation index was decreased as six F0 females and three F1females lost their litters in the immediate perinatal period. One control female of the F1 generation lost her first as well as second litter. The mean litter size at birth was smaller in both generations at the highest dietary concentration due to increased pup mortality. The mean percentage of pup loss at birth was greater than the control value for both matings of the F0 generation and for the first mating of the F1 generation, however none of the differences from the control values was statistically significant. At 625 ppm, impaired growth of the offspring up until weaning was noted. The incidence of structural anomalies recorded at terminal macroscopic examination of the remaining F1a and F2a pups and all F1b and F2b offspring provided no indication of any adverse effect due to treatment. Increased mean liver weights compared to controls were recorded in weanling (by 14%) and adult F1a males (by 13%) at 625 ppm and in F2a weanling females at all three dietary concentrations (by 9%, 9% and 14%, respectively at the low, mid and high concentrations). At the highest concentration the mean thymus weight was lower than the control value in F1a weanlings of both sexes, and the mean brain weight of F1 female weanlings was also lower than the control value. None of the histopathological changes in the tissues examined was considered to be attributable to treatment.

The NOAEL for parental toxicity was 150 ppm (equal to 13 mg/kg bw per day) based on clinical signs and decreased body weight gain at 625 ppm (equal to 57 mg/kg bw per day).

The NOAEL for offspring toxicity was 150 ppm (equal to 13 mg/kg bw per day) based on reduced body weight gain, and effects on liver, thymus and brain weights at 625 ppm (equal to 57 mg/kg bw per day).

The NOAEL for reproductive toxicity was 150 ppm (equal to 16 mg/kg bw per day) based on the effects on gestation index, gestation length and dystocia at 150 ppm (equal to 14 mg/kg bw per day) (Cozens et al., 1982).

**(b) Developmental toxicity**

***Rat***

In a study conducted in compliance with the principles of GLP (QA audit of final report), groups of 20 mated Charles River CD rats were given technical grade prochloraz (purity 96.2%, batch 17) orally at doses of 6, 25 or 100 mg/kg bw per day on days 1–20 after mating, day 1 after mating being the day on which sperm were detected in a vaginal smear. A further 31 rats were given the vehicle (10% aqueous acacia solution) as controls. The doses used were determined in a preliminary study in which pregnant rats given the highest dose of 100 mg/kg bw per day showed increased salivation and liver enlargement. Body weight, food consumption and overt signs of toxicity were recorded throughout the study. The dams were killed on day 21 after mating and necropsied. Their reproductive organs were removed and examined, corpora lutea were counted, and the placentas weighed. Fetuses were weighed, dissected and examined; half were then decapitated and the heads subsequently sectioned and examined. The carcasses of all fetuses were processed and the skeletons stained and examined.

Maternal toxicity was seen at 100 mg/kg bw per day, manifested as increased salivation, reduced food consumption, lower body weight gain (by 12% compared with controls) and liver enlargement. At 25 mg/kg bw per day, increased salivation and a slightly higher relative liver weight (by 6%) were also observed. Dams gained marginally less body weight at both 6 mg/kg bw per day (suppressed by 6%) and 25 mg/kg bw per day (suppressed by 5%). There were no macroscopic or microscopic findings attributable to treatment. The number of corpora lutea was similar in all groups (Table 21). The highest dose appeared to be embryotoxic, as the litter size, implantation index and viability index were slightly

reduced and the incidence of dead fetuses was marginally increased. In addition, the mean fetal weight was lower and the degree of calcification of sternebrae and vertebral arches was retarded. The mean weight of placentas from the mid- and high-dose dams was significantly higher than that of controls, and the difference was dose-related.

***Table 21. Summary of litter parameters in developmental toxicity study in rats***

| | Dose level (mg/kg bw per day) | | | |
|---|---|---|---|---|
| **Parameter** | **0** | **6** | **25** | **100** |
| Number of litters | 27 | 18 | 18 | 19 |
| Living fetuses | 397 | 245 | 243 | 232 |
| Number of corpora lutea | 16.7 | 15.9 | 16.5 | 15.5 |
| Mean litter size at birth; alive | 14.7 | 13.6 | 13.5 | 12.2 |
| Mean litter size at birth; dead | 0.11 | 0.06 | 0.17 | 0.47 |
| Implantation index (%) | 93.8 | 88.5 | 89.6 | 84.9 |
| Viability index (%) | 94.1 | 96.5 | 91.4 | 87.2 |
| Mean placental weight, male pups (g) | 0.42 | 0.45 | 0.49* | 0.56*** |
| Mean placental weight, female pups (g) | 0.41 | 0.44 | 0.49** | 0.57*** |
| Mean fetal weight, males (g) | 3.57 | 3.48 | 3.55 | 3.24* |
| Mean fetal weight, females (g) | 3.34 | 3.27 | 3.34 | 3.13 |
| Number of dead fetuses | 3 | 1 | 3 | 9 |
| Sex ratio (M:F) | 1.13 | 0.88 | 0.74 | 1.00 |

Statistical difference from control at: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; Source: Beswick, 1980

There was no evidence of a teratogenic response.

The NOAEL for maternal toxicity was 6 mg/kg bw per day, based on clinical signs and mild effects on body weight gain, food consumption and liver weight at 25 mg/kg bw per day.

The NOAEL for developmental toxicity was 25 mg/kg bw per day, based on reductions in litter size, implantation index and viability index, an increased incidence of dead fetuses and retarded ossification at 100 mg/kg bw per day (Beswick, 1980).

In a developmental toxicity study, groups of 22 female Charles River CD rats received gavage doses of prochloraz (purity 97.5%, batch DCM-1) in corn oil at 0, 30, 60 or 120 mg/kg bw per day from gestation day (GD) 6 to GD 15. All groups were observed daily for mortality, clinical signs, food consumption, water consumption and body weight development. Animals were euthanized and caesarean section was performed on GD 20. Maternal (placenta weight and abnormalities, number of corpora lutea and implantations, early and late resorptions) and litter data (number of dead and live fetuses, individual weights of live fetuses, sex ratio) were recorded. All fetuses were examined for external malformations. Half of the number of fetuses in each litter were subjected to examination of skeletal malformations and the remaining half given visceral examinations.

With the exception of some slight effects on food and water intake, oral administration of prochloraz to pregnant rats had no adverse effects on maternal condition at dose levels up to 120 mg/kg bw per day.

The number of pregnant females at termination was 22, 22, 22, and 22 with mean numbers of live fetuses of 13.2, 14.3, 15.1, and 14.5 in the control, low-, mid- and high-dose groups respectively. No treatment-related effects were observed on the number of females becoming pregnant, the number of corpora lutea, implantation rates, embryonic resorptions or number of living fetuses per dam. Placental weight at 60 mg/kg bw per day was significantly increased, but not in the high-dose group and this was therefore regarded as not treatment-related.

A dose-related, significant reduction in mean fetal weights was observed in the mid- (by 4%) and high-dose (by 6%) groups. However, effects were only small and therefore not considered to be adverse.

At 30 mg/kg bw per day, fetal weight was slightly higher than that of the controls, reaching statistical significance only for male fetuses. However, this finding was not attributed to treatment as no similar finding was observed at the higher dose. No treatment-related effect on type and incidences of external, visceral and skeletal malformations was observed at any dose level.

The NOAEL for both maternal and fetal toxicity was 120 mg/kg bw per day, the highest dose tested (Reader, 1991).

### (c) Public literature studies on reproductive and developmental toxicity in rats

Additionally a number of literature references concerning the developmental and reproductive toxicity of prochloraz, were found to be available, as follows.

***Rat***

*Study 1*

In a peripostnatal developmental and reproductive toxicity study from public literature prochloraz (purity 98% ± 1%) was administered by gavage at doses of 0, 0.01, 5 and 30 mg/kg bw per day to groups of 20 pregnant and lactating Wistar rats from GD 6 to postnatal day (PND) 21. The study also investigated three subsets of offspring. Offspring subset 1 received no treatment and were killed with dams on PND 21. The other offspring received prochloraz by gavage from PND 21 until sexual maturity (subset 2) or young adulthood (subset 3). The animals were observed for mortality, clinical signs, body weight and food consumption. Other investigations consisted of sperm analysis, measurement of anogenital distance, nipple/areola retention at PND 12 and sexual maturation, hormone levels, morphology and histopathology of reproductive organs.

In dams at 30 mg/kg bw per day reduced feed consumption and body weight (gain), and increased gestation lengths were seen. At this dose there was a reduced number of live pups and increased number of stillborn pups and a statistically significantly decreased pup survival during PNDs 0–4, reduced litter weight from birth to weaning, as well as decreased live birth and viability indices. Prochloraz caused a small decrease in individual offspring body weight during lactation at the top dose. High-dose female pups showed increased anogenital distances (PND 1) and anogenital index. No other developmental or reproductive effects were seen in females and the onset of female puberty in these animals was not affected. At the mid and high dose (5 and 30 mg/kg bw per day) male pups revealed transient nipple/areola, which was present on PND 12 but absent on PND 20. Delayed preputial separation on PND 1 was observed in males at 30 mg/kg bw per day, which may be due to a combination of both general toxicity (impaired body weight development) and specific endocrine-mediated effects.

In addition to the foregoing effects, male offspring at 30 mg/kg bw per day displayed reduced absolute weights of reproductive organs (cauda epididymis and elevator ani and bulbocavernosus muscles), but no statistically significant decreases in corresponding relative organ weights were observed, nor were histopathological correlates identified. Increased testosterone and androstenedione levels, as well as decreased estradiol levels, were observed in females (dams after weaning and adult female offspring on PND 83) in the mid- and high-dose groups (5 and 30 mg/kg bw per day).

Sperm analysis revealed a small but statistically significant reduction in sperm motility at 30 mg/kg bw per day, which fell within the historical control range and was thus not considered an anti-androgen effect at this dose. Furthermore, no effects were seen on other sperm parameters such as sperm numbers, numbers of motile sperm cells or sperm velocities. Serum hormone levels were not altered at the dose levels tested (Melching-Kollmuss et al., 2016).

*Study 2*

Toxic effects on reproduction were investigated after exposure during gestation and lactation to prochloraz (purity 99.4%) at 30 mg/kg bw per day. During the study, pregnant Wistar dams were dosed from GD 7 until PND 16. Some dams were taken for caesarean section at GD 21, others were allowed to give birth. Hormone levels were measured in fetuses at GD 21 and in fetuses during the lactation period, as well as after weaning. Malformation, sexual maturation, neurodevelopment and organ weights were also examined. Results showed that prochloraz significantly reduced plasma (by about 80%) and testicular testosterone levels (by about 60%) in GD 21 male fetuses, whereas testicular progesterone was increased (by about 600%). Gestational duration was increased by half a day by prochloraz. Chemical

analysis of the rat breast milk showed that prochloraz was transferred to the milk. In males a significant increase in nipple retention was found, and the bulbourethral gland weight was decreased, whereas other reproductive organs were unaffected. In addition cytochrome P450 (CYP)1A activity in livers was induced by prochloraz, possibly as a result of aryl hydrocarbon (Ah) receptor activation. Behavioural studies showed that the activity level and sweet preference of adult males were significantly increased by prochloraz. Overall these results strongly indicate that prochloraz feminizes the male offspring after perinatal exposure, and that these effects are due, at least in part, to diminished fetal steroidogenesis (Vinggaard et al., 2005).

*Study 3*
Wistar rats were dosed perinatally with prochloraz (purity 99.3% for in vivo studies; 99.4% for in vitro studies) at 0, 50 and 150 mg/kg bw per day from GD 7 to PND 16. Caesarean sections were performed on selected dams at GD 21, while others were allowed to give birth to pups that were followed until PND 16. Prochloraz caused mild dysgenesis of the male external genitalia as well as reduced anogenital distance and retention of nipples in male pups at both dose levels. An increased anogenital distance indicated virilization of female pups. Effects on steroidogenesis in male fetuses became evident as decreases in testicular and plasma levels of testosterone and increased levels of progesterone. Decreased seminal vesicles and bulbourethral gland weight was observed at both dose levels. Ex vivo synthesis of both steroid hormones was qualitatively affected in a similar way by prochloraz. Immunohistochemistry of fetal testes showed increased expression of 17α-hydroxylase/17,20-lyase (P450c17) and a reduction in 17β-hydroxysteroid dehydrogenase (type 10) expression, whereas no changes in expression of genes involved in testicular steroidogenesis were observed. Increased expression of P450c17 mRNA was observed in fetal male adrenals, and that of the androgen-regulated genes ornithine decarboxylase, prostatic binding protein C3 as well as insulin-like growth factor I mRNA was reduced in ventral prostates at PND 16. These results indicate that reduced activity of P450c17 may be a primary cause of the disrupted fetal steroidogenesis and that an altered androgen metabolism may play a role as well. In vitro studies on human adrenocortical carcinoma cells supported the findings in vivo, as reduced testosterone and increased progesterone levels were observed. Overall, these results together indicate that prochloraz acts directly on the fetal testis to inhibit steroidogenesis and that this effect is exhibited at a protein, and not at a genomic, level (Laier et al., 2006).

*Study 4*
The purpose of this study was to expand in vitro observations and to determine whether prochloraz (purity 99.4%) affected sexual differentiation. Prochloraz effects on androgen receptor-mediated gene expression were tested using a cell line (MDA-kkb2) containing endogenous androgen receptor and stably transfected with an MMTV-luc reporter. In vitro, prochloraz concentrations greater than 1 µM caused a dose-dependent inhibition of dihydrotestosterone-induced gene expression. In this respect prochloraz was about 10-fold less potent than the positive anti-androgen control, hydroxyflutamide. Prochloraz also inhibited R1881 binding to the rat androgen receptor with a half-maximal inhibitory concentration ($IC_{50}$) of around 60 µM. In vivo, pregnant rats received prochloraz by gavage from GD 14 to GD 18 at doses of 0, 31.25, 62.5, 125, and 250 mg/kg bw per day. Prochloraz delayed delivery in a dose-dependent manner and resulted in pup mortalities at the two highest doses. In male offspring, anogenital distance and body weight were slightly reduced at three days of age. Additionally, female-like areolas were observed at 13 days of age at frequencies of 31%, 43%, 41%, and 71% in the control, low-, mid- and high-dose groups, respectively. Weights of androgen-dependent tissues, including the testes, levator ani/bulbocavernosus muscle group, epididymides and seminal vesicle, showed dose-dependent reductions. Hypospadias and vaginal pouches were noted in all males treated with 250 mg/kg bw per day, whereas those defects were observed in 12.5% and 6.25%, respectively, of males treated with 125 mg/kg bw per day. Treatment did not affect age of preputial separation in animals without penile malformations. Despite severe malformations in males, no malformations were noted in females. Together, these results indicate that prochloraz alters sexual differentiation in an anti-androgenic manner (Noriega et al., 2005).

*Study 5*
The effect of prochloraz on cognitive behaviour was studied following exposure during the perinatal period. Pregnant Wistar rats were administered a 200 mg/kg bw dose of prochloraz (purity 99.5%) on

GD 7, GD 11 and GD 15. The social recognition test (SRT) was performed on seven-week-old male rat offspring. Findings revealed an increase in gestation length and a significantly reduced pup weight on PND 15 and PND 40 but no effect of prenatal prochloraz exposure on social investigation or acquisition of social-olfactory memory (Dmytriyeva et al., 2013).

*Study 6*

This study was composed of two assays, namely a male pubertal assay and a Hershberger assay. In the male pubertal assay, Sprague Dawley rats were dosed by gavage with 0, 31.3, 62.5 or 125 mg/kg bw per day of prochloraz (purity 99.5%) from PND 23 to PND 42 or 51. There was a significant delay in preputial separation at 125 mg/kg bw per day and several of the androgen-dependent organ weights were decreased significantly, but the significant organ weight effects were not consistent between the necropsy on PND 42 and the one on PND 51. At both ages, serum testosterone levels and ex vivo testosterone release from the testis were significantly decreased, whereas serum progesterone and 17α-hydroxyprogesterone levels were significantly increased at dose levels below those that affected preputial separation or reproductive organ weights. The hormone results suggested that prochloraz was inhibiting CYP17 activity. In a second pubertal study (0, 3.9, 7.8, 15.6, 31.3 or 62.5 mg/kg bw per day), serum testosterone levels and ex vivo testosterone production were significantly reduced at 15.6 mg/kg bw per day. However, neither reproductive organs were affected nor did a delay in preputial separation occur. A clear NOAEL for the most susceptible parameter of reduced serum and testis testosterone was proposed at 7.8 mg/kg bw per day.

In order to examine antagonistic effects of prochloraz on the androgen receptor, independent of its effects on testosterone synthesis, castrated, immature male rats were dosed daily by gavage with 0, 15.6, 31.3, 62.5 or 125 mg/kg bw per day prochloraz for 10–11 days (Hershberger assay). Each rat received 100 µg testosterone propionate by subcutaneous injection immediately after the gavage dose. This assay revealed only a very mild anti-androgenic effect. The most susceptible parameter was the increase in liver weight. The absolute weights of some reproductive organs were reduced at the top dose only, a dose that reduced serum luteinizing hormone (LH) levels. Serum testosterone was not affected at any dose level. These data from the pubertal assays demonstrate that prochloraz decreases testosterone levels and delays rat pubertal development. However, the fact that hormone levels were affected at a dosage eight-fold lower than that which delayed the onset of puberty suggests that rather large reductions in serum testosterone may be required to delay puberty and consistently reduce androgen-dependent tissue weights. (Blystone et al., 2007a).

*Study 7*

The sixth study included a Hershberger assay and an androgen receptor reporter (ARR) gene assay. In the Hershberger assay, male Wistar rats were castrated at the age of four weeks. Group 1 received the vehicle, peanut oil. The animals in the remaining eight groups were all dosed with 0.5 mg/kg bw per day testosterone propionate subcutaneously, with or without one of the test compounds. Group 2 animals were only administered testosterone propionate and this formed the control group with which all other groups were compared. Group 3 received positive control flutamide. Prochloraz (purity 99.4%) was administered orally to Group 6 at a dose of 25 mg/kg bw per day. A dose of 20 mg/kg bw per day of the known anti-androgen flutamide provided the positive control. Testosterone propionate was given 2–10 minutes after the test compounds. Blood samples were taken for hormone analysis. The ventral prostate, seminal vesicles/coagulation gland, levator ani/bulbocavernosus muscle, bulbourethral glands, liver, thyroid gland, paired adrenal glands, and paired kidneys were dissected and weighed. The ventral prostates were stored in 0.5 mL RNA*later*™ solution (a proprietary product to stabilize cellular RNA) at −20°C for gene expression analyses. In the ARR gene assay, prochloraz was tested using transfected Chinese hamster ovary (CHO) cells in the absence or presence of 0.1 nM R1881 (a known androgen receptor agonist) at concentrations of 0.025–50 µM. Incubation was performed for 20 hours. Afterwards cells were lysed and luciferin oxidation was measured by chemiluminescence.

In the Hershberger assay, organ weights and hormone levels in castrated rats treated with prochloraz and cotreated with testosterone propionate were unaffected except for an increase in liver weights. It was noted that the dose levels used in this study were lower than those used in the study by Vinggaard et al. (2002) in which effects on organ weights were observed. The positive control flutamide induced reduced weights of ventral prostate, seminal vesicles/coagulation gland, levator

ani/bulbocavernosus muscle, and bulbourethral glands. On the level of gene expression, reduction of the androgen-responsive genes ornithine decarboxylase (ODC), prostate binding protein subunit C3 (PBP C3) and testosterone-repressed prostatic message 2 (TRPM-2) were observed in the ventral prostate of prochloraz-treated animals. In vitro, prochloraz showed anti-androgenic effects in an ARR gene assay in transfected CHO cells, but had no effects on the gene expression of androgen receptor responsive genes, whereas flutamide affected both parameters (Birkhøj et al., 2004).

*Study 8*

This study tested prochloraz (purity 99.4%) for anti-androgenic effects in vitro and in vivo. The study consisted of Hershberger assays and an ARR gene assay. In the Hershberger assays groups of six male Wistar rats received prochloraz orally for seven days at doses of 0, 50, 100, 200 or 250 mg/kg bw per day. Rats were treated with testosterone propionate with or without flutamide. All the animals from each group underwent a thorough autopsy. The testis (for intact animals), both lobes of the ventral prostate, combined seminal vesicles and coagulating glands including fluids, elevator ani/bulbocavernosus muscles, paired bulbourethral glands, pituitary, liver, and paired kidneys were dissected and weighed. The ventral prostates were subject to gene expression analysis. Blood serum determinations were made and LH, follicle-stimulating hormone (FSH), thyroxine (T4), thyroid-stimulating hormone (TSH) and testosterone concentrations were determined. In the ARR gene assay prochloraz was tested using transfected CHO-K1 cells in the absence or presence of 0.01 nM R1881 (an androgen receptor agonist) at concentrations of 0.025–50 µM. Incubation was performed for 20 hours. Afterwards cells were lysed and luciferin oxidation was measured by chemiluminescence.

Under the conditions of the study, prochloraz (but not the metabolites 2,4,6-trichlorophenoxyacetic acid or 2,4,6-trichlorophenol) inhibited the R1881-induced response in an ARR gene assay. In the Hershberger assay, prochloraz exposure at all dose levels (50, 100, and 200 mg/kg bw per day) given orally to castrated testosterone-treated males markedly reduced weights of the ventral prostate, seminal vesicles, levator ani/bulbocavernosus muscles, and bulbourethral gland. These effects were accompanied by an increase in LH and a reduction in the T4 and TSH levels. The effects on seminal vesicles, LH, T4 and TSH were also evident in intact prochloraz-exposed young adult rats. Body weights were unaffected, whereas liver weights were increased in prochloraz-treated animals. Changes in androgen-regulated gene expression were determined in ventral prostates by real-time polymerase chain reaction (RT-PCR). A pronounced decrease in ornithine decarboxylase and PBP C3 mRNA levels was observed for both prochloraz and flutamide. These results indicate that prochloraz antagonises the peripheral androgen receptors resulting in decreased growth of androgen-dependent tissues and that it antagonises central androgen receptors blocking the negative feedback mechanism of testosterone, resulting in increased LH secretion from the pituitary. The anti-androgenic effects of prochloraz were in many ways qualitatively comparable, although weaker, to the effects of flutamide. However, differential effects on levels of FSH, T4, and TSH indicated that other modes of action apart from the pure androgen receptor antagonism might play a role in vivo (Vinggaard et al., 2002).

*Study 9*

In another study the sensitivity of fetal testicular steroidogenesis to prochloraz was investigated to test the hypothesis that the reported morphological effects from maternal exposure were associated with reduced testosterone synthesis. During the study, groups of eight pregnant Sprague Dawley rats were dosed by gavage with prochloraz (purity 99.5%) at doses of 0, 7.8, 15.6, 31.3, 62.5 or 125 mg/kg bw per day from GD 14 to GD 18. On GD 18, the effects of prochloraz on fetal steroidogenesis were assessed by measuring hormone production from ex vivo fetal testes after a three-hour incubation. Lastly, prochloraz levels in amniotic fluid and maternal serum were measured using liquid chromatography/mass spectroscopy and correlated to the inhibition of steroidogenesis. Fetal progesterone and 17α-hydroxyprogesterone production levels were increased significantly at every prochloraz dose, whereas testosterone levels were significantly decreased only at the two high doses. These results suggest that prochloraz inhibits the conversion of progesterone to testosterone through the inhibition of CYP17. To test this hypothesis an evaluation was made of the effects of prochloraz on CYP17 gene expression and in vitro CYP17 hydroxylase activity. Prochloraz had no effect on testicular CYP17 mRNA levels as measured by quantitative RT-PCR. However, microsomal CYP17 hydroxylase activity was significantly inhibited by the fungicide ($K_i$ = 865 nM). Prochloraz concentrations in amniotic fluid ranged from 78 to 1512 ppb (207–4014 nM) and testosterone production was reduced when prochloraz reached around

500 ppb, which compares favourably with the determined CYP17 hydroxylase $K_i$ (326 ppb). These results demonstrated that prochloraz lowers testicular testosterone synthesis by inhibiting CYP17 activity which likely contributes to the induced malformations in androgen-dependent tissues of male offspring (Blystone et al., 2007b).

***Rabbit***

*Study 1*

In a dose range-finding study of embryotoxicity and teratogenicity, groups of five, mated female chinchilla rabbits were given technical grade prochloraz (purity 95.2%) by gavage on days 6–18 after mating at doses of 0, 25, 50, 100 or 200 mg/kg bw per day. The vehicle alone, distilled water with 8% carboxymethyl cellulose, was given to the controls. On day 28 after mating, all females were killed by cervical dislocation, and their fetuses removed surgically.

No effects on clinical signs or mortality were observed. Does receiving 200 mg/kg bw per day lost weight on days 7–13 after mating (days 2–7 of treatment). Thereafter, slight body-weight gain was noted but the rate was lower than that in the control group. The mean food consumption of does in this group during treatment was distinctly reduced, and this was confirmed by the compensatory increase in food consumption after treatment. Mean liver weights (absolute and relative) compared to controls were dose-dependently increased (by 5.5%, 10%, 22% and 36% in absolute terms with increasing dose, and by 8.1%, 17%, 24% and 42%, respectively in relative terms). No treatment-related pathological changes were noted in the liver. The small number of females per group, wide variations within and between the groups and the insufficient number of pregnant does (two) at the highest dose prevented a definitive conclusion, but no dose-related differences were seen in reproductive parameters. No malformed or anomalous fetuses were found in any group. Reduced fetal weight was observed at 100 and 200 mg/kg bw per day, but a definitive conclusion cannot be drawn due to the low number of animals and the observed variation (Becker, Muller & Vogel, 1989a).

*Study 2*

In another dose range-finding study of embryotoxicity and teratogenicity, groups of six mated female chinchilla rabbits were given technical grade prochloraz (purity 95.2%) by gavage on days 6–18 after mating at doses of 0, 200 or 250 mg/kg bw per day. The vehicle alone, distilled water with 1% carboxymethyl cellulose (CMC), was given to controls. On day 28 after mating all females were killed by cervical dislocation, and their fetuses removed surgically.

Four of the six does at 250 mg/kg bw per day died between days 10 and 13 after mating. All four lost weight from the start of dosing. Somnolence, ataxia and dyspnoea were observed in one doe, and sedation, catalepsy and dyspnoea were seen in a second before death. At necropsy, macroscopic changes were observed in the livers of two of the four does that died at the highest dose. The liver to body weight ratios varied from 4.4% to 7.0% (control mean, 2.2%), and histological examination of the livers showed moderate to severe increases in hepatocellular lipid in three of four animals and severe fatty changes with minimal to moderate multifocal necrosis in two of four animals. None of the does at 250 mg/kg bw per day had viable young at termination of the study. Of the two surviving does, one did not become pregnant, and the second lost its litter. Of the does at 200 mg/kg bw per day, three had viable young, two aborted and one did not become pregnant. The overall pregnancy rates (including animals that died) were 100% in controls, 83% at 200 mg/kg bw per day and 33% at 250 mg/kg bw per day. Animals at 200 mg/kg bw per day showed a mean weight loss during the first five days of dosing; thereafter, some recovered and there was a slight compensatory increase in weight gain after treatment, by comparison with concurrent controls. At this dose, food and water consumption was reduced throughout the dosing period. No macroscopic changes were observed in any doe surviving to termination on day 28 after mating. The mean liver weights for does surviving to termination were increased by 37% and 34% at 200 and 250 mg/kg bw per day, respectively, in comparison with concurrent controls, and the mean relative liver weights were increased by 53% and 46% at the two doses, respectively. No treatment-related histological changes were observed in the livers of does surviving to termination. In the three does at 200 mg/kg bw per day with viable young, there was no evidence of an adverse effect of treatment on reproductive or fetal parameters.

These results indicate a marked maternally toxic effect, which, in some animals, either

prevented implantation or caused early post-implantation loss, so that at termination on day 28 after mating there was no evidence of implantation and the does were categorized as not pregnant. In other animals, pregnancy was maintained slightly longer, but, because of the stress to the maternal system, total resorption or abortion occurred. In those does that maintained pregnancy to term, there did not appear to be an obvious adverse effect on the fetuses. On the basis of the results of these two studies, a dose less than 200 mg/kg bw per day was considered an appropriate highest dose for the main study (Becker, Muller & Vogel, 1989b).

*Study 3*

In a study conducted in compliance with the principles of GLP (QA certification), groups of 16 mated female chinchilla rabbits were given technical grade prochloraz (purity 95.2%) by gavage at doses of 10, 40 or 160 mg/kg bw per day on days 6–18 after mating. A group of 17 mated rabbits receiving only the vehicle, 1% CMC in distilled water, served as controls. The does were killed on day 28 after mating and their fetuses removed surgically. Both does and fetuses were examined.

No effect on clinical signs nor mortality was observed. Does at 160 mg/kg bw per day showed significantly reduced food consumption during the dosing period and reduced water consumption during the first five days. Weight gain was retarded during the first five days of dosing (32 g versus 84 g for controls), but the differences from control values did not attain statistical significance. Overall body weight gain during gestation was reduced by 12%. The liver weights were significantly increased in comparison with controls, by 19% (absolute) and 22% (relative). There was a slightly increased incidence of non-pregnant animals and animals with total litter loss; these effects were considered treatment-related in view of the results obtained at higher doses in the dose range-finding studies. Does with viable young at termination had a significantly increased incidence of fetal resorption. None of the other reproductive parameters and none of the developmental parameters appeared to be adversely affected by treatment.

The NOAEL for maternal toxicity was 40 mg/kg bw per day, based on reduced body weight gain, increased liver weights, increased incidences of non-pregnant animals and animals with total litter loss at 160 mg/kg bw per day.

The NOAEL for developmental toxicity was 40 mg/kg bw per day, based on the increased incidence of fetal resorptions at 160 mg/kg bw per day (Becker et al., 1988).

*Study 4*

In a second main developmental study in rabbits, prochloraz (purity 95.4%) was administered orally, daily, via gavage, to groups of 16 mated female New Zealand White rabbits from GD 7 to GD 19 at dosages of 0, 10, 40 or 160 mg/kg bw per day. All females were examined for clinical signs, body weight and food consumption. Does were sacrificed on GD 29 and uterine contents examined. The number of corpora lutea, gravid uterus weight, number of live and dead fetuses and resorptions were recorded. Fetal weight and crown–rump length measured. Skeletal and visceral examinations of the fetuses were conducted.

No preterminal deaths occurred during the study and no treatment-related effects on clinical symptoms were observed. Food intake was significantly reduced in the high-dose group throughout the dosing period (days 8–20) in comparison to controls. No treatment-related effects on body weight were observed. Occult faecal blood was detected in a single high-dose female. Similar effects were observed in the range-finding part of the study at doses of 200 mg/kg bw per day and higher.

The number of pregnant females at termination was 15, 14, 15 and 13, with mean live fetus counts of 9.3, 9.0, 8.9 and 8.8 in the control, low-, mid- and high-dose groups respectively. The average number of corpora lutea, implantation losses, early and late resorptions were comparable among groups. No significant differences were seen in mean gravid uterus weight or placental weight compared to controls.

Crown–rump length was slightly, but significantly, lower in high-dose animals than the controls (91.7 mm compared with 95.0 mm for controls). Fetal weight compared to controls was slightly decreased (by 5%) in the high-dose group. However, this difference did not reach statistical significance. Fetal viability was similar to controls in all treated groups. No treatment-related effect on type and incidences

of external, visceral and skeletal malformations was observed at any dose level. In the low- and mid-dose groups the proportion of fetuses with reduced or incomplete ossification of the epiphyses was significantly lower than for controls.

The NOAEL for maternal toxicity was 40 mg/kg bw per day, based on decreased food consumption and a single case of occult faecal blood observed at 160 mg/kg bw per day.

The NOAEL for developmental toxicity was 40 mg/kg bw per day, based on a slight reduction in fetal crown–rump length at 160 mg/kg bw per day (Gal et al., 1990).

*Study 5*

In a developmental toxicity study that was performed before the publication of OECD TG 414, groups of 15 female New Zealand White rabbits were given prochloraz (purity 96.2%–97% according to data from other studies, batch 17) at doses of 0, 3, 12 or 48 mg/kg bw per day by gavage on GDs 1–28. All females were examined daily for clinical signs and body weight. Food consumption was measured every three days. Does were terminated on GD 29 and uterine contents examined. The number of corpora lutea, number of live and dead fetuses and fetal weight were recorded. Skeletal and visceral examinations of the fetuses were conducted.

Maternal toxicity was observed at the highest dose, as evidenced by a significant increase in liver weight, with liver discolouration. No evidence of embryotoxicity, fetotoxicity or teratogenicity was found.

The maternal NOAEL was 12 mg/kg bw per day based on increased liver weight and liver discolouration at 48 mg/kg bw per day.

The NOAEL for developmental toxicity was 48 mg/kg bw per day, the highest dose tested (Palmer Bottomley & Billington, 1980).

## 2.6 Special studies

### (a) Neurotoxicity

Groups of five male and five female Boots Wistar rats were given prochloraz (purity, 98.2%, batch 7 part 2) as a single gavage dose of 100 mg/kg bw, and control groups received the vehicle, 10% aqueous acacia solution. A blood sample was obtained from the tail vein of each rat before dosing, and a further sample was collected by heart puncture immediately after the rats were killed 0.25, 1.5 or 6 hours after dosing. The samples were analyzed for erythrocyte and plasma cholinesterase (ChE) activity. Prochloraz did not affect ChE activity in either sex at any interval after treatment (Smithson, 1979).

A single oral dose of 20 mg/kg bw of prochloraz (purity, 98.2%, batch 7 part 2) was given by gavage to three groups of one male and one female beagle dogs; a similar group was given an equivalent volume of the vehicle (10% aqueous acacia solution) and served as controls. A blood sample was taken from each dog immediately before and at 0.75, 1.5, 3, 6 and 24 hours after dosing and analyzed for erythrocyte and plasma Che activity. There was no consistent difference in ChE activity between treated and control animals (Morgan & Stobart, 1979).

In an acute neurotoxicity study, groups of 10 male and 10 female 4–5-week-old Crl:CD (SD) rats received a single gavage dose of 0, 20, 90 or 405 mg/kg bw of prochloraz (purity: 97.5%, batch 311-39) dissolved in corn oil. Animals were observed for 14 days following treatment. Animals were checked daily for clinical signs. Body weight and food consumption were measured pretreatment and on days 1, 8 and 15. A functional observational battery (FOB; clinical condition, observations in the home cage, in-the-hand, standard arena, manipulation observations, motor activity, body temperature) was performed pretreatment, on day 1 (six hours after dosing) and on days 8 and 15. At termination brains were weighed and anatomical measurements, macroscopy and neurohistopathological investigations were undertaken.

No treatment-related effects on mortality, body weight or food consumption were observed. At 90 and 405 mg/kg bw, treatment-related neurobehavioural effects were confined to the day of administration, particularly at six hours after dosing when the plasma levels of prochloraz might be expected to peak. There were no treatment-related findings from the home cage investigation. In-the-hand observations

about six hours after dosing at 405 mg/kg bw revealed piloerection in the majority of males and females, brown perianal staining in three females, reduced or slightly reduced body tone in six males and seven females and coldness to touch in two males and five females.

Testing in the standard arena at the time of peak effect revealed hunched posture in the majority of animals given 405 mg/kg bw and in some animals at 90 mg/kg bw. Abnormal gait (mainly elevated, slow and/or unsteady) was displayed by many animals given 405 mg/kg bw and some females at 90 mg/kg bw. There was a low level of arousal in seven females given 405 mg/kg bw and a reduction in motor activity and rearing counts in females at 90 and 405 mg/kg bw. No findings on days 8 or 15 were attributed to treatment.

At about six hours after dosing, body temperature was markedly reduced (2.1–3°C) in males and females at 405 mg/kg bw, and moderately reduced (1.1°C) in females at 90 mg/kg bw. Approximately half of the males and females given 405 mg/kg bw showed a slow righting reflex and the majority of females given 405 mg/kg, and half of the females given 90 mg/kg bw, failed to show an approach response. Seven females given 405 mg/kg failed to show a touch response and three had a weak tail pinch response. No findings on days 8 or 15 of the observation period were attributed to treatment.

Assessments of brain weight, anatomical measurements, macropathology and neurohistopathological investigations revealed no effects due to treatment.

The NOAEL for general toxicity was 20 mg/kg bw, based on clinical signs, effects on motor activity and rearing counts, righting reflex and approach response and on reduced body temperature, observed at 90 mg/kg bw.

The NOAEL for acute neurotoxicity was 405 mg/kg bw, the highest dose tested (Rees, 2009).

**(b) Immunotoxicity**

No data were available.

**(c) Studies with metabolites and impurities**

***Acute toxicity of metabolites***

Three male and three female rats were given [*phenyl*-$^{14}$C-]*N*-formyl-*N'*-propyl-*N'*-2-(2,4,6-trichlorophenoxy)ethylurea, also known as metabolite M2 or BTS 44596. This is a major plant metabolite of prochloraz. A single oral dose of 89 mg/kg bw was administered and the excretion of radiolabel followed for 96 hours, after which the rats were killed and dissected. The study was conducted before GLP regulations were issued in the testing facility, but the protocol and results were well reported.

Excretion of radiolabelled material was virtually complete within 72 hours. Urinary excretion accounted for 53%–63% of the total dose, the remainder being excreted in faeces. The concentration of the compound appeared to decline rapidly in the tissues, the highest concentrations being detected in liver (2.6–3.4 µg/g tissue) and kidney (1.3–5.3 µg/g tissue) and the lowest in muscle and brain (0.05–0.2 µg/g tissue). The remaining tissues revealed concentrations less than 1 µg/g tissue, except for the skin. There were no apparent sex differences (Needham, 1981).

Groups of five male Boots Wistar rats were given metabolite M1 (BTS 44595) or M2 (BTS 44596), both major plant metabolites, as a single oral dose of 3200 mg/kg bw or prochloraz at 1600 mg/kg bw. After dosing with prochloraz or M1, the overt signs of toxicity were similar and were seen within one hour. Rats in both groups showed signs of central nervous system depression, adopted a hunched posture, had slowed respiration, piloerection and diarrhoea, were cool and limp to touch, tremorous, ataxic and had increased nasal exudate and salivation; in addition, rats given prochloraz had increased lachrymation. Animals had generally recovered within seven days of dosing with M1 and within nine days of dosing with prochloraz. Four rats given prochloraz died within two days, and one rat given M1 died on the day after dosing. At autopsy, they were found to have GI irritation. After dosing with M2, one rat was inactive, adopted a hunched posture and had a wasted appearance, piloerection, staining around the eyes, a nasal exudate and urine staining around the genital region; these signs were seen 2–8 days after dosing. None of the rats died after treatment with M2.

The median lethal dose ($LD_{50}$) for prochloraz was below 1600 mg/kg bw while the $LD_{50}$ for metabolites M1 and M2 were greater than 3200 mg/kg bw (Carter & Smithson, 1979).

Groups of four or five male rats were given 2,4,6-trichlorophenol (M3, BTS 45186) as a single oral dose of 3200 mg/kg bw. Ten controls received the vehicle. After treatment, a clear nasal exudate was observed in three rats, and diarrhoea and increased salivation were each seen in one animal; these signs occurred on the day of dosing. The rats had recovered by the following day, except for one rat that had a wasted appearance on day 6. No mortality occurred and therefore the $LD_{50}$ was greater than 3200 mg/kg bw (Carter & Smithson, 1979).

Groups of five male rats were given 2-(2,4,6-trichlorophenoxy)ethanol (BTS 3037) as a single oral dose of 800, 1600 or 3200 mg/kg bw, and a group of controls received the vehicle. Overt signs of toxicity were seen within 30 minutes of dosing. Rats at all doses showed evidence of central nervous system depression, were tremorous and displayed piloerection, increased nasal exudate and urine staining around the genital region. In addition, some rats at 1600 or 3200 mg/kg bw were ataxic, convulsive and cool to touch, and some given 1600 mg/kg bw showed increased salivation and closed eyes. All rats given 3200 mg/kg bw and four given 1600 mg/kg bw died within four days of dosing, and one control lost condition and died 13 days after dosing. The survivors in the treated groups generally recovered within seven days, although some remained in poor condition throughout the study. Autopsy of the decedents revealed macroscopic and microscopic evidence of GI irritation. None of the autopsy findings were statistically significant. The $LD_{50}$ value was found to be between 800 and 1600 mg/kg bw (Carter, Lancaster & Smithson, 1979a).

In an acute oral toxicity study, prochloraz (purity 98.6%) and its metabolite BTS 19036 (imidazole; purity 99.6%), were administered to groups of five male Boot Wistar rats, as a single oral gavage dose in a 10% aqueous acacia suspension at dose levels of 200, 400, 800, 1600 or 3200 mg/kg bw and a dosing volume of 10 mL/kg bw. A group of 15 rats served as the control and received only the vehicle. After dosage, the animals were observed continually for six hours and twice a day thereafter until the rats died or were killed on day 15. At termination animals were killed and examined macroscopically, except for those that had received 200 or 400 mg/kg bw of BTS 19036 or 200 mg/kg bw prochloraz.

Several deaths were observed within 48 hours of dosing, and included 1, 1 and 5 rats exposed to 800, 1600 and 3200 mg/kg bw prochloraz respectively, and 3 and 5 rats exposed to 1600 and 3200 mg/kg bw of BTS 19036 respectively. At or above a dose of 800 mg/kg bw prochloraz and BTS 19036 induced similar signs of toxicity including CNS depression, slow or rapid respiration rate, diarrhoea, coolness to touch, tremor, ataxia, increased lacrimation, excitability, convulsions and tremor, within half an hour. In addition, prochloraz induced a hunched posture, increased salivation, red tears, urine stains on the abdomens, limpness to touch and ataxia. Rats treated with BTS 19036 returned to normal within two days. Rats treated with 400 or 800 mg/kg bw of prochloraz returned to normal within four days, or at 1600 mg/kg bw of prochloraz within eight days. In rats treated with 200 mg/kg bw of prochloraz, or 200 or 400 mg/kg bw of BTS 19036 no clinical signs of toxicity were observed. In rats treated with prochloraz, GI irritation was observed during autopsy. The acute oral $LD_{50}$ for metabolite BTS 19036 was between 800 and 1600 mg/kg bw, and the $LD_{50}$ for prochloraz was between 1600 and 3200 mg/kg bw (Shaw et al., 1979b).

Pairs of male and female dogs received metabolite M1 (BTS 44595) as single oral doses (in gelatin capsules) of 50 mg/kg bw (on day 1) and 150 mg/kg bw (on day 36). Similar groups were administered metabolite M2 (BTS 44596) at 500 mg/kg bw (on day 1) and 1000 mg/kg bw (on day 36). Similarly, imidazole (BTS 190 36) was administered to further groups at 50 mg/kg bw (on day 1), 50 mg/kg bw (on day 36) and 10 mg/kg bw (on day 50). The control group (one male and one female) received empty gelatin capsules. No effect was seen in dogs given M1 at 50 mg/kg bw or in males at 150 mg/kg bw, but the female at the latter dose appeared subdued and had slightly increased serum ALP activity the following day. Metabolite M2 at 500 mg/kg elicited neutrophilia and increased serum ALP activity in the male, and M2 at 1000 mg/kg bw caused marginal increases in serum ALP activity and plasma glucose concentration in the female. The female treated with imidazole at 50 mg/kg bw on day 1 vomited and retched within 1.5 hours of dosing, but no toxic effect was seen in the male. On day 36 at the same dose, the male retched but no effect was seen in the female. On day 50, no toxic effect was elicited in either animal after 10 mg/kg bw was administered (Watson, Lancaster & Robinson, 1980a).

Pairs of male and female dogs were given a single oral dose of 250 mg/kg bw of prochloraz, M1 (BTS 44595), M2 (BTS 44596), imidazole (BTS 19 036) or 2,4,6-trichlorophenol (BTS 45186) on day 1 of the study, while two males and two females received empty gelatin capsules and served as controls. On day 1, emesis and diarrhoea were observed in the pairs given prochloraz or M1, and both dogs given imidazole showed emesis, increased salivation and appeared subdued. On day 2, the male given prochloraz had lost weight, and this dog and the female given M1 had slightly increased serum ALP activity; these parameters returned to normal by day 5. No effects from treatment with M2 was detected.

The toxic signs elicited by imidazole appeared to be different from those induced by prochloraz and were further investigated. The pairs given prochloraz or imidazole were given a second oral dose of the same test article on day 29. The female given prochloraz and both dogs given imidazole salivated during administration of the dose and subsequently vomited. In addition, the female given imidazole had excessive diarrhoea, increased salivation and appeared subdued. On day 30, the dogs given imidazole had increased salivation, and the female had a very slightly increased BUN concentration and increased serum ALP activity. These biochemical parameters were normal on day 36, but the female again showed increased salivation when examined clinically.

In order to investigate the different responses to doses of imidazole, one of the pairs of control dogs was given a single oral dose of imidazole at 250 mg/kg bw on day 43, and the other pair received empty gelatin capsules. Emesis and increased salivation were observed on day 43 in the naïve pair given imidazole, but no other effect of treatment was detected. These signs appear to be the only consistent toxic effect of a single dose of imidazole. To investigate whether the instances of increased salivation seen in the first pair during administration of the dose and clinical examination were a conditioned response, these dogs were given a second dose consisting of empty capsules. No effect was elicited.

No effect of treatment was seen at autopsy or histopathological examination. No effect of treatment with 2,4,6-trichlorophenol was detected. Thus, M2 and 2,4,6-trichlorophenol were less acutely toxic than prochloraz. Compared to prochloraz M1 elicited a toxic response at the same dose, but imidazole appeared to be slightly more toxic (Watson, Lancaster & Robinson, 1980b).

***Genotoxicity of metabolites***

The mutagenic potential of M1 (BTS 44595) and M2 (BTS 44596) was evaluated in the Ames test using the *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538. During the study, the *S. typhimurium* strains were exposed to the metabolites dissolved in dimethyl sulfoxide (DMSO). All assays were conducted in the presence and absence of an in vitro microsomal activation system (S9) derived from the livers of male Charles River Wistar rats. Three replicates were made for each test. In preliminary toxicity studies, Metabolite M1 was shown to have antibacterial properties and consequently, dose levels of 15.6, 31.2, 62.5, 125 and 250 µg/plate were used. Metabolite M2 proved less toxic and was assayed at concentrations of 62.5, 125, 250, 500 and 1000 µg/plate. Positive and negative controls were included in all tests. At concentrations above 125 µg/plate, M1 exhibited antibacterial activity which precluded assessment of mutagenicity. In the case of M2, precipitation was noted at 1000 µg/plate, indicating that testing was carried out to the limit of solubility. A reduced bacterial lawn was observed at 1000 µg/plate for TA100, indicating some antibacterial activity towards the strain at this dose level. No substantial increase in the number of revertant colonies was observed with either compound. It was concluded that metabolites M1 and M2 are not mutagenic in this reverse mutation assay (Everest & Varley, 1979).

The mutagenic potential of 2-(2,4,6-trichlorophenoxy)ethanol (BTS 3037) was evaluated in an Ames test using the *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537, and *Escherichia coli* strain WP2. All assays were conducted in the presence and absence of S9 derived from the livers of male Charles River Wistar rats. Three replicate plates were tested for every test. Positive and negative controls were included in all tests. The preliminary toxicity study showed incomplete bacterial lawn at the highest tested concentration of 5000 µg/plate. For the main study concentrations of 0, 5, 15, 50, 150, 500, 1500 and 5000 µg/plate was tested. No evidence of mutagenic activity was seen at any dose level of BTS 3037. The concurrent positive controls demonstrated the sensitivity of the assay and the metabolizing activity of the liver preparations (Jones & Gant, 1992).

*In silico analysis of metabolites*

Quantitative structure–activity relationship (QSAR) evaluations were carried out using DEREK Nexus and the OECD QSAR Toolbox. Evaluations were run for prochloraz and a series of potentially relevant major plant metabolites: BTS 44595, BTS 54906, BTS 44770, BTS 40542, BTS 44596, BTS 45186 and BTS 9608.

For Derek Nexus v. 6.2.1 and Derek Knowledge base 2022 2.0 the end-point modules used were:

*Carcinogenicity (ALL)*

Carcinogenicity

Photocarcinogenicity

*Genotoxicity (ALL)*

Chromosome damage

Mutagenicity

Non-specific genotoxicity

For the OECD QSAR Toolbox v 4.5 2022 (local installation) and v. 2.5.3130a of the (beta) online version (https://toolbox.oasis-lms.org/WebClient/) the following profiles were used:

DNA binding by OASIS

DNA binding by DNA

Carcinogenicity (genotox and nongenotox) alerts by ISS

DNA alerts for AMES, CA and MNT by OASIS

In vitro mutagenicity (Ames test) alerts by ISS

In vivo mutagenicity (Micronucleus) alerts by ISS

Oncologic primary classification

Protein binding alerts for chromosomal aberration by OASIS

Based on the QSAR analyses, none of the metabolites are predicted to be genotoxic.

**(d) Mode of action studies**

Several mechanistic studies are available to elucidate the mode of action responsible for the increased incidence of liver tumours in mice.

***In vitro***

*Study 1*

The effects of prochloraz on isolated male CD-1 mouse hepatocyte cytotoxicity was investigated, to select suitable concentrations for a future study. During the study, hepatocyte cultures isolated from unexposed male CD-1 mice, were exposed to prochloraz (purity 98.1%) at test concentrations of 0 (vehicle control), 0.03, 0.1, 0.3, 1, 3, 10, 30, 100 and 300 µM in DMSO, for 96 hours in CL15 medium, for measurement of cytotoxicity.

Exposure of hepatocytes to prochloraz (0.03, 0.1, 0.3 and 1 µM) resulted in similar statistically significant reductions in intracellular ATP, by approximately 9%. There was no effect on ATP content with 3 and 10 µM prochloraz when compared to the vehicle control. However, exposure to 30, 100 and 300 µM prochloraz resulted in significant decreases in ATP content to 65.3%, 0.31% and 0.073% of vehicle control values respectively.

Based on the results of this preliminary study, 0, 0.3, 1, 3 and 10 µM prochloraz were recommended as concentrations to be used for the main CD-1 mouse investigative study, as some cytotoxicity is observed at the highest proposed dose level (CXR Biosciences, 2015a).

*Study 2*
In the main study, hepatocyte cultures isolated from male CD-1 mice were exposed to prochloraz (purity 98.1%) at 0.3, 1, 3 and 10 µM in DMSO for 96 hours in CL15 medium, in humidified air at 37°C. Vehicle and positive control, phenobarbital sodium salt, (PB) and epidermal growth factor) controls were run concurrently. Measurements of cytotoxicity, replicative DNA synthesis and measurement of Cyp2B10 and Cyp3A11 mRNA expression were performed.

The exposure of hepatocytes to prochloraz (0.3 µM) and positive controls resulted in similar statistically significant reductions in intracellular ATP. A small, but statistically significant decrease in ATP concentration was also observed with 1 µM prochloraz, while no changes in ATP concentrations occurred at concentrations of 3 or 10 µM. These reductions in ATP concentrations were considered to reflect normal variability and considered not to be indicative of cytotoxicity due to the lack of a concentration–response pattern for ATP content in the treated groups and historical control values. The reduction in ATP level in PB-exposed cells was significant, although in the same range as for the prochloraz-exposed cells. The results of the range-finding study (R-34436B) indicate that the cytotoxic range was approached at the high dose.

Prochloraz produced a concentration-dependent, significant increase in replicative DNA synthesis at all concentration levels, with the largest increase (2.76-fold) observed at 10 µM. The positive controls produced the expected significant increases in the labelling index, consistent with historical data.

Prochloraz additionally produced significant increases in CYP2B10 mRNA at 1, 3 and 10 µM. However these were not clearly concentration-dependent as the largest increase (2.33-fold) occurred at 1 µM. Similarly, prochloraz produced significant increases in CYP3A11 expression at all concentrations, with the largest increase (7.75-fold) at 1 µM. The positive control produced the expected increase in CYP3A11 expression confirming the responsiveness of the cells. No increase in CYP2B10 occurred in PB-exposed cells. Historical control data from the testing laboratory indicated variable responses to PB, therefore the lack of effect of PB in this study was considered to reflect normal variability.

It was concluded that prochloraz induces both an increase in CYP2B10/CYP3A11 mRNA expression and hepatocyte proliferation in CD-1 mouse hepatocytes, indicating that prochloraz may be a constitutive androstane receptor/pregnane X receptor (CAR/PXR) activator in mice (CXR Biosciences, 2015b).

*Study 3*
A study was conducted to investigate the effects of prochloraz on cytotoxicity in cryopreserved male human hepatocyte cultures to select suitable concentrations for a future investigative study. During the study, hepatocyte culture isolated from a human volunteer (male, aged 68 years) was exposed to prochloraz (purity 98.1%) at test concentrations of 0 (vehicle control), 0.03, 0.1, 0.3, 1, 3, 10, 30, 100 and 300 µM in DMSO for 96 hours, for measurement of cytotoxicity.

The exposure of hepatocytes to up to 10 µM prochloraz resulted in statistically significant increases of up to 1.2-fold in ATP content. However, exposure to 30, 100 and 300 µM prochloraz resulted in statistically significant decreases in ATP content to 62.9%, 2.0% and 0.3% respectively of the vehicle control value.

Based on the results of this preliminary study, it was recommended to select concentrations of 0.3, 1, 3 and 10 µM prochloraz for the main human investigative study, as cytotoxicity was observed at higher dose levels (CXR Biosciences, 2015c).

*Study 4*
In the main study, the effects of prochloraz on cytotoxicity, hepatocyte proliferation and expression of CYP2B6 and CYP3A4 mRNA in isolated male human hepatocyte cultures was investigated. During the study, hepatocyte culture isolated from a human volunteer (male, aged 68 years) was exposed to prochloraz (purity 98.1%) at test concentrations of 0.3, 1, 3 and 10 µM in DMSO, for 96 hours in CL15 medium, in humidified air at 37°C. Vehicle and positive (PB and epidermal growth factor) controls were run concurrently. Measurements of cytotoxicity and replicative DNA synthesis (S-phase) were performed, the data for the latter expressed as a labelling index, percentage of total hepatocytes that have incorporated BrdU. Expression of CYP2B6 and CYP3A4 was also assessed.

There was no change in ATP content in hepatocytes exposed to levels of 3 µM and above of prochloraz, when compared with the vehicle controls. At the highest exposure level of 10 µM prochloraz a statistically significant reduction in ATP content to 73% of control level was observed. The reduction in ATP content of hepatocytes exposed to 10 µM prochloraz was considered to be at the threshold of cytotoxicity. The positive control, PB, did not cause cytotoxicity in the hepatocytes.

Prochloraz did not produce any significant increases in the labelling index, compared to the vehicle control, indicating a lack of cell proliferation potential. The labelling index was also unchanged compared with the vehicle controls for hepatocytes treated with PB, while epidermal growth factor produced a 5.29-fold increase, demonstrating the ability of these hepatocytes to enter S-phase in response to a proliferative stimulus.

Treatment with prochloraz caused increases in CYP2B6 mRNA expression at all concentrations, with the largest effect (2.03-fold) observed at 3 µM. Significant increases in CYP3A4 mRNA expression were also observed at all concentration levels. However, these were not concentration-dependent, as the largest increase (3.47-fold) occurred at 0.3 µM. The positive control PB produced an increase in CYP2B6 and CYP3A4 expression at 1000 µM.

Findings suggest that prochloraz may be a CAR activator and thus may possibly be a PXR activator in humans. However, the lack of effect on replicative DNA synthesis (S-phase response) in the study shows that this compound is unlikely to cause hyperplasia (CXR Biosciences, 2015d).

*Study 5*

Prochloraz (purity 96.3%) was tested for its ability to induce morphological transformation in C3H/10$T_{1/2}$ clone 8 mouse embryo fibroblasts in the presence and absence of a rat liver preparation (S9 mix). Positive and negative controls were included in all tests. Based on the results of a preliminary toxicity test the concentrations tested in the cell transformation assay were 7.5, 15 and 30 µg/mL in both the presence and absence of S9 mix.

The vehicle control groups were within the normal laboratory range, and concurrent positive controls demonstrated the sensitivity of the assay and the metabolising system of the S9 mix. No evidence of cell-transforming activity due to prochloraz was obtained in the presence of S9 mix. In the absence of S9 mix, prochloraz induced significant incidence of transformed foci at 7.5 and 15 µg/mL (McGregor & Riach, 1984).

***In vivo***

*Study 1*

Prochloraz technical grade (purity 95% or higher) and analytical grade (purity 99.5%) were investigated for their effects on the initiation and promotion stages of hepatocarcinogenesis and for their ability to inhibit gap-junctional intercellular communication in the scrape-loading dye-transfer assay in IAR 20 rat liver epithelial cells. In the test for tumour promotion, female Sprague Dawley rats were initiated with *N*-nitrosodiethylamine (30 mg/kg bw, intraperitoneally) 24 hours after partial hepatectomy to maximize any interaction between proliferation and the effects of prochloraz. Two weeks later, they were given prochloraz on five days a week by gavage at 30 or 150 mg/kg bw per day for 10 weeks. Groups of uninitiated and/or non-hepatectomized rats were included. The control group was given the vehicle (corn oil) only. A positive control group was given phenobarbital (500 ppm) in drinking-water for 10 weeks, starting two weeks after initiation. In the test for tumour initiation, animals were given prochloraz at 150 mg/kg bw per day by gavage for 12 days, and a control group was given the vehicle according to the same schedule. On day 8 of treatment all the animals were partially hepatectomized. After two weeks of recovery, promotion was started by giving all groups phenobarbital (500 ppm) in drinking-water for 10 weeks. The rats were killed one week after the promotion period. Altered hepatic foci were counted by quantitative stereology in liver sections stained for GGTP and glutathione-*S*-transferase placental form (GST-P).

Prochloraz inhibited cell to cell communication in the test system used. It had no effect on the initiation of GGTP-positive foci, but significant increases in the percentage of liver tissue occupied by these foci and the number of GST-P-positive altered hepatic foci square centimetre were recorded at the lower dose. The absence of effects on plasma transaminase activity and on body weight gain in the

prochloraz-treated animals suggested that the doses used in the study were not overtly hepatotoxic. A significantly increased relative liver weight was observed in rats given prochloraz at 150 mg/kg bw per day. The data suggest that prochloraz acts as a weak promoter of hepatocarcinogenesis but does not initiate the process (Kato, Flodström & Wärngard,1998).

*Study 2*

Eight pesticides, including prochloraz, were tested in a bioassay based on the induction of preneoplastic lesions in the liver. Rats were divided into three groups of 15 or 16 animals. Group 1 was given a single intraperitoneal injection of *N*-nitrosodiethylamine dissolved in 0.9% saline at 200 mg/kg bw to initiate hepatocarcinogenesis. After two weeks on a basal diet they received a diet containing prochloraz (purity 94.8%) at a concentration of 625 ppm for six weeks, being subjected to partial hepatectomy at week 3. Group 2 was given *N*-nitrosodiethylamine and subjected to partial hepatectomy in the same way but received the basal diet throughout the test period. Group 3 received only the 0.9% saline vehicle and was then given a diet containing prochloraz from week 2, with partial hepatectomy at week 3. The experiment was terminated at week 8. Tumour-promoting potential was assessed by comparing the number and area of GST-P-positive foci (of diameter 0.2 mm or greater) in the liver, with those in controls given *N*-nitrosodiethylamine alone. Statistical analysis was performed using Student's t-test. The results were scored on the basis of the difference between the first two groups: a result was considered positive when there was an increase in both the number and area of foci at $p$ below 0.05; a borderline result was an increase in either the number or the area of foci at $p$ below 0.05; and a negative result was no or a nonsignificant increase ($p = 0.05$ or above) in both parameters.

No treatment-related deaths were seen. Body weight gain at week 8 was impaired in Group 1 (by 12 g; not reported for Group 3). A slight but statistically significant increase ($p < 0.01$) in relative liver weight was noted, a slight (not statistically significant) increase in the number of GST-P-positive foci per unit area of liver was found in sections from animals fed prochloraz ($8.0 \pm 3.3$ foci/cm$^2$ in Group 1, $6.5 \pm 2.2$ foci/cm$^2$ in Group 2), and a slight increase ($p < 0.05$) was found in the total area of GST-P-positive foci ($0.71 \pm 0.35$ mm$^2$/cm$^2$ in Group 1, $0.44 \pm 0.22$ mm$^2$/cm$^2$ in Group 2). In Group 3, no GST-P-positive foci greater than 0.2 mm in diameter were seen. Thus, prochloraz was not considered to be an inducer of hepatocarcinogenesis (Cabral et al., 1991).

*Study 3*

Male and female C57BL/6J mice were administered prochloraz (purity 98.0%) at 325 or 1300 ppm in the diet for 14 days. At termination blood was taken and plasma prepared for clinical chemistry (ALT, AST and ALP activity). Liver tissue was processed for Taqman® analysis of gene expression (Cyp1a1, Cyp1a2, Cyp2b10, Cyp3a11, Cyp4a10, Cyp4a14 and Acyl-CoA oxidase), and histological analysis. The remaining liver was processed to: heavy pellets, assayed for total protein content and the activity of CN-insensitive acyl CoA oxidase (PCoA); and microsomes, assayed for total protein content, total cytochrome P450 content, and the activities of ethoxyresorufin-*O*-deethylase (EROD), benzyloxyresorufin-*O*-debenzylation (BROD), lauric acid 12-hydroxylation, pentoxyresorufin-O-deethylase (PROD) and benzyloxyquinoline debenzylation (BQ) enzymes. Microsomal samples were also used for western blot analysis of Cyp1a1/1a2, Cyp2b, Cyp3a and Cyp4a).

There was no effect on either body weight or body weight gain following administration of prochloraz. Increases in liver weight were observed in both males (1.1–1.4 fold) and females (1.4–1.5 fold). No effect on clinical signs of food consumption was observed. Clinical chemistry parameters did not reveal hepatotoxicity at 325 ppm but increased ALT was observed at 1300 ppm in both males (1.7 fold) and females (3.3 fold). Regarding histopathology, hepatocellular hypertrophy was observed in all male animals treated with 325 ppm or 1300 ppm prochloraz and in 2/5 female animals treated with 325 ppm and all female animals treated with 1300 ppm prochloraz, which correlated well with the observed weight changes. Hepatocellular hypertrophy was accompanied by fatty change in all male and female animals at 1300 ppm prochloraz. The effects on the liver were accompanied by a marked induction of Cyp2b10 as demonstrated by increased mRNA, increased protein and increased PROD and BROD), which are markers for the induction of the Cyp2b family. Similarly, induction of Cyp3a expression, characterized by increased benzyloxyquinoline-*O*-debenzylation, mRNA and protein, was also demonstrated. These data suggest that prochloraz is an activator of CAR and perhaps PXR.

Prochloraz exhibited no properties to suggest that it was a peroxisome proliferator-activated

receptor-α (PPAR-α) activator. Indeed, it generally decreased PPAR-α-mediated effects such as peroxisomal β-oxidation, lauric acid 12-hydroxylation and Cyp4a expression.

Despite the observed increases in Cyp1a mRNA and the respective Cyp1a protein, the study author considered that the corresponding EROD enzyme activity was not relevantly increased. They concluded that the observed slight increase in enzyme induction had no relevance to tumour induction via an aryl hydrocarbon receptor (AHR) pathway and remarked that a slightly increased CYP1A activity was also observed for other CAR activators without indicating any relevance to a major mode of action via AHR (CXR Biosciences, 2012a).

*Study 4*

Male C57BL/6J wild-type mice were fed diets containing prochloraz (purity 98.0%) at dose levels of 0, 325 and 1300 ppm for 3, 7, 14 and 28 days. The animals that were treated with prochloraz for 7, 14 and 28 days were implanted (subcutaneously in the dorsal region) with Alzet osmotic pumps containing approximately 200 µL of bromodeoxyuridine (BrdU) solution, concentration 15 mg/mL in phosphate-buffered saline (PBS) at pH 7.4. The animals that were treated with prochloraz for 3 days received the Alzet osmotic pumps 3 days before termination. A positive control group was fed a diet containing 1000 ppm phenobarbital. Biochemical measurements were performed, including: clinical chemistry, peroxisome proliferation, total cytochrome P450, BROD, PROD, BQ, LAH and protein assays, and TaqMan® analysis for Cyp2b10, Cyp3a11, Cyp4a10 and Cyp4a14 mRNA expression. On the day of termination livers were removed from each animal, weighed and samples taken for histological analysis: BrdU immunohistochemical analysis, haemotoxylin/eosin staining and Taqman® analysis.

Body weight was reduced at a prochloraz dose of 1300 ppm, but not at 325 ppm nor with phenobarbital. Liver weight was increased compared to vehicle controls in all treated animals. At 325 ppm no increases in ALT or AST were observed. At 1300 ppm of prochloraz and in the phenobarbital group, increases in ALT were observed. Significant increases between 21- and 83-fold in PROD and BROD (as markers for Cyp2b activity) were observed after treatment with 325 or 1300 ppm prochloraz for seven and 14 days. Increases (3.1- to 5.8-fold) were observed in BQ, serving as a marker for Cyp3a activity, with mice treated the same way. Marked upregulation in Cyp2b10 mRNA expression (50- to 400-fold) was seen after treatment with prochloraz at dietary concentrations of 325 and 1300 ppm. in addition Cyp3a11 mRNA levels were increased four- to nine-fold compared to controls at 325 and 1300 ppm respectively. The induction of Cyp2b and Cyp3a in the absence of induced peroxisomal β-oxidation, lauric acid hydroxylation or Cyp4a expression therefore strongly suggested that the mode of action (MOA) for prochloraz is via activation of CAR (and possibly PXR). There is no evidence, that prochloraz is a peroxisome proliferator acting via PPAR-α. Any potential effect via an AhR pathway was not investigated.

Prochloraz at 325 ppm induced hepatocellular hypertrophy at all time points; increased proliferation was, however, only observed at day 7. At 1300 ppm prochloraz induced hepatocellular hypertrophy, fatty changes as well as proliferation at all time points, peaking at days 7 and 14. The mean of all zones values for day 3 and for day 28 were not increased to a statistically significant extent compared to controls. In conclusion, prochloraz caused hypertrophy and increased S-phase response of mainly centrilobular (zone 3) hepatocytes (CXR Biosciences, 2012b).

*Study 5*

Male C57BL/6J wild type mice were implanted (subcutaneously in the dorsal region) with Alzet osmotic pumps containing approximately 200 µL BrdU solution, at 15 mg/mL in PBS at pH 7.4, 7 days before termination. Animals were fed diets containing prochloraz (purity 98.0%) at dose levels of 0 or 325 ppm for seven days. A positive control group was fed a diet containing 500 ppm phenobarbital. Biochemical measurements were performed, including: clinical chemistry, peroxisome proliferation, total cytochrome P450, BROD, PROD, BQ, LAH and protein assays, and TaqMan® analysis for Cyp2b10, Cyp3a11, Cyp4a10 and Cyp4a14 mRNA expression. On the day of termination, the livers were removed from each animal, weighed and samples taken for histological analysis: BrdU immunohistochemical analysis, haemotoxylin/eosin staining and Taqman® analysis. Plasma markers of liver function demonstrated no hepatotoxicity associated with the administration of prochloraz at 325 ppm for seven days.

The administration of prochloraz, at 325 ppm for seven days to male C57BL/6J wild-type mice induced total cytochrome P450, PROD, BROD and BQ, and increased the expression of Cyp2b10 and Cyp3a11 mRNA and protein. Only small increases in these parameters were observed following the administration of prochloraz to male hPXR/hCAR mice. Prochloraz had nearly no effect on these parameters in male PXR KO/CAR KO mice. There was little if any biologically relevant effect of prochloraz on absolute or relative liver weights in the different mouse strains. In conclusion, the data generated using mice nulled for CAR and PXR clearly demonstrates that the MOA of prochloraz is via activation of CAR (and possibly PXR). Additionally, the data obtained utilising the mice humanized for CAR and PXR suggest that prochloraz is a less potent activator of human CAR than of mouse CAR (CXR Biosciences, 2012c).

*Study 6*
In order to investigate the effect of prochloraz on the hepatic mixed-function oxidase system of male mice after oral administration, prochloraz (purity 97.3 ± 1% and greater than 95%) was administered by gavage to groups of 10–12 mice, at dose levels of 0, 10 and 100 mg/kg bw, twice daily for four days. A separate group of 12 mice were administered phenobarbitone at a level of 80 mg/kg bw per day as a single intraperitoneal injection for four days. At the end of the dosing period all animals were killed and a supernatant containing microsomal enzymes was prepared from each liver. Analysis was carried out for microsomal protein concentration and cytochrome P450 and b5 levels.

Liver weights were significantly increased compared to controls in the 100 mg/kg bw prochloraz dose group. Microsomal protein concentration and cytochrome levels were also significantly increased. Thus, prochloraz was shown to be a potent enzyme inducer at this dose level. Supernatent prepared from the 10 mg/kg bw prochloraz group revealed significant increases in only the cytochrome P450 and microsomal protein levels, while liver weights were unaffected. Thus prochloraz also caused induction at this dose level. No evidence of cytochrome P448 was found in any of the samples (Challis & Campbell, 1983).

*Study 7*
Prochloraz (purity 98% or higher) was administered to three groups of six male rats at dose levels of 0, 10 or 100 mg/kg bw, by oral gavage twice daily for four days. A separate group of six male rats were administered phenobarbitone at a dose level of 1 g/L in the drinking water for seven days. At the end of the dosing period all animals were killed and a supernatant containing microsomal enzymes was prepared from each liver. Analysis was carried out for levels of aniline hydroxylase and *p*-nitroanisole demethylase activity and for concentrations of cytochrome P450 and b5.

Prochloraz significantly increased the microsomal protein concentration, enzyme activity, and concentration of cytochrome P450 at a dose level of 100 mg/kg bw. At this dose level the liver weights were also significantly increased. Thus prochloraz was shown to be a potent enzyme inducer at this dose level. At a dose level of 10 mg/kg bw only an increase in cytochrome P450 was noted, indicating that this dose is close to the threshold level for induction to occur. Liver weights were similar to controls at 10 mg/kg bw. The spectrum of induction was similar to that caused by phenobarbitone with the levels of cytochrome P450 being increased at both dose levels. No evidence of cytochrome P448 was found in any of the samples (Needham, 1983a).

*Study 8*
In order to examine the effect of prochloraz on the hepatic mixed-function oxidase system of the mouse at comparable dietary dose levels to those used in the two-year mouse study, prochloraz (purity 95.7%) was administered in the diet to CD-1 mice (five/sex per dose) at 0, 80, 325 and 1300 ppm for 2, 6 and 14 weeks. A separate group of five male and five female mice received four daily intraperitoneal doses of phenobarbitone at 80 mg/kg bw five days prior to being used for the assay. Microsomal fractions were prepared and analyzed for cytochrome P450 and b5 in addition to protein concentrations.

Prochloraz was found to be a potent phenobarbitone-type inducer in mice at dietary dose levels of 1300 and 325 ppm, with increased levels of cytochrome P450 and microsomal protein concentration and significantly increased liver weights. Prochloraz at 80 ppm appeared to be close to the threshold level that produces induction of the liver enzyme system. At this dose level the changes in hepatic enzyme activity were considered to be adaptive changes and not indicative of an adverse effect. There was no

evidence of cytochrome P448 in any of the livers examined. There was no significant sex difference at any dose level (Needham, 1983b).

*Study 9*
Prochloraz was examined on its enzyme induction potential in rats, following dietary administration to IOPS/OPA female rats at dietary dose levels of 0, 100, 500 and 2500 ppm for seven days.

Body weight was decreased dose-dependently and relative liver weights were increased. At 500 ppm, and to a much higher extent at 2500 ppm, prochloraz increased the levels of microsomal protein and cytochrome P450 and b5. At these dose levels prochloraz proved to be a potent enzyme inducer, with NADPH-cytochrome-c reductase, aniline hydroxylase, 7-ethoxycoumarin dealkylase, 7-ethoxyresorufin dealkylase and epoxide hydrolase being slightly induced at 500 ppm and significantly induced at 2500 ppm. At 100 ppm only cytochrome P450 and 7-ethosyresorufin activity were increased. Prochloraz was therefore found to be a potent inducer of the rat hepatic microsomal cytochrome P450 system (Rivière, 1983).

*Study 10*
Prochloraz and the three metabolites BTS 3037, BTS 44595, and BTS 9608 were examined on their respective profiles of enzyme induction. Prochloraz was administered to two groups of male Sprague Dawley rats at different dosage regimes (50 or 100 mg/kg bw) twice daily either in solution or suspension in corn oil. The metabolites were dosed on a molecular equivalent basis to prochloraz.

At the higher dose level prochloraz and its metabolites clearly increased liver weights. The overall induction profile of prochloraz reflected the contribution of the individual metabolites. There was a slight increase of LAH, but by far the major induction was seen in the activity of aldrin epoxidase and 7-pentoxyresorufin-*O*-dealkylase (7-and 14-fold respectively). Metabolite BTS 44595 was a phenobarbitone-type inducer, increasing the activity of the aldrin epoxidase and 7-pentoxyresorufin-*O*-dealkylase by 120% and 8-fold respectively. The metabolites BTS 3037 and BTS 9608 were both inducers of the clofibrate type, increasing the activity of LAH. Overall, the predominant characteristics were those of phenobarbitone-type induction (Needham, Creedy & Dawson, 1992).

*Summary of the MOA studies*
In summary, the mechanistic studies described above, performed in order to elucidate the non-genotoxic MOA responsible for increased incidence of liver tumours in mice, show increased liver weight, hepatocellular hypertrophy and hepatocellular proliferation in mice treated with prochloraz. Activation of CAR is indicated by the prochloraz-induced increases in liver cytochrome P450 mono-oxygenases Cyp2b as demonstrated by increased mRNA expression, protein content and increased PROD and BROD. Similarly, induction of Cyp3a was indicated by increased mRNA, protein content and benzyloxyquinoline-O-debenzylation. Whereas P450, PROD, BROD, BQ, mRNA and protein were induced in wild-type mice, virtually no effect on these parameters were seen in PXR KO/CAR KO (knock-out) mice. The studies indicate that the MOA for tumour formation by prochloraz in mice is through activation of CAR and possibly PXR. In addition there are indications that prochloraz may act as a weak tumour promoter, which is consistent with the CAR MOA. However, it is noted that a full investigation of the MOA was not performed.

**(e) Endocrine disruption studies**

A number of in vitro screening studies were conducted in various assays, for instance cell proliferation, estrogen receptor (ER), estrogen receptor transactivation (ERTA), androgen receptor, androgen receptor (AR) transactivation (ARTA), aromatase assays and hormone alteration assays. A summary of the available studies is provided below. Additionally, in vivo data, investigating the potential anti-androgenic properties of prochloraz were available together with a mechanistic study about the sensitivity of fetal rat testicular steroidogenesis to maternal prochloraz exposure, and the underlying mechanism of inhibition; for this see section 2.5 Reproductive toxicity.

### *Metabolome investigation*

Changes in metabolite profiles were monitored in the human adrenocarcinoma cell line (H295R) after exposure to prochloraz using a metabolomics approach. The H295R cells were exposed to prochloraz (5 µM) for 48 hours. After exposure, enzyme immunoassay analysis was performed to determine progesterone, 17β-testosterone and 17β-estradiol levels. Additionally, the concentration of nine steroids were determined by quantitative targeted tandem gas–liquid chromatography/mass spectrometry (GC-MS/MS) and compared to the concentration of six steroids measured by ultra-performance liquid chromatography-time-of-flight mass spectrometry (UPLC-ToF-MS). Cell viability was measured using the WST-1 and ATPlite assay. Exposure to prochloraz (0.0001–100 µM) resulted in a decreased cell viability at concentrations above 10 µM. Therefore a maximum concentration of 5 µM was used to ensure optimal cell viability. In the enzyme immunoassay prochloraz induced levels of progesterone and decreased levels of 17β-testosterone and 17β-estradiol compared to the control. The maximum increase in level of progesterone was 2.2-fold at a concentration of 1 µM. The maximum decreases in 17β-testosterone and 17β-estradiol level were 21-fold and 13-fold respectively, and these were observed at a concentration of 5 µM. This is likely the result of the CYP17A1 inhibitory properties of prochloraz. Examination using GC-MS/MS also revealed an increase in progesterone levels and a decrease in 17β-testosterone and 17β-estradiol levels. Moreover, prochloraz increased the level of 11-deoxycorticosterone by 5.6-fold as measured by UPLC-ToF-MS. This is likely to have been the result of the CYP21A1-inhibiting properties of prochloraz. Overall, the results of GC-MS/MS and UPLC-ToF-MS were comparable (Rijk et al., 2012).

### *AR binding assay*

The ability of prochloraz (purity 99.4%) to compete with [$^3$H]R1881, a synthetic ligand for the androgen receptor, for binding to the rat androgen receptor (AR) was studied in ventral prostate tissue obtained from 90-day-old rats. The cells were exposed to concentrations of 1, 3, 10, 30, 100 and 300 µM, and 1 and 3 mM prochloraz, and 83.5 Ci/mmol of [$^3$H]R1881 for 24 hours. Incubations with a 100-fold molar excess of unlabelled R1881 were used to estimate non-specific binding. The samples were washed to remove unbound ligand and receptor-bound ligand was separated so radioactivity could be determined by LSC. Prochloraz inhibited [$^3$H]R1881–AR binding in a dose-dependent manner with an $IC_{50}$ of approximately 60 µM (Noriega et al., 2005).

In a second study, 0.1, 0.3, 1, 3, 10, 30, 100 and 300 µM prochloraz (purity 99.5%) was incubated with AR and [$^3$H]R1881 overnight. Radioactivity was determined LSC after ligand-bound receptor was separated from unbound ligand. Prochloraz showed affinity to the AR. The AR binding affinity of prochloraz had an $IC_{50}$ of 40.6 ± 3 µM, and was 0.0121 ± 0.0030% relative to the positive control dihydrotestosterone (Freyberger et al., 2010a).

### *AR transactivation assays*

The AR CALUX reporter gene assay was used to test the androgenic and anti-androgenic activity of prochloraz in vitro. For agonist testing, calux cells were incubated with prochloraz in DMSO at concentrations of 0.1 pM–10 µM for 24 hours. Afterwards, cells were lysed and measured for luciferase activity in a luminometer. For antagonist testing, a receptor agonist, at a concentration around the half-effective concentration value ($EC_{50}$) was included during the incubation. Under the conditions of the study, prochloraz had no androgenic potential when tested up to 10 µM. Prochloraz showed an anti-androgenic effect, yielding an $IC_{50}$ of 1.5 and 2 µM respectively in tests carried out in two different laboratories (Van der Burg et al., 2010).

In another AR reporter gene assay, transfected Chinese hamster ovary cells (CHO K1) were exposed for 20 hours to prochloraz (purity 99.4%) at concentrations ranging from ca 40 nM–50 µM with (antagonist testing) or without (agonist testing) 0.01 nM AR agonist R1881. Afterwards, the medium was removed, cells were lysed and measured for luciferase activity in a luminometer after automatic addition of luciferin and ATP. Cytotoxicity was determined and controls were included. Prochloraz showed an anti-androgenic effect in this in vitro assay, and inhibited the R1881-induced response at 1.6 µM (the lowest observed effect concentration, LOEC; a 20% reduction) and up to 25 µM (the maximum-observed-effect concentration, MOEC; a 90% reduction) to a statistically signicant extent.

The $IC_{50}$ was 3.6 µM. By contrast, the major metabolite, 2,4,6-trichlorophenoxyacetic acid, and a minor metabolite, 2,4,6-trichlorophenol, metabolite of prochloraz, caused no statistically significant inhibition of the R1881-induced response. Prochloraz showed no androgenic potential when tested up at to 50 µM. Moreover, prochloraz decreased the transcriptional activity at a concentration of 50 µM, whereas no effects were observed at concentrations at or below 25 µM (Vinggaard et al., 2002).

In another AR reporter gene assay, transfected CHO K1 cells were exposed for 20 hours to prochloraz at 12 concentrations ranging from 25 nM to 50 µM, with 0.01 nM AR agonist R1881. Afterwards, the medium was removed, cells were lysed and measured for luciferase activity in a luminometer after addition of luciferin and ATP. Additionally, cytotoxicity was determined. Prochloraz was cytotoxic at a concentration of 50 µM. Prochloraz showed an anti-androgenic effect, yielding a LOEC of 3.1 µM, and an estimated $IC_{50}$ of 15–20 µM (Kjærstad et al., 2007).

In another AR reporter gene assay, AR-transfected CHO cells were exposed for 20 hours to prochloraz (purity 99.5%) at concentrations ranging from 25 nM to 50 µM with or without 0.1 nM AR agonist R1881. Afterwards the medium was removed, cells were lysed and measured for luciferase activity in a luminometer after addition of luciferin and ATP. Additionally, cytotoxicity was determined. Prochloraz showed an anti-androgenic effect, yielding a LOEC, $IC_{25}$, $IC_{50}$ and $IC_{75}$ of 6.3, 5.7, 13 and 18 µM, respectively. Cytotoxicity was observed at 50 µM and above (Kjærstad et al., 2010a).

In another AR reporter gene assay, AR-transfected CHO cells were exposed for 20 hours to prochloraz (purity 98.5%) at concentrations ranging from 25 nM to 50 µM with or without 0.01 nM AR agonist R1881. Afterwards the medium was removed, cells were lysed and measured for luciferase activity in a luminometer after addition of luciferin and ATP. Additionally, cytotoxicity was determined. Prochloraz showed an anti-androgenic effect, yielding a LOEC of 3.8 µM. The two highest concentrations tested were cytotoxic in the range 25–50 µM (Kjærstad et al., 2010b).

In another AR reporter gene assay, transfected CHO cells were exposed for 20 hours to prochloraz (purity 99.4%) at 12 concentrations ranging from 0.025 µM to 50 µM with or without 0.1 nM AR agonist R1881. Afterwards the medium was removed, cells were lysed and measured for luciferase activity in a luminometer after addition of luciferin and ATP. Additionally, cytotoxicity was determined. The AR antagonistic effects of prochloraz were observed at and above 1.6 µM, the $IC_{25}$ value was 3.5 µM and the $IC_{50}$ value 7.15 µM. Prochloraz did not show AR agonistic effects at the concentrations tested. Cytotoxicity was observed at 50 µM (Birkhøj et al., 2004).

In another AR reporter gene assay, transfected CHO K1 cells were exposed for 24 hours to prochloraz (purity 95% or greater) at concentrations 0.01–10 µM with or without 0.1 nM dihydrotestosterone (DHT), a AR receptor agonist. Afterwards the medium was removed, cells were lysed and measured for luciferase activity in a luminometer. Additionally, cytotoxicity was determined. No AR agonistic effects were observed for prochloraz. The 20% relative inhibitory concentration, (indicating the concentration of the test substance showing 20% inhibition of the activity induced by 100 pM DHT) observed for prochloraz was 3.4 µM. The relative luciferase activity was 43% when compared to the agonistic activity of DHT. Cytotoxicity was not observed at the selected doses (Kojima et al., 2004).

In another AR reporter gene assay, transfected MDA-kb2 cells were exposed overnight to prochloraz (purity 99.4%) at concentrations 0.03–30 µM, with or without 0.1 nM DHT. Cells were washed, lysed and luminescence measured in a plate luminometer. Cytotoxicity was additionally determined using a 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay. Controls were included. Prochloraz inhibited AR transcriptional activation induced by DHT at doses at and above 3 µM. The $IC_{50}$ for prochloraz was between 3 µM and 10 µM. Cytotoxicity was observed at 100 µM, when MTT activity was reduced to about 20% of the control value (Noriega et al., 2005).

In another AR reporter gene assay, MDA-kb2 cells were exposed for 24 hours to prochloraz at concentrations 0.1–10 µM, with and without 0.1 nM DHT. Afterwards, the medium was replaced by a phenol red-free medium containing D-luciferin and luminescence was measured in a luminometer. Cytotoxicity was determined using the MTT assay. Prochloraz did not show androgenic potential. However, prochloraz at 3 µM and above decreased the luciferase activity in a concentration-dependent manner in the presence of 0.1 nM DHT, showing an anti-androgenic potential. The $IC_{50}$ of prochloraz

was 3.09 µM. The relative potency of prochloraz compared to the known anti-androgen flutamide was also measured. However, the authors mention that the $IC_{50}$ value is more reliable since it is unknown if flutamide is metabolized to a more potent metabolite in this cell system or not. No cytotoxicity was observed at the doses tested (Aït-Aïssa *et al*., 2010).

In another AR reporter gene assay, androgen sensitive PALM cells were exposed in two different laboratories for 22–24 hours to prochloraz (purity 99.5%) at concentrations of 0.01, 0.033, 0.1, 0.33, 1, 3.3 and 10 µM, with 0.2 nM 17α-methyldihydrotestosterone (MDHT). Afterwards, cells were lysed, luciferase assay reagent was added and luminescence measured. Additionally, cytotoxicity was determined using the lactate dehydrogenase test. Prochloraz showed anti-androgenic properties, yielding an $IC_{50}$ value of 3.798–7.699 µM in one laboratory. In the other laboratory modest indication of antagonism was reported but without a clear concentration–response relationship, so no $IC_{50}$ was calculated. No cytotoxicity was observed at the tested doses (Freyberger et al., 2010b).

In a yeast (anti)androgenic screening assay, transfected yeast was exposed for 48 hours to prochloraz (purity 99.5%) at concentrations ranging from 100 pM to 100 µM, with or without 5 nM DHT. Afterwards, absorbance was measured at 540 nm with a plate reader. Additionally, cytotoxicity was determined and controls were included. Prochloraz showed an anti-androgenic response in the yeast screening assay. No androgenic activity was observed (Kolle et al., 2010).

In another AR reporter gene assay, transfected human T47D-ARE cells were exposed for 24 hours to prochloraz (purity 99.1%) at concentrations ranging from 100 pM to 100 µM, with and without 20 nM testosterone. Afterwards, cells were lysed, luciferase assay reagent was added and luminescence measured. Cytotoxicity was determined using the MTT assay. Prochloraz showed an anti-androgenic response in T47D-ARE cells with an $IC_{50}$ of 11.7 µM and a relative potency of 0.21 when compared to flutamide. No androgenic activity was observed. Cytotoxicity was not observed at the concentrations tested. It was not mentioned whether flutamide was metabolized by T47D-ARE cells (Roelofs et al., 2014).

***Cell proliferation assays***

Rat pituitary GH3 cells were exposed for six days to prochloraz (purity 95% or greater) at concentrations 0.01 0.1, 1, 10 or 50 µM, with and without 0.5 nM triiodothyronine (T3), a concentration of T3 that induced half of the maximal response. Afterwards, cell growth was measured by staining the fixed cells with sulforhodamine-B dye. Both negative (solvent) and positive controls were included. Additionally, the cytotoxicity was measured using the LDH test. Prochloraz had a weak decreasing effect on GH3 growth and only significantly decreased the cell proliferation to approximately 70% of its basal level at concentration ranging from 10 nM to 10 µM. Moreover, prochloraz dose-dependently inhibited the T3-induced GH3 growth down to 55% of the control value at 10 µM. Prochloraz yielded a LOEC of 10 nM and a MOEC of 10 µM in both the presence and absence of T3-induction, meaning prochloraz has the potential to disrupt the thyroid hormone system. Prochloraz was cytotoxic to GH3 cells at and above 10 µM (Ghisari & Bonefeld-Jørgensen, 2005).

In another cell proliferation assay, human breast cancer cells (MCF7) were exposed to prochloraz (purity 97% or greater) for six days at concentrations 0.001, 0.1, 1 and 10 µM. After six days, 0.1 mg/mL resazurin was added to each well and the plates incubated for four hours and read at an emission wavelength of 590 nm, with an excitation wavelength of 560 nm. Prochloraz did not induce cell proliferation, so showed no estrogenic potential in this study (Vinggaard, Breinholt & Larsen, 1999).

In another cell proliferation assay, MCF7 cell were exposed for six days to prochloraz at concentrations of 0.001–150 µM, with or without 10 pM 17β-estradiol (the concentration that induced half of the maximum response). Afterwards, cells were fixed and stained and the number of cells measured with an enzyme-linked immunosorbent assay reader. Additionally, cytotoxicity was measured using the LDH test. Prochloraz showed anti-estrogenic properties yielding LOEC, $IC_{25}$, $IC_{50}$ and $IC_{75}$ values of 12.5, 21, 28 and 38 µM respectively. Cytotoxicity was only detected at the highest concentration and thus cannot explain the decreased cell proliferation at lower concentrations of prochloraz. No estrogenic activity was observed for prochloraz (Kjærstad et al., 2007).

In another cell proliferation assay, MCF7 cells were exposed to prochloraz (purity 99.5%) for six days at 20 concentrations of 0.001–150 µM, with or without 10 pM 17β-Estradiol. Afterwards,

cells were fixed and stained and the number of cells measured with an ELISA reader. Additionally, cytotoxicity was measured using the LDH test. Prochloraz showed anti-estrogenic properties yielding LOEC, $IC_{25}$, $IC_{50}$ and $IC_{75}$ values of 3.1, 21, 29 and 38 µM respectively. Cytotoxicity was detected at and above 75 µM and cannot explain the decreased cell proliferation at lower concentrations of prochloraz. No estrogenic activity was observed for prochloraz (Kjærstad et al., 2010a).

In another cell proliferation assay, MCF7 cells were exposed to prochloraz for six days at concentrations ranging from 10 nM to 50 µM, with or without 10 pM 17β-estradiol. Afterwards, cells were fixed and stained and the number of cells measured at 492 nm using a plate reader. Additionally. cytotoxicity was measured using the LDH test. No estrogenic potential was observed for prochloraz, however, prochloraz showed anti-estrogenic properties in a dose-related manner at 1 µM and above. The LOEC and MOEC were 1 µM and 25 µM respectively. Prochloraz showed a potency of 47% when compared to10 pM 17β-estradiol. Cytotoxicity was detected at concentrations above 25 µM (Andersen et al., 2002).

***ER transactivation assays***

In this estrogen receptor transactivation assay, transfected MCF7 cells were exposed for 24 hours to prochloraz at concentrations ranging from 10 nM to 50 µM, with and without 10 nM 17β-estradiol, the concentration of ER agonist which induces the maximum response. Afterwards the cells were lysed and β-gal and luciferase activity were measured using a luminometer. Cytotoxicity was determined using a LDH test and controls were included. No estrogenic potential was observed for prochloraz, however, prochloraz showed anti-estrogenic properties in a dose-related manner at 10 µM and above. The LOEC and MOEC were 10 µM and 25 µM, respectively. Prochloraz showed a potency of 51% when compared to 10 nM 17β-estradiol. Cytotoxicity was detected at prochloraz concentrations above 25 µM (Andersen et al., 2002).

In a ER-CALUX assay, MVLN cells were exposed to prochloraz at concentrations of 0.5–50 µM. For agonist testing, prochloraz was incubated in the presence or absence of 50 nM ICI 182,780, an antiestrogen. For antagonist testing, prochloraz was incubated in the presence of 25 pM of 17β-estradiol. After 24 hours of incubation, cells were lysed, the luciferase activity measured and protein content determined, to correct for cell density. Cytotoxicity was determined according to the CellTiter 96 cell proliferation assay and controls were included. No estrogenic potential was observed for prochloraz, however, prochloraz showed anti-estrogenic properties in a dose-related manner. Prochloraz at a concentration of 25 µM impaired the 17β-estradiol response to 70% of the control containing17β-estradiol alone. Cytotoxicity was detected at concentrations above 25 µM prochloraz (Bonefeld-Jørgensen, Grünfeld, & Gjermandsen, 2005).

In another ER transactivation assay, MCF7 cells were exposed to prochloraz (purity 98.5%) for 48 hours at concentrations of 1 or 25 nM, with or without 0.06 nM 17β-estradiol. Afterwards, total RNA was isolated, to perform a polymerase chain reaction (PCR) based on fluorescence monitoring. Controls were included. Prochloraz did not have any significant effect on ERα and ERβ mRNA when tested alone. Prochloraz at 1 µM counteracted the 17β-estradiol-induced decrease in the ERα mRNA level. No effect was observed at a concentration of 25 µM prochloraz. Prochloraz increased the ERβ mRNA level counteracting the effect of 17β-estradiol only at 25 µM prochloraz. This study demonstrated that prochloraz has the ability to interfere with the ERα and ERβ mRNA steady state levels (Grünfeld & Bonefeld-Jørgensen, 2004).

In a RT-PCR assay, MCF7 cells were exposed to 10 µM prochloraz (purity 98.5%) with and without 100nM ICI 182,780, for 3, 24 or 48 hours. Afterwards, total RNA was isolated to perform PCR based on fluorescence monitoring. Moreover, a Western Blot analysis was conducted. MCF-7cells were treated with 17β-Estradiol, ICI 182,780 (an antiestrogen) or prochloraz (10 µM) for 3 or 24 hours. Exposure to prochloraz for 48 hours significantly down-regulated the mRNA levels of ERα and ERβ compared to the control cells. The impairing effect of prochloraz on ERα and ERβ mRNAs was abolished by ICI 182,780. Prochloraz decreased the ERα protein level after three hours of exposure, but the level had recovered to its basal level after 24 hours. The effect on ERβ was not measured in a western blot procedure. This study demonstrated that prochloraz had the ability to interfere with ERα and ERß mRNA and ERα protein levels in MCF7 cells (Hofmeister & Bonefeld-Jørgensen, 2004).

***ER transactivation (YES) assays***

In a yeast estrogen screen (YES) assay, yeast cells were exposed for four days to prochloraz (purity 97% or greater) at concentrations of 0.24–500 µM. Afterwards, β-galactosidase activity was quantified by measuring absorption with an ELISA reader. Prochloraz showed no estrogenic response in the YES assay. Cytotoxicity was observed as starting somewhere between 30 µM and 63 µM, but this was not specified for each compound individually (Vinggaard, Breinholt & Larsen, 1999).

In a YES assay, recombinant yeast cells were exposed for 48 hours to prochloraz (purity 99.5%) at concentrations ranging from 100 pM to 100 µM, with and without 1 nM 17β-estradiol. Afterwards, absorbance was measured using a plate reader. Positive controls were included. Prochloraz showed an anti-estrogenic response in the YES assay. It is not reported at which concentrations prochloraz exerted this anti-estrogenic effect. No estrogenic activity was observed (Kolle et al., 2010).

***Aromatase assays***

Prochloraz (purity greater than 97%) was added to placental microsomes at concentrations of 0.1–100 µM, and after pre-incubation for 20 minutes at 37°C, the reaction was started by the addition of NADPH. The reaction was stopped after 15 minutes by adding trichloromethane and sodium chloride. After vortexing and centrifugation, a portion of the water phase was transferred to a scintillation vial, mixed with Hionic-Fluor scintillation cocktail, and scintillation counting performed. The positive control, 1 µM 4-hydroxyandrostendione, caused a statistically significant inhibition of CYP19 aromatase activity (by 74%) in human placental microsomes. Prochloraz caused a statistically significant inhibition of aromatase activity at a concentration of 50 µM (down to ca 8% of control value) in human placental microsomes with an $IC_{50}$ of 0.34 µM (Vinggaard et al., 2000).

Prochloraz was added to MCF-7 cells at 16 concentrations between 0.001 µM and 100 µM. Following a six day incubation in the presence or absence of 1 µM testosterone, cells were fixed and stained and the number of cells measured using an ELISA reader. Cytotoxicity was determined using an LDH test. Prochloraz caused a statistically significant inhibition of aromatase activity in MCF-7 cells at 1 µM and above. The $IC_{25,}$ $IC_{50}$ and $IC_{75}$ values were calculated to be 3, 9 and 25 µM, respectively. Cytotoxicity was observed at 75 µM and above. The concentrations needed to reduce the testosterone-induced response were lower than those needed to reduce the 17β-estradiol-induced response, indicating that prochloraz possesses both aromatase inhibiting and anti-estrogenic properties but that aromatase inhibition dominates at low concentrations (Kjærstad et al., 2007).

Prochloraz (purity 99.5%) was added to MCF-7 cells at 20 concentrations between 0.001 µM and 150 µM. Following a six day incubation in the presence or absence of 1 µM testosterone, cells were fixed and stained and the number of cells was measured using an ELISA reader. Cytotoxicity was determined using an LDH test. Prochloraz caused a statistically significant inhibition of aromatase activity in MCF-7 cells at 1 µM and above. The $IC_{25}$, $IC_{50}$ and $IC_{75}$ values were calculated to be 7, 15 and 27 µM, respectively. Cytotoxicity was observed from at 75 µM and above (Kjærstad et al., 2010a).

Prochloraz was tested for its ability to modulate aromatase activity using the JEG-3 human choriocarcinoma cell line. Experiments were conducted with different exposure times. In the first experiment, the directly competitive effects of prochloraz (at 1, 3 and 10 µM) alongside 80 nM [*1β*-$^{3}$H]androst-4-ene-3,17-dione on the aromatase enzymic complex was studied after a two-hour incubation. In the second experiment, prochloraz was incubated in a 24 hour exposure prior to changing the medium and beginning the aromatase assay described in the first experiment. This was done to take into account the possible effects of chemical aromatase gene expression through interference at the transcription or (post)translation level. After incubation with [*1β*-$^{3}$H]androst-4-ene-3,17-dione for two hours at 37°C, plates were vortexed and centrifuged and a portion of the water phase was transferred to a scintillation vial, mixed with scintillation fluid and counted by LSC. Cytotoxicity was determined in cells by means of the methylthiazol tetrazolium (MTT) assay. Prochloraz caused a statistically significant inhibition of aromatase activity at concentrations of 1 µM and above in JEG-3 cells, with an $IC_{50}$ of greater than1 µM (two hour exposure) and 2.4 µM (24 hour exposure). The basal aromatase activity was inhibited by 95% and 83% after a two hour and a 24 hour exposure to 10 µM prochloraz, respectively (Laville et al., 2006).

H295R cells were treated with prochloraz (0.1–100 µM) for 24 hours. Medium containing

54 nM [*1β*-$^3$H]androstenedione was added following prochloraz treatment, and cells were incubated for 1.5 hours at 37°C. Aromatase activity was measured using the titrated water release assay. Cytotoxicity was determined in cells by means of the MTT and neutral red uptake assay. Controls were run concurrently. Prochloraz caused inhibition of aromatase activity at concentrations of 0.1 µM and above in H295R cells with an $IC_{50}$ of 0.1 µM. The basal aromatase activity was inhibited by up to a value of 90% or greater after exposure to prochloraz. Prochloraz demonstrated mixed inhibition kinetics. Prochloraz and imazalil had $K_i$ values of 0.037 and 0.015 µM, and $K'_i$ values of 0.33 and 0.37 µM, respectively (Sanderson et al., 2002).

In another aromatase inhibition study a fluorimetric assay based on human recombinant CYP19 enzyme with dibenzylfluorescein (DBF) as a substrate was used to compare the inhibitory potency of 22 azole compounds. A reaction mixture containing potassium phosphate buffer (with cofactors) and prochloraz solution was prepared. To this 5 µL of enzyme was added and the sample pre-incubated for five minutes at 37°C. Reactions were started by the addition of DBF. Addition of sodium hydroxide (2 M) after 30 minutes stopped the enzymic reaction and resulted in cleavage of the oxidized DBF to a fluorescent product. After two hours fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Prochloraz caused inhibition of recombinant aromatase activity with an $IC_{50}$ of 0.047 µM. Of the azoles tested only two breast cancer adjuvant drugs and one medicinal antifungal azole had lower $IC_{50}$ values than prochloraz (Trösken et al., 2004).

In a second study by the same author aromatase inhibition was studied in human CYP19 cells using a new LC-MS/MS method for the analysis of estradiol production. A reaction mixture containing potassium phosphate buffer (with cofactors) was added to testosterone solution and azole dilution. The reaction mixture was pre-incubated for five minutes at 37°C, after which the reaction was started by addition of CYP19. After 40 minutes propan-2-ol, containing d6-cholesterol as an internal standard, was added to stop the reaction. Protein was removed by centrifugation at 15 000*g* for five minutes and the supernatant analyzed by LC–MS/MS. Prochloraz caused inhibition of recombinant aromatase activity with an $IC_{50}$ of 0.44 µM. (Trösken et al., 2006).

In another study, the potential of a number of azole compound to inhibit the aromatase activity of human placental cells and fetal liver was investigated. To this end [$^3$H]androstendione (22 Ci/mmol) and $NADP^+$ were incubated in the presence of a NADPH-regenerating system. The reaction was initiated by the addition of androstenedione and proceeded for 30 minutes at 37°C. Determination of the reaction was performed by the addition trichloroacetic acid. After the addition of trichloromethane and centrifugation, radioactivity in the supernatant was quantified by liquid scintillation spectrometry. Prochloraz caused inhibition of aromatase activity in human placental microsomes with an $IC_{50}$ of 0.7 µM (Mason, Carr & Murry, 1987).

In a study aimed at comparing human H295R and rat R2C cell lines as in vitro screening tools for effects on aromatase, cells were treated with prochloraz at 0.1–100 µM. Following incubation for 24 hours, H295R and R2C cells were exposed to 54 and 25 nM [*1β*-$^3$H]androstendione respectively, and incubated for 1.5 hours at 37°C and 5% carbon dioxide. Aromatase activity was measured using the titrated water release assay. Cytotoxicity was determined in cells by means of the MTT assay. Controls were run concurrently. Exposure of both cell lines at up to 100 µM inhibited aromatase activity. The H295R cells were about 10 times less sensitive than R2C cells. Kinetics showed that prochloraz is a mixed type inhibitor. For H295R and R2C cells $K_i$ values were determined to be 0.04 µM and 0.004 µM respectively, with corresponding $K'_i$ values of 0.33 µM and 0.41 µM, respectively (Heneweer, Van den Berg & Sanderson, 2004).

Another study investigated the effect of prochloraz on aromatase expression in the canine mammary CMT-U27 cell line. After growing up to 100% confluency, cells were treated with 50 µM prochloraz for 24 hours at 37°C and 5% carbon dioxide. Thereafter, total RNA was extracted and cDNA reverse transcribed. Prior to reaction, a DNase treatment was performed with the RNA sample. Quantitative real-time PCR was performed using primers chosen for CYP19 and 16S rRNA by software assistance. From the results Ct values (threshold cycles) were derived and the level of expression was calculated as $1/2^{Ct}$. Exposure of CMT-U27 cells for 24 hours to prochloraz decreased the aromatase (CYP19) mRNA levels (Mavridis & Pappas, 2010, event abstract – only limited information was available).

In another study, H295R cells were exposed to three different concentrations of prochloraz in the range 0.01–10 μM, in three different tests: indirect aromatase activity (cells exposed for 48 hours prior to rinsing); combined aromatase activity (cells exposed for 48 hours prior to rinsing and cells were also exposed during tritium-release assay); and direct aromatase activity (prochloraz was added during the conduct of the tritium-release assay). At the end of treatment the medium underwent hormone analysis. After treatment for 48 hours cells were washed with PBS and then supplemented medium containing 54 nM [*1β*-$^3$H]androstenedione, and finally incubated for 1.5 hours at 37°C. The reaction was then stopped and an aliquot of the medium removed and all remaining [*1β*-$^3$H]androstenedione removed. Aromatase activity was determined using a tritiated water-release assay in which the rate of conversion of [*1β*-$^3$H]androstenedione to estrone by aromatase. The quantity of tritium in extracts of medium was determined by LSC. The specificity of the reaction for the substrate was determined by use of a competitive test with non-labelled androstenedione and the use of the specific aromatase inhibitor fadrozole. Hormone quantification was performed by extracting samples prior to phase separation by centrifugation. The solvent was evaporated and the residue dissolved in ELISA assay buffer and measured by competitive ELISA. Cytotoxicity was determined in cells by means of the MTT assay prior to exposure. Prochloraz caused a dose-dependent inhibition of direct and combined aromatase activities from control levels, statistically significant at 0.3 μM and above. The response in the indirect aromatase activity experiment was not monotonic; activity increased dose-dependently up to 0.3 μM and then decreased at greater concentrations. At 0.3 μM, aromatase activity was approximately four-fold greater than in the controls, but was significantly less in cells treated with 10 μM prochloraz. Exposure to prochloraz resulted in a dose-dependent decrease in the production of 17β-estradiol and testosterone at all test concentrations. A statistically significant correlation was observed between 17β-estradiol and testosterone but not between the hormones and indirect aromatase activity. The decrease in testosterone levels may indicate that prochloraz affects multiple enzymes within the steroidogenesis pathway, including aromatase and CYP17 (Higley et al., 2010).

***Steroidogenesis assays***

Prochloraz was tested for its ability to affect steroidogenesis in H295R cells. The effect of cell confluency on steroid hormone production was evaluated at two different seeding densities, 30% confluency (ca 200 000 cells/mL), and 70% confluency (ca 470 000 cells/mL). To examine the possible influence of hormones and other substances in the cell culture medium on hormone production, the medium was stripped with dextran-coated charcoal prior to test initiation and the results compared to normal supplemented medium. In the timecourse experiment, H295R cells were cultured under standard conditions and basal hormone production determined in medium collected at 3, 6, 12, 24, 48, and 72 hours. For the chemical tests, cells were exposed to prochloraz at 0.001–1 μM for 48 hours, with controls run concurrently. For hormone quantification, medium samples were extracted prior to phase separation by centrifugation. The solvent was evaporated and the residue was dissolved in ELISA assay buffer and measured by competitive ELISA. Prior to measuring hormone concentrations, cell viability was determined in the solvent control, blank control and the three highest tested concentrations by means of the MTT assay. Cytotoxic concentrations were not included in the hormone measurements. All further experiments were conducted with regular medium using cells seeded at 30% confluency (200 000 cells/mL) for 48 hours, since these conditions better to monitored positive and negative effects. Exposure to prochloraz produced significant effects on progesterone, testosterone, and estradiol. Progesterone concentrations increased in a dose-dependent manner with levels being significantly greater than the control at concentrations of 0.01 μM and above. Testosterone and estradiol decreased in a dose-dependent manner at concentrations greater than 0.03 μM and 0.003 μM, respectively. Maximal changes in hormone concentrations were observed at 1 μM, the greatest non-cytotoxic dose of prochloraz. At this dose, progesterone concentrations were five-fold greater than the solvent control while estradiol and testosterone concentrations were 5.5- and 3-fold lower respectively, than their corresponding solvent controls. Cytotoxicity was observed at concentrations above 3 μM (Hecker et al., 2006).

H295R cells were exposed to prochloraz (0.01, 0.03, 0.1, 0.3, 1 or 3 μM) dissolved in DMSO. After 48 hours the medium was removed and steroid hormones measured. Cytotoxicity was determined using resazurin solution. Prochloraz did inhibit the production of testosterone and estradiol from concentrations 0.01 μM and 0.1 μM respectively. Estradiol levels were also reduced at 0.01 and 0.03 μM prochloraz, but without reaching statistical significance. Prochloraz led to increased progesterone

levels from 0.3–1 μM, whereas at 3 μM prochloraz the progesterone was slightly decreased compared to 0.3 and 1 μM incubations, but still higher than for control, with statistical significance. This effect was not due to cytotoxicity, since no cytotoxicity was observed at any of the tested concentrations (Kjærstad et al., 2007).

In one study to investigate and compare the endocrine disrupting properties of conazole compounds (often used as pharmaceutical antifungals or pesticides), prochloraz was the positive control. H295R cells were exposed to prochloraz (purity 99.5%) dissolved in DMSO, at concentrations of 0.01, 0.03, 0.1, 0.3, 1 or 3 μM. After 48 hours the medium was removed and steroid hormones measured. Cytotoxicity was determined using resazurin solution. Prochloraz inhibited the production of both testosterone and estradiol at the lowest concentration measured, 0.01 μM. For testosterone the $IC_{25}$, $IC_{50}$ and $IC_{75}$ were 0.0004, 0.005 and 0.06 μM respectively. For estradiol the $IC_{25}$, $IC_{50}$ and $IC_{75}$ were 0.005, 0.02 and 0.08 μM respectively. Prochloraz led to increased progesterone levels at 0.01 μM and above; the largest increase was seen at 0.3 μM. No cytotoxicity was observed for prochloraz up to 3 μM (Kjærstad et al., 2010a).

H295R cells were exposed to prochloraz (purity 99.3%) dissolved in DMSO, at concentrations of 0.01, 0.03, 0.1, 0.3, 1 or 3 μM. After 48 hours the medium was removed and steroid hormones measured. Similarly, mLTC-1 cells were exposed to prochloraz, but at 6.25, 12.5, 25, 50 or 100 μM. After 48 hours the cells were washed in PBS and stimulated with human chorionic gonadotropin (hCG). After three hours of incubation the stimulation the medium was removed and the steroid hormones measured by fluorescence. Cytotoxicity was determined using resazurin solution. In H295R cells prochloraz inhibited the production of testosterone by 50% at the lowest concentration 0.01 μM and nearly completely at 1 and 3 μM. Estradiol levels were also reduced at 0.1 μM and above, showing a dose-dependent correlation. Prochloraz led to increased progesterone levels from 0.1–3 μM. In control and hCG-induced mLTC-1 cells, prochloraz caused a dose-dependent decrease in progesterone production. Preliminary data indicated that the effect manifests itself at a level at or after protein kinase A activation. No cytotoxicity was observed for prochloraz up to 3 μM (Laier et al., 2006).

In another study, the effect of prochloraz on human 5α-reductase activity in prostate and LNCaP cells was investigated. Prostate tissue was homogenized and centrifuged. Protein concentration was determined for the supernatant containing the microsomal fraction. Prostate homogenates were then exposed to prochloraz for 30 minutes incubation. Pre-incubation was stopped by chilling, and 100 μL of [$^3$H]androstenedione solution was added. The enzyme reaction was started by adding 50 μL homogenization buffer containing 3 mM NADPH. After 30 minutes the reaction was stopped by chilling on ice. Separately, LNCaP cells were exposed to prochloraz. The enzyme assay was started by adding medium containing 0.5 μM of [$^3$H]androstenedione and stopped after 60 minutes by chilling on ice. Solvent controls were included in the experiment. Protein concentrations were determined after lysing cell samples with sodium hydroxide. Prochloraz inhibited 5 α-reductase activity in both prostate tissue and LNCaP cells. The $IC_{20}$ values derived with prostate tissue and LNCaP cells were 4.6 and 14.4 μM respectively. The respective $IC_{50}$ values were 12.4 and 53.2 μM in prostate tissue and LNCaP cells (Lo et al., 2007).

In a novel steroidogenic model, mouse Leydig BLTK1 cells were treated with DMSO or prochloraz (0.1, 0.3, 1, 3, 10 or 30 μM) for four hours. BLTK1 cells were also treated with 30 μM prochloraz in the presence of recombinant hCG for four hours. Testosterone levels were analyzed by enzyme immunoassay. Cytotoxicity was assessed using the MTT assay. Prochloraz showed marked reduction of MTT activity at 100 mM and above. Prochloraz blocked testosterone induction by recombinant hCG, but did not alter basal testosterone levels, most likely due to low basal level of production in BLTK1 cells (Forgacs et al., 2012).

H295R cells were exposed to prochloraz (purity 98% or greater) dissolved in ethanol at 0.001, 0.01, 0.03, 0.09, 0.2, 0.33 and 1 μM. After 72 hours the medium was removed and samples spiked with six deuterated analogues. The samples were enriched by solid phase extraction cartridges, derivatized, dried and the solutions analyzed by GC-MS/MS for seven key steroid hormones. Cytotoxicity was determined using resazurin solution. In the quality control plates prochloraz inhibited testosterone by 1.5- to 6.3-fold and 17β-estradiol was not detected in any of the quality control samples. Prochloraz led to concentration-dependent, increased pregnenolone and progesterone upstream of P450c17. Downstream concentration-dependent reductions were observed for dehydroepiandrosterone, androstenedione and

estrone, while no clear trends were visible for testosterone and 17β-estradiol. No cytotoxicity was observed for prochloraz up to 1 µM (Nielsen et al., 2012).

H295R cells were treated with 4 µM prochloraz for 24 hours in medium with or without forskolin or angiotensin to mimic adrenocorticotrophic hormone (ACTH) stimulation and the activation of the pathway leading to synthesis and secretion of aldosterone. After exposure the medium was removed. Cortisol and aldosterone levels in culture medium were measured by ELISA. Cytotoxicity was determined using the CellTiter96®AQueos assay. Prochloraz led to decreased basal or forskolin-induced cortisol levels (decreased by 75%–100%). Similarly, prochloraz led to decreased basal or angiotensin-II-induced aldosterone levels (decrease by 25%–50%). No cytotoxicity (less than 10% reduction in viability) was observed for prochloraz up to 4 µM (Ullerås, Ohlsson & Oskarsson, 2008).

H295R cells were treated in medium for 24 hours with 0.01, 0.1, 1 or 10 µM prochloraz. After exposure the medium was removed and cortisol, aldosterone, progesterone, 17α-OH-progesterone, and corticosterone levels in culture medium were measured by ELISA. Additionally, following treatment of the cells, total RNA was isolated, cDNA was synthesised and gene expression of StAR, CYP11A1, CYP17A1, 3β-HSD2, CYP21A2, CYP11B1 and CYP11B2 were quantified using real-time PCR. Separately, following the 24 hour treatment period, the effects of prochloraz on CYP17A1 (hydroxylase and lyase) activity were determined in cells incubated with pregnenolone and trilostane (to inhibit 3β-hydroxysteroid dehydrogenase activity). After 24 hours the production of dihydroepiandrosterone (DHEA) was measured by ELISA. The combined CYP21A2 and CYP11B1 activity was determined by cortisol measurement after 24 hours incubation of the cells with prochloraz and 17α-OH-progesterone. The CYP11B1 activity was determined by measurement of cortisol secretion after incubation with prochloraz and 11-deoxycortisol. Additionally, cytotoxicity was determined using the CellTiter96®AQueos assay. No cytotoxicity was observed for prochloraz up to 10 µM. Prochloraz treatment displayed a biphasic effect on aldosterone secretion with a low-dose stimulation followed by an inhibition at higher doses. A two-fold increase in aldosterone secretion was observed at 0.1 µM of prochloraz followed by a sharp decrease at concentrations above 0.3 µM. Prochloraz inhibited aldosterone secretion to 23% of the control level at a concentration of 10 µM. In contrast to aldosterone, a dose-dependent inhibition was observed for cortisol secretion. Starting at 80% of the control level after treatment with 0.01 µM prochloraz, cortisol secretion was down to approximately 20% of the control level at 0.3 µM prochloraz. At this concentration the aldosterone secretion peaked following prochloraz treatment. Prochloraz treatment displayed a biphasic effect on progesterone, 17α-OH-progesterone and corticosterone secretion with a low-dose stimulation followed by an inhibition at higher doses. Progesterone secretion was stimulated to a maximum of 5.3-fold of the control level at 1 µM prochloraz. In contrast to the other steroids, progesterone secretion increased in the presence of 10 µM prochloraz to 3.6-fold the control level. The secretion of 17α-OH-progesterone reached a maximum 1.6-fold control levels at the 0.3 µM prochloraz treatment. Following 10 µM prochloraz treatment, 17α-OH-progesterone secretion was reduced to about 50% of control levels. Corticosterone secretion showed a similar pattern as aldosterone. The maximum increase in corticosterone of 2.3-fold, reached at 0.1 µM prochloraz, was followed by at least a 50% inhibition at 10 µM prochloraz. Dose-dependent inhibition of gene expression (RNA in the cell) following prochloraz treatment was statistically significant for StAR, CYP11A1, CYP17A1, 3β-HSD2, CYP21A2 and CYP11B1. No dose-dependent pattern could be observed for CYP11B2. The activity of CYP17A1 and CYP21A2, as measured by conversion of several substrates, revealed a dose-dependent inhibition of hormone secretion following prochloraz treatment. No dose-dependent pattern could be observed for CYP11B1. Overall, the study showed that prochloraz exhibits specific effects on the secretion of adrenal aldosterone and cortisol, and this is mediated by inhibition of the steroidogenic enzymes CYP17A1 and CYP21A2 (Ohlsson, Ullerås & Oskarsson, 2009).

H295R cells were treated with prochloraz in medium at 0.003, 0.01, 0.03, 0.1, 0.3, 0.7, 1, 3 and 10 µM for 24 hours. After exposure the medium was removed and cortisol and aldosterone levels in the culture medium were measured by ELISA. After treatment of cells, total RNA was isolated, cDNA was synthesised and gene expression of StAR, CYP11A1, CYP17A1, 3β-HSD2, CYP21A2, CYP11B1 and CYP11B2 was quantified by real-time PCR. Cytotoxicity was determined using the CellTiter96®AQueos assay. No cytotoxicity was observed for prochloraz up to 10 µM. Prochloraz treatment displayed a biphasic effect on aldosterone secretion with a low-dose stimulation followed by inhibition at higher doses; $EC_{50}$ for inhibition was 11.2 ± 3.8 µM. Stimulation maximum was 2.1-fold control level with 0.06 µM prochloraz. In contrast to aldosterone, a dose-dependent inhibition was

observed for cortisol secretion with $IC_{50}$ of 0.13 ± 0.02 μM). Gene expression following prochloraz treatment (0.03 μM) slightly decreased the gene expression of StAR, 3β-HSD2 and CYP11B1 although only the difference for 3β-HSD2 was statistically significant. No effects were observed for CYP11B2, CYP11A1, CYP17A1 or CYP21A2 (Ohlsson et al., 2010).

H295R cells were treated with prochloraz dissolved in methanol at 0.001, 0.01, 0.03, 0.1, 0.3, 1 and 10 μM for 48 hours. After exposure, the medium was removed and samples extracted and analyzed by LC-MS/MS to quantify cortisol and corticosterone. Cytotoxicity was determined using the alamarBlue® viability assay. Prochloraz induced a concentration-dependent inhibition of cortisol and corticosterone secretion. No effect was observed at 0.001 μM. Exposure to 1 μM prochloraz resulted in an 11.8 ± 4.4-fold inhibition of cortisol and a 6.2 ± 2.1-fold inhibition for corticosterone (if one outlier is removed).The $IC_{50}$ values of 0.06 ± 0.01 μM and 0.09 ± 0.02 μM respectively were observed for cortisol and corticosterone secretion after treatment with prochloraz. No cytotoxicity was observed the concentrations tested (Winther et al., 2013).

H295R cells were treated for 48 hours with 1 μM prochloraz dissolved in DMSO. After exposure, the medium was removed and levels of pregnenolone, 17α-OH-pregnenolone, progesterone, 17α-OH-progesterone, dehydroepiandrosterone, androstenedione, testosterone, 11-deoxycorticosterone, corticosterone, 11-deoxycortisol and cortisol were measured by LC-MS/MS. Levels of 17β-estradiol and aldosterone were determined by means of enzyme immunoassays. In addition, total RNA was isolated, cDNA was synthesised and gene expression of StAR, CYP1A1, MC2R, CYP11A1, 3β-HSD2, CYP11B1, CYP11B2, and CYP19A1 was determined by real-time PCR. Prochloraz was the negative control in this study and it is not mentioned if cytotoxicity was also determined using the WST-1 cytotoxicity assay and the ATPlite assay. Prochloraz significantly reduced all hormone levels, except for progesterone, pregnenolone and 11-deoxycorticosterone. Progesterone levels were increased by 10-fold. None of the gene expression levels were decreased, however CYP1A1 and CYP19A1 mRNA levels were statistically significantly increased (Van den Dungen et al., 2015).

Jeg-3 cells were treated for 48 hours with prochloraz dissolved in DMSO at 0.01–40 μM. After exposure, the medium was removed and hormone levels of progesterone and 17β-estradiol were determined by ELISA. After treatment of cells, total RNA was isolated, cDNA was synthesised and gene expression of 18sRNA, GAPDH, CYP1A1, CYP11A1, CYP17A, CYP19, CYP1A2, ABCC1, UGT2B4 and UGT2B17 was quantified by real-time PCR. Cytotoxicity was determined using the WST-1 cytotoxicity assay. Prochloraz significantly reduced progesterone levels at 2.5 μM and above (to 60% of control levels), and increased 17β-estradiol levels to about 250% of control values at 40 μM. No gene expression changes except for CYP19 were observed regarding enzymes relevant to steroid biosynthesis. Prochloraz increased CYP19 mRNA 2.5-fold. Furthermore, CYP1A1 mRNA was increased up to a maximum of 40-fold at 40 μM prochloraz (Rieke et al., 2014).

Murine Leydig tumour cells (MA-10) were exposed to 10 μM prochloraz (purity 99.1) dissolved in DMSO. The compound was tested alone or in combination with luteinizing hormone. After 48 hours the medium was removed and samples analyzed for testosterone secretion, by testosterone radioimmunoassay. To analyze gene expression, cells were exposed to 10 μM prochloraz for six hours. Total RNA was then isolated, cDNA prepared and gene expression of Cyp51, HMG-CoA reductase, StAR, Cyp11A1, Cyp17A1, 3β-HSD1, 17βHSD3 and cytochrome P450 oxidoreductase determined by real-time PCR using β-actin as a reference gene. Cytotoxicity was measured using the MTT assay. Prochloraz at 10 μM did inhibit the production of testosterone to 23% of the control level. Furthermore, prochloraz increased the gene expression of Cyp51 by1.7-fold and HMG-CoA reductase by 1.9-fold. No change in gene expression was observed for StAR, Cyp11A1, Cyp17, 3β-HSD1, 17βHSD3 or cytochrome P450 oxidoreductase. No cytotoxicity was observed (Roelofs et al., 2014).

***Mechanistic study***

The antiandrogenic mechanism by which prochloraz acts was investigated in human prostate carcinoma cells (LNCaP). Cytotoxicity was tested using a WST-1 assay. LNCaP cells were exposed to prochloraz (purity 99.1%) at 1, 3, 10 or 30 μM in the presence or absence of 0.1 nM DHT for 24 hours. For the prostatic-specific antigen (PSA) secretion study, PSA levels were determined using ELISA following 24 hours of exposure to 1, 3, 10 or 30 μM prochloraz, with or without 0.3 nM DHT. Moreover, to measure AR protein expression and phosphorylation, cytoplasmic and nuclear proteins were extracted after

24 hours of exposure to 1 or 10 µM prochloraz, both with 10 nM DHT, and analyzed.. Phosphorylated AR levels were determined in total lysates of LNCaP cells exposed for 1, 6 or 24 hours to pesticides with or without DHT. The same study also exposed human adrenocortical carcinoma cells (H295R) to prochloraz. Cytotoxicity was tested by exposing cells to concentrations 1, 3, 10, 30 or 100 µM prochloraz for 24 hours. Also, CYP17 gene and protein expression, SRD5A gene expression and CYP17 activity were assessed with H295R cells. H295R cells were exposed to fresh medium containing 10 µM prochloraz for 24 hours. To measure gene expression, RNA was extracted and quantified with a SpectraMax M5 spectrophotometer, reverse transcription and PCR. For measuring protein expression, protein was extracted and quantified using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. To assess CYP17 catalytic activity, H295 cells were exposed to 1 and 10 µM prochloraz for 24 hours. The medium was replaced with fresh medium lacking supplements, but containing 100 nM of the CYP17 substrate pregnenolone and 3β-hydroxysteroid dehydrogenase-inhibitor trilostane. After a 90-minute incubation, the reaction medium was removed and dehydroepiandrosterone content was quantified by ELISA. Prochloraz did not affect basal LNCaP cell proliferation at concentrations between 1 and 30 µM. However, prochloraz at concentrations at or above 10 µM did reduce DHT-stimulated LNCaP cell proliferation in a dose-dependent manner, and to a statistically significant extent. At a level of 30 µM, prochloraz reduced DHT-stimulated cell proliferation by 88%. Prochloraz, at concentrations between 1 and 30 µM did not affect basal secretion of PSA in LNCaP cells. However, prochloraz at concentrations at or above 1 µM did reduce DHT-stimulated PSA secretion in a dose-dependent manner by 90% or more, which was statistically significant. Prochloraz significantly reduced the DHT-stimulated increase in nuclear AR levels after 24 hours of exposure to 1 or 10 µM prochloraz. Prochloraz at 30 µM decreased the DHT-mediated phosphorylation of AR on Ser81 and Ser213 in LNCaP cells. Prochloraz was cytotoxic at concentrations greater than 10 µM in H295R cells. Exposure to 10 µM prochloraz decreased expression of the CYP17 gene, as well as protein expression, in H295R cells. Pregnenolone-stimulated dehydroepiandrosterone production, a measure of CYP17 activity, was decreased in H295R cells exposed to 1 or 10 µM prochloraz (Robitaille, Rivest & Sanderson, 2015).

## 3. Observations in humans

### Medical surveillance on manufacturing plant personnel

An annual tonnage of ca 2400 tonnes of prochloraz has been produced at Jiangsu, China, since 1997.

During production, workers are potentially exposed to prochloraz during loading, quality control and packaging operations. During these operations, workers are shielded from prochloraz by structural measures. These workers additionally undergo regular general medical examinations by a professional physician (subcontracted). The medical examinations include, but are not limited to, the following: examination of general health, haematology tests, clinical chemistry determinations, audiometry test, eye test, lung function test and an abdominal test. Additionally, cases of accidental exposures are also followed up, if they occur.

Overall, there is no symptomology observed in known cases of exposure to prochloraz. No consistent prochloraz-related ill health has been detected.

## 4. Microbiological aspects

The possible impact of prochloraz residues on the human intestinal microbiome was evaluated considering two end-points of concern for human health: disruption of the colonization of the human intestinal microbiome and increases in populations of resistant bacteria in the human intestinal microbiome.

Conjugative plasmid transfer is a major contributor to the spread of antibiotic resistance genes (ARGs). A publication by Guo et al., (2022) described a study to evaluate the effects of the fungicide prochloraz alone, or in combination with copper oxide nanoparticles (nano-CuO), on the conjugation of a plasmid between *Escherichia coli* strains. In this non-GLP study, four different concentrations of prochloraz (0, 5, 50 and 500 µg/L) with or without nano-CuO, were tested in an *E. coli* conjugation mating system to investigate the effects on ARG transfer. The authors reported that prochloraz alone

increased cell membrane permeability, extracellular polymeric substances, polysaccharides secretion, and cell adhesion, thus contributing to the conjugative transfer of ARGs. They concluded that the increased conjugative transfer frequency under the single fungicide suggested that the potential risk of prochloraz fungicides on ARG transfer should not be ignored. However, this information is insufficient to complete an evaluation of the end-point of concern for human health related to the increases in populations of resistant bacteria in the human intestinal microbiome.

Therefore a search of literature available in the public domain was made and this identified some, but insufficient, information in the form of direct or indirect experimental evidence which addressed the possible impact of prochloraz residues on the human intestinal microbiome.

No experimental data were submitted by the sponsor in this regard.

# Comments

## Biochemical aspects

Following oral administration, prochloraz was rapidly absorbed, with peak plasma levels of radiolabel reached on average two hours after a low dose (5 mg/kg bw) and about 10 hours following a high dose of 100 mg/kg bw (Reynolds, 1995, 1996). Half-lives in blood and plasma ranged between 11 and 17 hours (Reynolds, 1995). Excretion was rapid, with virtually complete elimination of radioactivity within 96 hours (Challis & Creedy, 1988). Small sex differences in plasma levels, blood and plasma half-lives (Reynolds, 1995) and excretion patterns (Challis & Creedy, 1988; Needham & Challis, 1991) were observed. Excretion of radioactivity through expiration was negligible at both the low dose (Challis & Creedy, 1988) and the high dose (Dawson, 1989). Studies with bile duct-cannulated rats administered a single oral dose of 5 mg/kg bw showed that 48%–49% of the radioactivity was excreted in bile in both males and females. Based on the biliary and urinary excretion, an average of 74% of the administered dose (AD) was absorbed (D'Souza, 1995). Tissue distribution was widespread, but levels in tissues were low. The highest levels of radioactivity were found in liver and kidney (Challis & Creedy, 1988; Dawson, 1989; Reynolds, 1995). Repeated administration of prochloraz (daily oral doses of unlabelled prochloraz at 10 mg/kg bw, followed by a single oral dose of 10 mg/kg bw of labelled prochloraz) had no appreciable influence on the rates of absorption, elimination, nor on the distribution, as judged on the basis of radioactivity levels in excreta and tissues compared to those resulting from a single dose of radiolabelled prochloraz (Crawley, 1994).

Studies in mice and dogs showed the kinetics of prochloraz to be similar in these species (Needham, 1982a; Needham & Campbell, 1982).

Prochloraz is metabolized by modification of the phenyl ring moiety through oxidative hydroxylation (sometimes accompanied by substitution of chlorine) and sulfation, and through stepwise degradation of the imidazole ring and the resulting *N*-alkyl chains. The latter step is often followed by oxidative deamination to eliminate the nitrogen atoms, and conjugation of the resultant oxyacyl hydroxyl group. The main metabolite in the urine of rats was BTS 9608 (2,4,6-trichlorophenoxyacetic acid). Unchanged prochloraz was not found in urine. In faeces unchanged prochloraz, BTS 44596 (*N*-formyl-*N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy)ethyl]urea) and BTS 3037 (2-(2,4,6-trichlorophenoxy)ethanol) were found (Campbell & Needham, 1981; Gedik & Kidd, 2003; Needham, 1989).

## Toxicological data

The acute oral median lethal doses ($LD_{50}$) for prochloraz in rats ranged in different studies between 300 mg/kg bw and greater than 4000 mg/kg bw (Dreher, 1990). The acute dermal $LD_{50}$ was greater than 2000 mg/kg bw (Bioassay Labor für biologische Analytik, 2012a; Hounsell & Ogle, 1987) and the acute inhalation median lethal concentration ($LC_{50}$) was greater than 2.1 mg/L (Jackson & Hardy, 1987). Prochloraz was not irritating or, in another study, mildly irritating to the skin of rabbits (Cuthbert & D'Arcy-Burt, 1984a; Kenan, 1989a). Prochloraz was not irritating to the eyes of rabbits (Bioassay Labor für biologische Analytik, 2012a; Cuthbert & D'Arcy-Burt, 1984; Kenan, 1989b). Prochloraz was not a skin sensitizer in two Magnusson & Kligman tests and a Buehler test (Frey-Tox, 2012; Marom & Crown, 1989; Shaw, 1979).

Acute toxicity studies with prochloraz–zinc complex and prochloraz–copper showed similar results to those with prochloraz. Acute oral $LD_{50}$ values for prochloraz–copper complex in rats ranged between 800 mg/kg bw (Stobart, Lancaster & Smithson, 1979a), and greater than 2000 mg/kg bw (Dreher, 2002b). The dermal $LD_{50}$ was greater than 2000 mg/kg bw (Chevalier, 2004a). Prochloraz–copper complex was not irritating to the skin of rabbits (Jackson & Wood, 1990) and was moderately irritating to the eyes of rabbits (Davies, 1990). Prochloraz–zinc complex was not a skin sensitizer in a Magnusson & Kligman test (Dreher, 2002c).

In repeat-dose oral toxicity studies with prochloraz in mice and rats the main target for toxicity was the liver, whereas in dogs the main target for toxicity was the prostate.

In a 13-week study in mice using dietary prochloraz concentrations of 0, 6, 25, 100 or 400 mg/kg bw per day, the no-observed-adverse-effect level (NOAEL) was 25 mg/kg bw per day, based on reduced body weight gain and effects on the liver at 100 mg/kg bw per day (Gale, 1980; Keene, 1988a; Mallyon, 1988a).

In a 90-day study in rats using dietary prochloraz concentrations of 0, 50, 500 or 1500 ppm (equal to 0, 4.4, 43.3 and 137.2 mg/kg bw per day for males, 0, 4.7, 46.6 and 144.9 mg/kg bw per day for females), the NOAEL was 50 ppm (equal to 4.4 mg/kg bw per day), based on effects on the thyroid, kidney and liver weights and on histopathological changes in the liver at 500 ppm (equal to 43.3 mg/kg bw per day) (Marom et al., 1990).

In a 13-week study in dogs using gavage doses of prochloraz at 0, 1 2.5, 7 or 20 mg/kg bw per day, the NOAEL was 2.5 mg/kg bw per day, based on reduced prostate weight at 7 mg/kg bw per day (Gale, 1980; Jackson, 1989; Keene, 1988b; Lancaster, Morgan & Stobart, 1979; Markham, 1988b).

In a one-year study in dogs using gelatin capsules delivering prochloraz doses of 0, 1, 5 or 25 mg/kg bw per day, the NOAEL was 1 mg/kg bw per day, based on reduced prostate weights and histopathological changes in prostate and adrenals at 5 mg/kg bw per day (East, 1994).

The overall NOAEL for the 13-week and the one-year studies in dogs using gavage or gelatine capsule administration was 2.5 mg/kg bw per day, and the overall lowest-observed-adverse-effect level (LOAEL) was 5 mg/kg bw per day.

In a 104-week study in dogs using dietary prochloraz concentrations of 0, 30, 135 or 600 ppm (equal to 0, 0.94, 4.5 and 18 mg/kg bw per day for males, 0, 0.90, 4.1 and 18 mg/kg bw per day for females), the highest dose was increased at week 57 to 1000 ppm (equal to 29 and 28 mg/kg bw per day for males and females respectively). The NOAEL was 135 ppm (equal to 4.1 mg/kg bw per day), on the basis of decreased prostate weight, increased liver weight, changes in alkaline phosphatase (ALP) activity, and histopathological changes in the prostate and liver, all at 600/1000 ppm (equal to 18 mg/kg bw per day) (Chesterman et al., 1981).

In a 106- or 121-week (for males and females respectively) study in mice using dietary prochloraz concentrations of 0, 78, 325 or 1300 ppm (equal to 0, 7.5, 33 and 134 mg/kg bw per day for males, 0, 8.8, 36 and 149 mg/kg bw for females), the NOAEL was 78 ppm (equal to 7.5 mg/kg bw per day), based on increased liver weights and liver tumours (unspecified) at 325 ppm (equal to 33 mg/kg bw per day) (Colley et al., 1983).

In a 78-week study in mice using dietary prochloraz concentrations of 0, 40, 220 or 1200 ppm (equal to 0.0, 4.7, 26.3 and 151.2 mg/kg bw per day for males, 0.0, 6.0, 31.5 and 182.5 mg/kg bw per day for females), the NOAEL was 40 ppm (equal to 4.7 mg/kg bw per day), based on the increased incidence of liver tumours (adenomas and carcinomas) at 220 ppm (equal to 26.3 mg/kg bw per day) (Chapman et al., 1993a).

The overall NOAEL for the two chronic toxicity studies in mice was 78 ppm (equal to 7.5 mg/kg bw per day), and the overall LOAEL was 220 ppm (equal to 26.3 mg/kg bw per day).

In a 111- or 115-week (for males and females respectively) study in rats using dietary prochloraz concentrations of 0, 37.5, 150 or 625 ppm (equal to 0, 1.3, 5.1 and 21.5 mg/kg bw per day for males, 0, 1.6, 6.4 and 28.1 mg/kg bw per day for females), the NOAEL was 37.5 ppm (equal to 1.3 mg/kg bw per day), based on macroscopic and microscopic signs of liver toxicity at 150 ppm (equal to 5.1 mg/kg bw per day). No effect on tumour incidence due to prochloraz was observed (Colley et al., 1982).

In a two-year study in rats using dietary prochloraz concentrations of 0, 40, 220 or 1200 ppm (equal to 0, 1.7, 9.4 and 50.3 mg/kg bw per day for males, 0, 2.1, 11.8 and 64.1 mg/kg bw per day for females) the NOAEL was 40 ppm (equal to 1.7 mg/kg bw per day), based on histopathological changes in the liver at 220 ppm (equal to 9.4 mg/kg bw per day). No effect of prochloraz on tumour incidence was observed (Chapman et al., 1993b).

The overall NOAEL for the two chronic toxicity studies in rats was 40 ppm (equal to 1.7 mg/kg bw per day), and the overall LOAEL was 150 ppm (equal to 5.1 mg/kg bw per day).

The Meeting concluded that prochloraz is carcinogenic in mice, but not in rats.

Mechanistic studies indicated that the mode of action (MOA) for tumour formation by prochloraz in mice is through activation of CAR and possibly PXR (CXR Biosciences, 2012a, b, c, 2015b, d), a MOA that is not relevant to humans. However, a full investigation of the MOA was not performed.

Prochloraz was tested in an extensive range of in vitro and in vivo assays for genotoxicity. These studies gave consistently negative results.

The Meeting concluded that prochloraz is unlikely to be genotoxic.

In view of the lack of genotoxicity, the absence of carcinogenicity in rats, and the induction of liver tumours in mice through a CAR-mediated MOA, the Meeting concluded that prochloraz is unlikely to pose a carcinogenic risk to humans from the diet.

In a multigeneration study in rats using dietary prochloraz concentrations of 0, 37.5, 150 or 625 ppm (equal to 0, 3.1, 13 and 57 mg/kg bw per day for F0 males, 0, 3.5, 14 and 58 mg/kg bw per day for F0 females, 0, 3.7, 16 and 70 mg/kg bw per day for F1 males, and 0, 4.5, 18 and 81 mg/kg bw per day for F1 females), the NOAEL for parental toxicity was 150 ppm (equal to 13 mg/kg bw per day) based on clinical signs and decreased body weight gain at 625 ppm (equal to 57 mg/kg bw per day). The NOAEL for offspring toxicity was 150 ppm (equal to 13 mg/kg bw per day) based on reduced body weight gain, and effects on liver, brain and thymus weights at 625 ppm (equal to 57 mg/kg bw per day). The NOAEL for reproductive toxicity was 150 ppm (equal to 14 mg/kg bw per day) based on the effects on gestation index, gestation length and dystocia at 625 ppm (equal to 58 mg/kg bw per day) (Cozens et al., 1982).

In another multigeneration study in rats using dietary prochloraz concentrations of 0, 50, 150 or 450 ppm (equal to premating intake levels of 0, 3.8, 10.9 and 34.0 mg/kg bw per day for males, 0, 4.5, 13.7 and 43.0 mg/kg bw per day for females), the NOAEL for parental toxicity was 50 ppm (equal to 3.8 mg/kg bw per day) based on decreased body weight gain at 150 ppm (equal to 10.9 mg/kg bw per day). The NOAEL for offspring toxicity was 150 ppm (equal to 10.9 mg/kg bw per day) based on reduced body weight gain at 450 ppm (equal to 34 mg/kg bw per day). The NOAEL for reproductive toxicity was 50 ppm (equal to 4.5 mg/kg bw per day) based on the effects on gestation index, gestation length and dystocia at 150 ppm (equal to 13.7 mg/kg bw per day) (Reader, 1993).

In a developmental toxicity study in rats using oral doses of prochloraz at 0, 6, 25 or 100 mg/kg bw per day on days 1 to 20 after mating, the NOAEL for maternal toxicity was 25 mg/kg bw per day, based on clinical signs, decreased body weight gain, reduced food consumption and increased liver weight at 100 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 25 mg/kg bw per day, based on reductions in litter size, implantation index and viability index, an increased incidence of dead fetuses and retarded ossification at 100 mg/kg bw per day (Beswick, 1980).

In another developmental toxicity study in rats using gavage doses of prochloraz at 0, 30, 60 or 120 mg/kg bw per day from gestation days (GDs) 6 to 15, the NOAELs for both maternal and embryo/fetal toxicity were 120 mg/kg bw per day, the highest dose tested (Reader, 1991).

In a developmental toxicity study in rabbits using gavage doses of prochloraz of 0, 10, 40 or 160 mg/kg bw per day from GD 6 to GD 18, the NOAEL for maternal toxicity was 40 mg/kg bw per day, based on reduced body weight gain, increased liver weights, increased incidences of animals with total litter loss, and increased incidence of fetal resorptions, at 160 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 40 mg/kg bw per day based on the increased incidence of fetal resorptions at 160 mg/kg bw per day (Becker et al., 1988).

In another developmental toxicity study in rabbits using gavage doses of prochloraz at 0, 10, 40 or 160 mg/kg bw per day from GDs 7 to 19, the NOAEL for maternal toxicity was 40 mg/kg bw per day, based on decreased food consumption and a single case of occult faecal blood observed at 160 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 40 mg/kg bw per day, based on a slight reduction in fetal crown–rump length at 160 mg/kg bw per day (Gal et al., 1990).

In a third developmental toxicity study in rabbits using gavage doses from GD 1 to GD 28 of prochloraz at 0, 3, 12 or 48 mg/kg bw per day, the maternal NOAEL was 12 mg/kg bw per day based on increased liver weight and liver discolouration at 48 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 48 mg/kg bw per day, the highest dose tested (Palmer, Bottomley & Billington, 1980).

In a study from published literature, pregnant rats were treated by gavage with prochloraz from GD 14 until GD 18 at doses of 0, 31, 62, 125 or 250 mg/kg bw per day. Hypospadias and vaginal pouches were noted in males at 125 mg/kg bw per day and above, indicating that prochloraz alters sexual differentiation in an anti-androgenic manner (Noriega et al., 2005).

Various studies from public literature investigated the effect of prochloraz on endocrine systems. In vitro, prochloraz antagonized estrogen and/or androgen receptors, inhibited aromatase and interfered with steroidogenesis at the level of CYP17. In vivo studies in rats showed that prochloraz induced signs that were indicative of anti-androgenic activity, such as retained nipples in males (at 5 mg/kg bw and above; Laier et al., 2006; Melching-Kollmuss et al., 2016; Vinggaard et al., 2005)), reduced plasma and testicular testosterone at 30 mg/kg bw per day and above (Laier et al., 2006; Vinggaard et al., 2005), increased progesterone levels at 7.8 mg/kg bw per day and above, and delayed entry into male puberty at 125 mg/kg bw per day (Blystone et al., 2007a). In addition, reduced androgen-sensitive gene expression and effects on anogenital distance were observed at 30 mg/kg bw per day and above (Melching-Kollmuss et al., 2016). In pregnant rats, prochloraz delayed parturition and caused dystocia (at 14 mg/kg bw per day and above) effects which are likely to have been secondary to aromatase inhibition (Cozens et al., 1982). In dogs prochloraz at 2.5 mg/kg bw per day and above led to reduced male reproductive organ weights (East, 1994; Jackson, 1989; Lancaster, Morgan & Stobart, 1979; Markham, 1988b).

The Meeting concluded that prochloraz is teratogenic, probably through an anti-androgenic mode of action.

In an acute neurotoxicity study in rats using gavage doses of prochloraz at 0, 20, 90 or 405 mg/kg bw, rats were observed for 14 days. The NOAEL for acute neurotoxicity was 405 mg/kg bw, the highest dose tested. The NOAEL for general toxicity was 20 mg/kg bw based on clinical signs, effects on motor activity and rearing counts, righting reflex and approach response, and reduced body temperature, observed at 90 mg/kg bw. (Rees, 2009). No repeat-dose neurotoxicity studies were available, but no evidence of neurotoxicity was observed in other routine repeat-dose studies.

The Meeting concluded that prochloraz is unlikely to be neurotoxic.

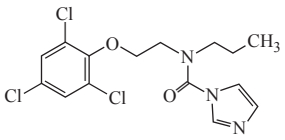
No studies on immunotoxicity were available. In the available toxicity studies on prochloraz no indication of immunotoxic potential was seen.

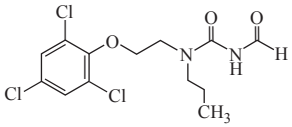
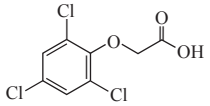
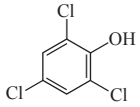
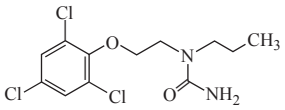
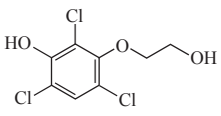
The Meeting concluded that prochloraz is unlikely to be immunotoxic.

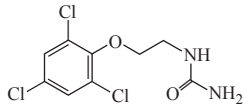
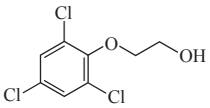
## Toxicological data on metabolites and/or degradates

Pending the periodic review of the residue definition, a number of potentially relevant major metabolites of prochloraz in crops and livestock were identified. The toxicological characteristics of these metabolites are listed in Table 22 below.

***Table 22. Summary of toxicological characterization of plant/livestock metabolites***

| **Compound, codes and structure** | **Major rat metabolite** (>10% AD) | **Genotoxicity assessment** (data, QSAR, read-across) | **General toxicity** | **Toxicological reference values** |
|---|---|---|---|---|
| **Prochloraz**  | Parent | Unlikely to be genotoxic in humans<br>(data) | Full data set | ADI: 0–0.02 mg/kg bw<br>ARfD: 0.2 mg/kg bw |

| Compound, codes and structure | Major rat metabolite (>10% AD) | Genotoxicity assessment (data, QSAR, read-across) | General toxicity | Toxicological reference values |
|---|---|---|---|---|
| **BTS 44596**<br>(*N*-formyl-*N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy) ethyl]urea;<br>M201-03)<br> | This is an intermediate formed early in rat metabolism; its downstream metabolites BTS 3037, BTS 9608, BTS 45186, M18 found in urine, at a combined level of ≥ 28% | Not genotoxic in Ames test<br>No genotoxic alert in QSAR analysis | Oral $LD_{50}$: > 3200 mg/kg bw<br>After oral dose of BTS 44596 to rats excretion of radiolabel virtually complete within 72 hours, with 53–63% of the total dose excreted in urine and the remainder in faeces | Covered by parent |
| **BTS 9608**<br>(2,4,6-trichlorophenoxyacetic acid; M201-13)<br> | Yes<br>BTS 9608 and its glucuronide, 13–35% in urine | No genotoxic alert from QSAR | No data | Covered by parent |
| **BTS 45186**<br>(2,4,6-trichlorophenol; 2,4,6-TCP; M201-15; M3; Phenachlor)<br> | Yes<br>Up to 12% in urine (taking the partial absorption of parent into account) | No genotoxic alert in QSAR analysis | Oral $LD_{50}$: >3200 mg/kg bw.<br>WHO drinking water guideline value of 0.2 mg/L for 2,4,6-TCP<br>2,4,6-TCP is also a metabolite of pydiflumetofen, for which JMPR 2018 concluded that the toxicity of 2,4,6-TCP and its conjugates was covered by the parent | Covered by parent |
| **BTS 44595**<br>(*N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy)ethyl]urea;<br>M201-04)<br> | No | Not genotoxic in Ames test.<br>No genotoxic alert in QSAR analysis | Oral $LD_{50}$: >3200 mg/kg bw | TTC Cramer class III: 1.5 µg/kg bw per day |
| **BTS 54906**<br>(3-hydroxy-2,4,6-trichlorophenoxyethanol;<br>M15a)<br> | No | No genotoxic alert in QSAR analysis | | TTC Cramer class III: 1.5 µg/kg bw per day |

| Compound, codes and structure | Major rat metabolite (>10% AD) | Genotoxicity assessment (data, QSAR, read-across) | General toxicity | Toxicological reference values |
|---|---|---|---|---|
| **BTS 44770** (*N*-2-(2,4,6-trichlorophenoxy) ethylurea; M201-05)  | No | No genotoxic alert in QSAR analysis | | TTC Cramer class III: 1.5 µg/kg bw per day |
| **BTS 3037** (2-(2,4,6-trichlorophenoxy) ethanol)  | Yes<br>BTS 3037 is an intermediate in rat metabolism; its downstream metabolites BTS 9608 and BTS 45186 found in urine at a combined level of at least 17%. | Not genotoxic in Ames test. | Oral $LD_{50}$ 800–1600 mg/kg bw | Covered by parent |

ADI: Acceptable daily intake; RA: Read across; AD: Administered dose; TTC: Threshold of toxicological concern; QSAR: Quantitative structure–activity relationship

## Microbiological aspects

There is not sufficient information available in the public domain, and no experimental data were submitted that address the possible impact of prochloraz residues on the human intestinal microbiome.

## Human data

No health effects of prochloraz were detected during medical surveillance of manufacturing plant personnel.

The Meeting concluded that the existing database on prochloraz was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

# Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.02 mg/kg bw for prochloraz on the basis of an overall NOAEL of 1.7 mg/kg bw per day in two 2-year studies in rats. A safety factor of 100 was used. This ADI is supported by an overall NOAEL of 2.5 mg/kg bw per day from a 90-day and a one-year study in dogs. The upper bound of the ADI gives a margin of 1300 relative to the LOAEL for the observed hepatocellular adenomas and carcinomas in mice. The previous ADI of 0–0.01 mg/kg bw, based on increased ALP levels and minimal histopathological effects in one dog in a two-year study, was withdrawn because it is now established that isolated ALP changes in dogs are not adverse.

The Meeting established an acute reference dose (ARfD) of 0.2 mg/kg bw for prochloraz from a NOAEL of 20 mg/kg bw, based on an acute neurotoxicity study in rats. A safety factor of 100 was used. The Meeting withdrew a previous ARfD of 0.1 mg/kg bw, which had been based on a NOAEL of 10 mg/kg bw per day in a 14-day study in dogs, because it is now established that isolated ALP changes in dogs are not adverse.

***Levels relevant to risk assessment of prochloraz***

| Species | Study | Effect | NOAEL | LOAEL |
|---|---|---|---|---|
| Mouse | Eighteen-month studies of carcinogenicity[a,e] | Toxicity | 78 ppm, equal to 7.5 mg/kg bw per day | 220 ppm, equal to 26.3 mg/kg bw per day |
| | | Carcinogenicity | 78 ppm, equal to 7.5 mg/kg bw per day | 220 ppm, equal to 26.3 mg/kg bw per day |
| Rat | Acute neurotoxicity study[b] | Neurotoxicity | 405 mg/kg bw[c] | - |
| | | General toxicity | 20 mg/kg bw | 90 mg/kg bw |
| | Two-year studies of toxicity and carcinogenicity[a,e] | Toxicity | 40 ppm, equal to 1.7 mg/kg bw per day | 150 ppm, equal to 5.1 mg/kg bw per day |
| | | Carcinogenicity | 1200 ppm, equal to 50.3 mg/kg bw per day[c] | - |
| | Two-generation study of reproductive toxicity[a] | Reproductive toxicity | 50 ppm, equal to 4.5 mg/kg bw per day | 150 ppm, equal to 13.7 mg/kg bw per day |
| | | Parental toxicity | 50 ppm, equal to 3.8 mg/kg bw per day | 150 ppm, equal to 10.9 mg/kg bw per day |
| | | Offspring toxicity | 150 ppm, equal to 10.9 mg/kg bw per day | 450 ppm, equal to 34 mg/kg bw per day |
| | Developmental toxicity study[b] | Maternal toxicity | 25 mg/kg bw per day | 100 mg/kg bw per day |
| | | Embryo/fetal toxicity | 25 mg/kg bw per day | 100 mg/kg bw per day |
| Rabbit | Developmental toxicity study[b] | Maternal toxicity | 40 mg/kg bw per day | 160 mg/kg bw per day |
| | | Embryo/fetal toxicity | 40 mg/kg bw per day | 160 mg/kg bw per day |
| Dog | Thirteen-week\| and one-year studies of toxicity[b,e,f] | Toxicity | 2.5 mg/kg bw per day | 5 mg/kg bw per day |

[a] Dietary administration; [b] Gavage administration; [c] Highest dose tested;
[d] Lowest dose tested; [e] Two or more studies combined; [f] Capsule administration

*Acceptable daily intake (ADI) applies to prochloraz*

0–0.02 mg/kg bw

*Acute reference dose (ARfD) applies to prochloraz*

0.2 mg/kg bw

## Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure.

*Critical end-points for setting guidance values for exposure to prochloraz*

| | |
|---|---|
| ***Absorption, distribution, excretion and metabolism in mammals*** | |
| Rate and extent of oral absorption | Rapid;<br>$T_{max}$: 2 hours at 5 mg/kg bw, 10 hours at 100 mg/kg bw;<br>Absorption 74% in rats |
| Dermal absorption | No data |
| Distribution | Widely distributed, highest concentrations found in liver and kidneys |
| Potential for accumulation | Low |
| Rate and extent of excretion | Relatively rapid; virtually complete in 96 hours |
| Metabolism in animals | Extensively metabolized, major metabolites are: BTS 44596, BTS 9608 and BTS 3037, (see Table 22 for systematic and other names) |
| Toxicologically significant compounds in animals and plants | Prochloraz |
| ***Acute toxicity*** | |
| Rat, $LD_{50}$, oral | 300 to >4000 mg/kg bw |
| Rat, $LD_{50}$, dermal | >2000 mg/kg bw |
| Rat, $LC_{50}$, inhalation | >2.1 mg/L |
| Rabbit, dermal irritation | Not irritating to mildly irritating |
| Rabbit, ocular irritation | Not irritating to moderately irritating |
| Guinea pig, dermal sensitization | Not sensitizing (Magnusson & Kligman, Buehler) |
| ***Short-term studies of toxicity*** | |
| Target/critical effect | Prostate (dog), liver (rat) |
| Lowest relevant oral NOAEL | 2.5 mg/kg bw per day (dog) |
| Lowest relevant dermal NOAEL | 1000 mg/kg bw per day, highest dose tested (rat) |
| Lowest relevant inhalation NOAEC | No data |
| ***Long-term studies of toxicity and carcinogenicity*** | |
| Target/critical effect | Liver |
| Lowest relevant NOAEL | 1.7 mg/kg bw per day (rat) |
| Carcinogenicity | Carcinogenic in mice, but not in rats [a]<br>Mode of action for tumour formation by prochloraz in mice is probably through activation of CAR and possibly PXR |
| ***Genotoxicity*** | Unlikely to be genotoxic |
| ***Reproductive toxicity*** | |
| Target/critical effect | Clinical signs, body weight gain, gestation index, gestation length and dystocia |
| Lowest relevant parental NOAEL | 3.8 mg/kg bw per day (rat) |
| Lowest relevant offspring NOAEL | 10.9 mg/kg bw per day (rat) |
| Lowest relevant reproductive NOAEL | 4.5 mg/kg bw per day (rat) |
| ***Developmental toxicity*** | |
| Target/critical effect | Clinical signs, body weight gain, food consumption, liver, litter size, implantation index, viability index, incidence of dead fetuses and retarded ossification |
| Lowest relevant maternal NOAEL | 25 mg/kg bw per day (rat/rabbit) |
| Lowest relevant embryo/fetal NOAEL | 25 mg/kg bw per day (rat/rabbit) |

| | |
|---|---|
| ***Neurotoxicity*** | |
| Acute neurotoxicity NOAEL | 405 mg/kg bw (rat) |
| Subchronic neurotoxicity NOAEL | No data |
| Developmental neurotoxicity NOAEL | No data |
| ***Other toxicological studies*** | |
| Immunotoxicity | No data |
| **Studies on toxicologically relevant metabolites** | |
| ***BTS 45595*** | Acute oral $LD_{50}$: >3200 mg/kg bw (rat) |
| ***BTS 44596*** | Acute oral $LD_{50}$: >3200 mg/kg bw (rat)<br>Not genotoxic (Ames) |
| ***BTS 45595*** | Acute oral $LD_{50}$: >3200 mg/kg bw (rat) |
| ***BTS 45186*** | Acute oral $LD_{50}$: >3200 mg/kg bw (rat) |
| ***BTS 3037*** | Acute oral $LD_{50}$: 800–1600 mg/kg bw (rat)<br>Not genotoxic (Ames) |
| ***Microbiological aspects*** | Insufficient information |
| ***Human data*** | No health effects detected during medical surveillance of manufacturing plant personnel |

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

## Summary

| | Value | Study | Safety factor |
|---|---|---|---|
| ADI | 0–0.02 mg/kg bw | Two-year study of toxicity and carcinogenicity (rat) | 100 |
| ARfD | 0.2 mg/kg bw | Acute neurotoxicity study (rat) | 100 |

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# Thiophanate-methyl (addendum)

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## Explanation

Thiophanate-methyl (TM) is the International Organization for Standardization (ISO)-approved common name for dimethyl 4,4′-(*O*-phenylene)-bis(3-thioallophanate) (IUPAC). It has the Chemical Abstracts Service number 23564-05-8. Thiophanate-methyl is a systemically active fungicide that inhibits the synthesis of β-tubulin. Thiophanate-methyl was previously evaluated by the Joint Meeting on Pesticide Residues (JMPR) in 1973, 1975, 1977, 1995, 1998, 2006 and 2017. In 1998, an acceptable daily intake (ADI) of 0–0.08 mg/kg body weight (bw) was established and this was maintained until 2017 when a new ADI of 0–0.09 mg/kg bw was established on the basis of a no-observed-adverse-effect level (NOAEL) of 8.8 mg/kg bw per day, based on reduction in body weight gain and clinical chemistry, urine analysis and histopathological changes in the kidney, thyroid, liver and adrenals in a two-year study in rats and the application of a safety factor of 100. In addition, in 2017, an ARfD of 1 mg/kg bw was established on the basis of a NOAEL of 125 mg/kg bw for transient reductions in body weight

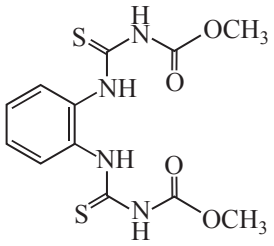
gain (including body weight losses) and food consumption in an acute neurotoxicity study in rats, and the application of a safety factor of 100. In 2017 the Meeting did not receive any information on the toxicology of carbendazim, a significant plant and livestock metabolite of thiophanate-methyl and an active substance in its own right, and for this reason the Meeting was unable to determine the residue definition of thiophanate-methyl and complete its evaluation.

With the purpose of addressing the data gap on carbendazim identified by the 2017 Meeting, and following the submission of additional information on thiophanate-methyl and its metabolites by the sponsor, the compound has been re-evaluated by the present Meeting. This addendum includes the evaluation of newly submitted data alongside summary information taken verbatim from previous JMPR evaluations of thiophanate methyl (here presented in smaller type and indented). New studies include two in vivo kinetic studies, an in vitro interspecies comparative metabolism study, four in vivo mutagenicity studies (two in somatic cells and two in germ cells), four in vitro thyroid peroxidase (TPO) inhibition assays in different species, two in vivo micronucleus tests (one each on metabolites 4-OH-TM and 5-OH-MBC) and genotoxicity in silico analyses of other metabolites.

A literature search performed by the monographers identified two potentially relevant publications, also briefly summarized here.

All the newly submitted studies were compliant with GLP and relevant OECD guidelines.

***Figure 1. Chemical structure of thiophanate-methyl***



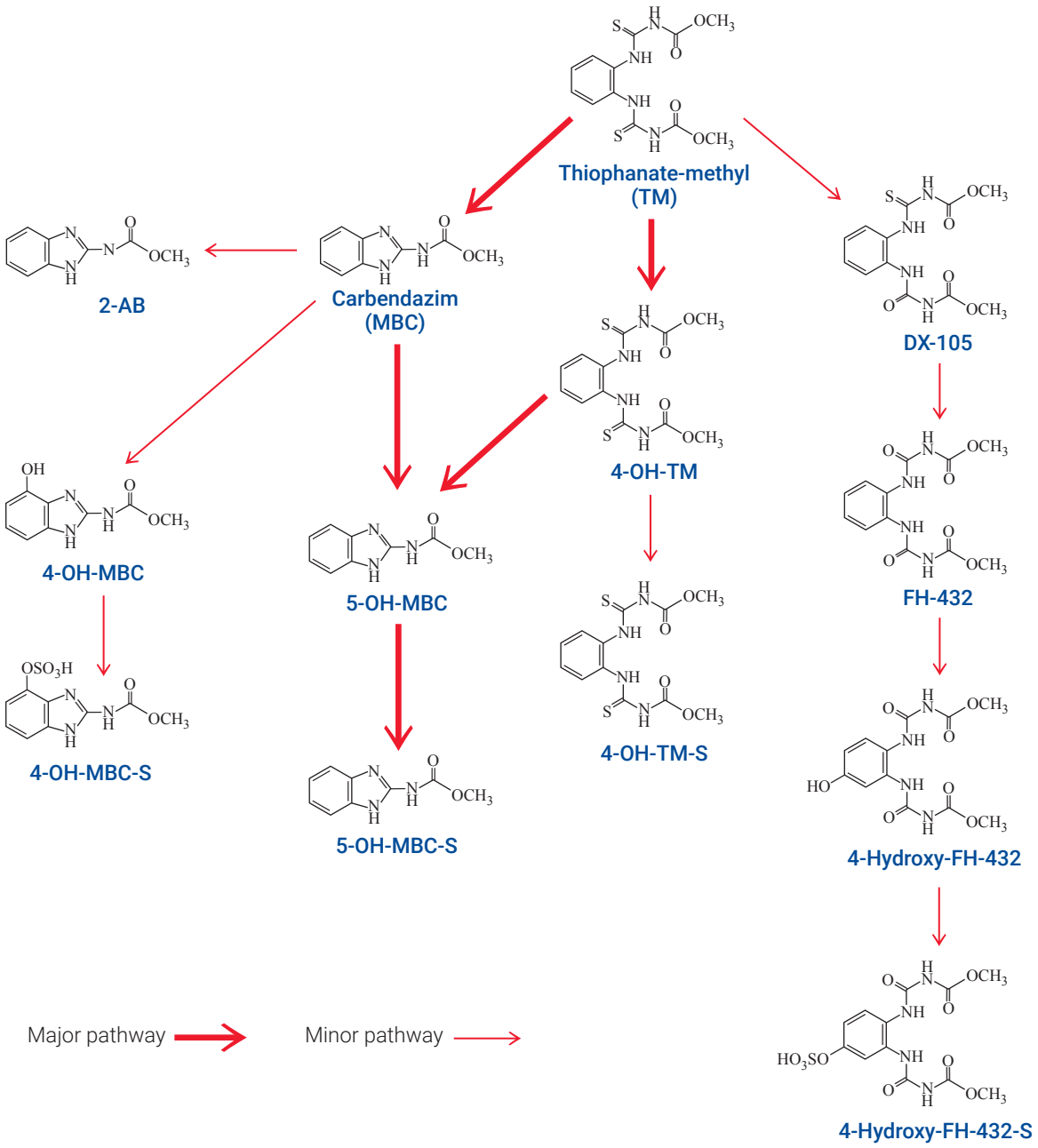

# Evaluation for acceptable daily intake

## 1 Biochemical aspects

The absorption, distribution, metabolism and excretion (ADME) of thiophanate methyl (Fig. 1), as well as its toxicokinetics, were described in the 2017 report item (JMPR, 2017).

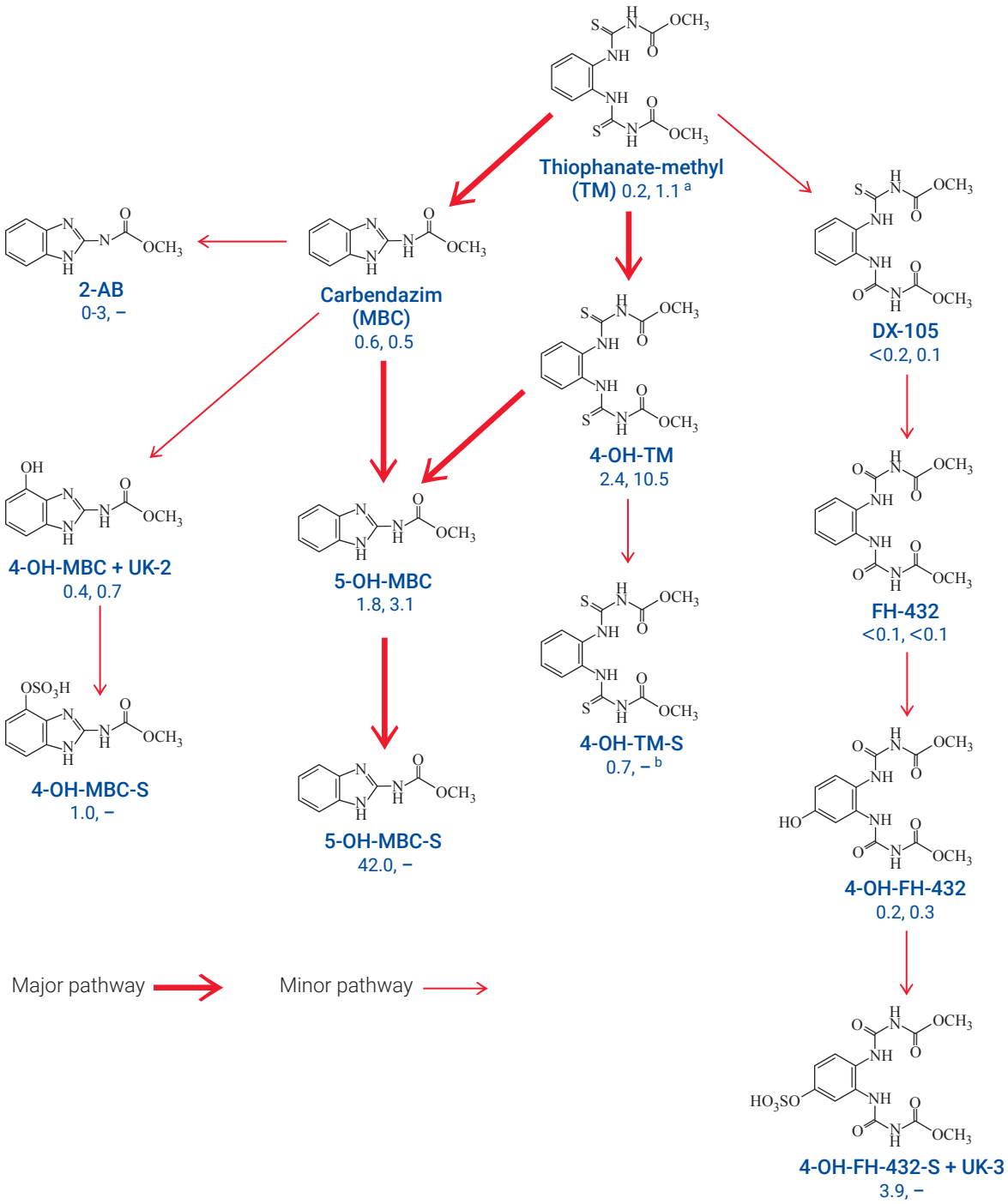
> "Thiophanate-methyl is rapidly and almost completely absorbed (88–89%) after oral administration of a dose of 14 mg/kg bw in mice (Noguchi, 1970; Noguchi & Kosaka, 1971). Thiophanate-methyl is rapidly excreted (approximately 47% in urine and approximately 40% in bile) within 48 hours of administration in rats (Bernard, 2011a). Plasma half-lives were 1.6–2.8 hours after a dose of 13 mg/kg bw and 2.4–7.8 hours after a dose of 140–170 mg/kg bw in rats (Tanoue, 1992a, b). Absorption and excretion patterns were similar in male and female rats (Bernard, 2011b). There is no potential for accumulation (Kosaka et al., 1975). Thiophanate-methyl is widely distributed in rats, with highest levels in liver and thyroid (Tanoue, 1992a, b). The major urinary metabolite in rats was 5-hydroxycarbendazim sulfate (5-OH-MBC-S). Minor metabolites were 5- and 4-hydroxythiophanate-methyl, each representing approximately 2% of the administered radiolabel. The major faecal metabolites were 4-hydroxythiophanate-methyl (6–10%), 5-OH-MBC-S (2–5%) and carbendazim (MBC) (2–3%). Unchanged thiophanate-methyl accounted for approximately 20–24% and 50% of the administered radiolabel after repeated low and high doses, respectively (Nabetani & Mori, 1993). The proposed metabolic pathways of thiophanate-methyl in mice and rats are presented below."

*Figure 2. Proposed metabolic pathway of thiophanate-methyl in mice*




(Redrawn from Nabetani & Mori, 1993)

*Figure 3. Proposed metabolic pathway of thiophanate-methyl in rats*




1 Percentage of radioactivity found in urine and faeces of male rats in Group B (Redrawn from Tanoue, 1992a)

2 Metabolites in parenthesis of this type < > were not identified by spectrometry

Free type: UK-1 (2.2/0.7); conjugated type: UK-4 (2.6/–)

## 1.1 Absorption, distribution and excretion

Two oral single-dose kinetic studies in mice, one in ICR mice (Kuroiwa, 2017) and one in B6D2F1 mice (Kuroiwa, 2018) have were submitted for the purposes of this evaluation. Both were performed with unlabelled thiophanate-methyl to evaluate systemic exposure and distribution to the testes of thiophanate-methyl and its major metabolites, and to ensure the validity of the in vivo somatic and germ cell mutagenicity tests with the substance.

**(a) Oral route**

*Study 1*

A toxicokinetic study in accordance with GLP, and broadly consistent with OECD TG 417 was conducted with unlabelled thiophanate-methyl (TM) to evaluate the systemic exposure and distribution to the testis in ICR mice of the tested substance and its metabolites 4-hydroxy-thiophanate-methyl (4-OH-TM), 5-hydroxy-thiophanate-methyl (5-OH-MBC) and carbendazim (MBC). Thiophanate-methyl (purity 97.9%; Batch no. TBC-035G) was administered to nine male ICR mice, once, orally by gavage, using aqueous methylcellulose 400 as a vehicle, at 2000 mg/kg bw. The concentrations of TM and its metabolites 5-OH-MBC, 4-OH-TM and MBC were determined in plasma and in the testis at 1, 6 and 24 hours after dosing (three animals samples at each time point). Quantification was by liquid chromatography/tandem mass spectrometry (LC-MS/MS).

The animals were observed for clinical signs (external appearance, nutritional condition, posture, behaviour, and excretions) once a day in the morning, following dosing.

There were no clinical signs of toxicity or mortality in any animal. Thiophanate-methyl and its metabolites 5-OH-MBC, 4-OH-TM and MBC were detected in the plasma and testis. In plasma, the time to peak concentration ($T_{max}$) values for TM, 4-OH-TM and MBC were all 1.0 hour; for metabolite 5-OH-MBC $T_{max}$ was 6.0 hours. Values for the peak concentration ($C_{max}$) and area under the concentration–time curve for the 24 hours following dosing ($AUC_{0–24\,h}$) were from the highest, in the following order: TM > MBC > 5-OH-MBC > 4-OH-TM, (see Table 1).

***Table 1. Plasma concentration and toxicokinetic parameters of TM and its metabolites following administration of a single oral dose of 2000 mg/kg bw TM to male ICR mice***

| | Plasma concentration (ng/g wet wt) | | | Toxicokinetics parameters | | |
|---|---|---|---|---|---|---|
| Analyte | At 1 hour (mean ± SD) | At 6 hours (mean ± SD) | At 24 hours (mean ± SD) | $C_{max}$[a] (ng/mL) | $T_{max}$[a] (hours) | AUC[a] (h × ng/mL) |
| Thiophanate-methyl | 10 600 ± 1500 | 4120 ± 900 | 543 ± 524 | 10 600 | 1.0 | 84 100 |
| 5-OH-MBC | 1200 ± 510 | 1270 ± 230 | 115 ± 200 | 1270 | 6.0 | 19 200 |
| 4-OH-TM | 265 ± 63 | 188 ± 26 | BLQ | 265 | 1.0 | 2960 |
| MBC | 2280 ± 560 | 1210 ± 300 | 252 ± 252 | 2280 | 1.0 | 23 000 |

BLQ: Below the limit of quantification (100 ng/g wet weight); Source: Kuroiwa, 2017

[a] Standard deviations not provided for the toxicokinetic parameters in the study report

In the testis, the $T_{max}$ values for TM, 4-OH-TM and MBC were all 1.0 hour; that for the metabolite 5-OH-MBC it was 6.0 hours. The $C_{max}$ and $AUC_{0–24\,h}$ were from the highest, in the following order: TM > 5-OH-MBC > MBC > 4-OH-TM, (see Table 2).

***Table 2. Testis concentration and toxicokinetic parameters of TM and its metabolites following administration of a single oral dose of 2000 mg/kg bw TM to male ICR mice***

| | Testis concentration (ng/g wet wt) | | | Toxicokinetics parameters | | |
|---|---|---|---|---|---|---|
| Analyte | At 1 hour (mean ± SD) | At 6 hours (mean ± SD) | At 24 hours (mean ± SD) | $C_{max}$[a] (ng/mL) | $T_{max}$[a] (hours) | AUC[a] (h × ng/mL) |
| Thiophanate-methyl | 13 100 ± 1800 | 5500 ± 1130 | 604 ± 799 | 13 100 | 1.0 | 109 000 |
| 5-OH-MBC | 417 ± 162 | 844 ± 215 | BLQ | 844 | 6.0 | 11000 |
| 4-OH-TM | BLQ | 212 ± 119 | BLQ | 212 | 1.0 | 2440 |
| MBC | 538 ± 154 | 447 ± 131 | BLQ | 538 | 1.0 | 6750 |

BLQ: Below the limit of quantification (100 ng/g wet weight); Source: Kuroiwa, 2017

[a] Standard deviations not provided for the toxicokinetic parameters in the study report

In conclusion, in this study in ICR mice, the concentrations of TM in plasma and the testis were comparable. However, the concentrations of its metabolites (5-OH MBC, 4-OH-TM and especially MBC), were lower in the testis than in plasma. This study, nevertheless demonstrated systemic exposure and distribution of TM and its metabolites to the testis in ICR mice (Kuroiwa, 2017).

*Study 2*

A similar toxicokinetic study in accordance with GLP and broadly consistent with OECD TG 417 was conducted with unlabelled TM to evaluate the systemic exposure and distribution to the testis in B6D2F1 mice of the tested substance and its metabolites 4-OH-TM, 5-OH-MBC and MBC. Thiophanate-methyl (purity 97.9%; Batch no. TBC-035G) was administered to nine male B6D2F1 mice, once, orally by gavage, using aqueous methylcellulose 400 as a vehicle at 2000 mg/kg bw. The concentrations of TM and its metabolites, 5-OH-MBC, 4-OH-TM and MBC were determined in plasma and in the testis at 1, 6 and 24 hours after dosing (three animals samples at each time point); quantification was by LC-MS/MS. The animals were observed for clinical signs (external appearance, nutritional condition, posture, behaviour, and excretions) once a day in the morning.

There were no clinical signs of toxicity or mortality in any animal. Thiophanate-methyl and its metabolites 5-OH-MBC, 4-OH-TM and MBC were detected in plasma and the testis. In plasma the $T_{max}$ values for TM, 5-OH-MBC and MBC were 1.0 hour; for 4-OH-TM it was 24.0 hours. The $C_{max}$ and $AUC_{0–24\,h}$ were from the highest, in the following order: TM > MBC > 5-OH-MBC > 4-OH-TM, (see Table 3).

Data from metabolite 4-OH-TM was not obtained in the testis since the mean values at each time point were below the limit of quantification (see Table 4).

***Table 3. Plasma concentration and toxicokinetic parameters of TM and its metabolites following administration of a single oral dose of 2000 mg/kg bw TM to male B6D2F1 mice***

| | Plasma concentration (ng/g wet wt) mean ± SD shown | | | Toxicokinetics parameters | | |
|---|---|---|---|---|---|---|
| Analyte | At 1 hour | At 6 hours | At 24 hours | $C_{max}$[a] (ng/mL) | $T_{max}$[a] (hours) | AUC[a] (h × ng/mL) |
| Thiophanate-methyl | 7360 ± 1610 | 6790 ± 2090 | 4130 ± 710 | 7360 | 1.0 | 137 000 |
| 5-OH-MBC | 601 ± 138 | 589 ± 98 | 326 ± 65 | 601 | 1.0 | 11500 |
| 4-OH-TM | 155 ± 13 | BLQ | 185 ± 16 | 265 | 24 | 2130 |
| MBC | 1710 ± 160 | 1250 ± 240 | 1020 ± 130 | 1710 | 1.0 | 28700 |

BLQ: Below the limit of quantification (100 ng/g wet weight); Source: Kuroiwa, 2018

[a] Standard deviations not provided for the toxicokinetic parameters in the study report

In the testis the $T_{max}$ value for TM and MBC was 1.0 hour and for 5-OH-MBC 6.0 hours. The $C_{max}$ and $AUC_{0–24\,h}$ were higher in the following order: TM > 5-OH-MBC > MBC. Data from 4-OH-TM was not obtained in the testis since the mean values of each time point were below the limit of quantification (see Table 4).

***Table 4. Testis concentration and toxicokinetic parameters of TM and its metabolites following administration of a single oral dose of 2000 mg/kg bw TM to male B6D2F1 mice***

| | Testis concentration (ng/g wet wt) mean ± SD shown | | | Toxicokinetics parameters | | |
|---|---|---|---|---|---|---|
| Analyte | At 1 hour | At 6 hours | At 24 hours | $C_{max}$[a] (ng/mL) | $T_{max}$[a] (hours) | AUC[a] (h × ng/mL) |
| Thiophanate-methyl | 8690 ± 1860 | 8690 ± 2290 | 3840 ± 890 | 8690 | 1.0 | 161 000 |
| 5-OH-MBC | 247 ± 55 | 454 ± 15 | 162 ± 20 | 454 | 6.0 | 7420 |
| 4-OH-TM | BLQ | BLQ | BLQ | NA | NA | NA |
| MBC | 403 ± 67 | 302 ± 76 | 262 ± 14 | 403 | 1.0 | 7040 |

BLQ: Below the limit of quantification (100 ng/g wet weight); Source: Kuroiwa, 2018

[a] Standard deviations not provided for the toxicokinetic parameters in the study report

In conclusion, in this study in B6D2F1 mice, the concentrations of TM in plasma and testis were comparable. However, the concentrations of its metabolites (5-OH-MBC, 4-OH-TM and especially MBC) were lower in the testis than in the plasma. This study demonstrated systemic exposure and

distribution to the testis of TM and its metabolites in B6D2F1 mice. This study also showed that MBC is a major plasma metabolite in the mouse (Kuroiwa, 2018).

Overall, the two studies showed systemic exposure in plasma and distribution to the testis of TM and its major metabolites in ICR and B6D2F1 mice when TM was dosed orally at 2000 mg/kg bw. However, the concentrations of its metabolites (5-OH-MBC, 4-OH-TM and especially MBC), were lower in the testis than in plasma in both mouse strains. These studies also demonstrated that MBC is a major plasma metabolite in the mouse. Despite similarities in the two strains of mice as regards the kinetic behaviour of TM and its major metabolites, some differences were noted. The plasma $T_{max}$ of metabolite 4-OH-TM was significantly different in the two strains (1 hour in ICR mice and 24 hours in B6D2F1 mice). In addition, this metabolite was not detected in the testes of B6D2F1 mice, but was present at low levels in the testes of ICR mice. The parent AUC in the testes of ICR mice was much lower (more than an order of magnitude) than in B6D2F1 mice. Clearance from plasma and testes of TM by 24 hours after dosing was almost negligible in B6D2F1 mice, whilst in ICR mice the concentration of parent in plasma and testes decreased at least 20-fold by 24 hours after dosing. The reasons for these differences are unclear; however, it should be noted that the relevance to humans of kinetic parameters measured at 2000 mg/kg bw in these two mouse strains is very tenuous.

### 1.2 Biotransformation

An in vitro comparative interspecies metabolism study was also submitted for this evaluation.

In an in vitro, GLP-compliant study, [*phenyl-U*-$^{14}$C]thiophanate-methyl (radiochemical purity 96.2%; batch XXIII/45/A/3) was added to CYP H-class human liver microsomes or female Fisher 344 rat liver microsomes, and incubated at 37ºC for 0, 0.5, 1 or 2 hours. Testosterone was used as a positive control substance to confirm the enzymic activity of the microsomes. After incubation, each sample was analyzed by high-performance liquid chromatography (HPLC) to characterize the metabolites formed.

Testosterone was stable in buffer solution. It was rapidly metabolized by human and rat microsomes and had almost disappeared after two hours of incubation, confirming the enzymic activity of the system. Thiophanate-methyl was metabolized and gradually decreased with time. Major metabolites 4-OH-TM, MBC and 5-OH-MBC were observed in both human and rat microsomal preparations. The metabolic pathways in both species were identical. No metabolite unique to the human microsome samples was detected. The maximum metabolic formation ratio of carbendazim was 0.7% with human microsomes and 3.2 % with rat microsomes, when compared to the control sample (absence of microsomes). Overall, it was demonstrated that the metabolic formation ratio of carbendazim was low, and it was lower with human microsome preparations than rat microsome preparations (Shimomura, 2017).

### 1.3 Effects on enzymes and other biochemical parameters

No studies were provided on this topic.

## 2 Toxicological studies

### 2.1 Acute toxicity

The acute toxicity of thiophanate-methyl was described in the 2017 monograph. No further acute toxicity studies were submitted for the current evaluation.

> "In rats, the oral $LD_{50}$ was greater than 5000 mg/kg bw (Nishibe, 1990b); in rabbits the dermal $LD_{50}$ was greater than 2000 mg/kg bw (Nishibe, 1990c); and in rats, the inhalation $LC_{50}$ was 1.7–1.98 mg/L (Nishibe, 1987). Thiophanate-methyl was not irritating to the skin or eyes of rabbits (Nishibe, 1986a, b). Thiophanate-methyl was a skin sensitizer in a Magnusson and Kligman test in Guinea pigs (Nishibe, 1989), but not in a Buehler test (Nishibe & Mochizuki, 1993)."

## 2.2 Short-term studies of toxicity

### (a) Oral administration

The short-term toxicity of thiophanate-methyl was described in the 2017 monograph (JMPR, 2021). No further acute toxicity studies were submitted for the current evaluation.

> "In repeated-dose oral toxicity studies with thiophanate-methyl in mice, rats and dogs, the most sensitive organs were the liver and thyroid.
>
> In a pre-guideline six-month dietary toxicity study (with limited investigations) in ICR mice administered dietary thiophanate-methyl concentrations of 0, 12.8, 64, 320, 1600 or 8000 ppm (equal to 0, 2, 10, 50, 250 and 1240 mg/kg bw per day for males and 0, 2, 11, 52, 231 and 1630 mg/kg bw per day for females, respectively), the NOAEL was 1600 ppm (equal to 231 mg/kg bw per day) based on decreased body weight gain and haematological changes indicative of slight anaemia in males and females at 8000 ppm (equal to 1240 mg/kg bw per day) (Noguchi & Hashimoto, 1970a).
>
> In a 13-week dietary toxicity study in Fisher 344 rats administered dietary thiophanate-methyl concentrations of 0, 200, 2200, 4200, 6200 or 8200 ppm (equal to 0, 13.9, 155, 293, 427 and 565 mg/kg bw per day for males and 0, 15.7, 173, 323, 479 and 647 mg/kg bw per day for females, respectively), the NOAEL was 200 ppm (equal to 13.9 mg/kg bw per day) based on haematological changes indicative of slight anaemia, increased thyroid and liver weights, follicular hyperplasia and hypertrophy of the thyroid and hepatocellular hypertrophy and increased lipofuscin pigment in both sexes and increased severity of glomerulonephrosis in males observed at 2200 ppm (equal to 155 mg/kg bw per day) (Nishibe & Takaori, 1990).
>
> In a pre-guideline six-month dietary toxicity study (with limited investigations) in Sprague-Dawley rats administered dietary thiophanate-methyl concentrations of 0, 12.8, 64, 320, 1600 or 8000 ppm (equal to 0, 1, 4, 20, 95 and 500 mg/kg bw per day for males and 0, 1, 5, 22, 110 and 660 mg/kg bw per day for females, respectively), the NOAEL was 1600 ppm (equal to 95 mg/kg bw per day) based on decreased body weight gain, haematological changes indicative of slight anaemia, decreased glucose levels and increased cholesterol levels, increased thyroid weights and histological changes in the thyroid in males and females at 8000 ppm (equal to 500 mg/kg bw per day) (Noguchi & Hashimoto, 1970b).
>
> In a three-month toxicity study in Beagle dogs, administering thiophanate-methyl by gelatine capsule at doses of 0, 50, 200 and 800/400 mg/kg bw per day, the LOAEL was 50 mg/kg bw per day based on the hypertrophy of the follicular epithelial cells of the thyroid at this dose level (Auletta, 1991).
>
> In a pre-guideline two-year toxicity study (with limited investigations) in Beagle dogs administered thiophanate-methyl by gelatine capsule at doses of 0, 2, 10, 50 or 250 mg/kg bw per day, the NOAEL was 10 mg/kg bw per day based on effects on thyroid weight and histopathology of the thyroid in both sexes at 50 mg/kg bw per day (Hashimoto et al., 1972).
>
> The overall NOAEL for the studies in dogs was 10 mg/kg bw per day, and the overall LOAEL was 50 mg/kg bw per day."

## 2.3 Long-term studies of toxicity and carcinogenicity

The long-term toxicity and carcinogenicity of thiophanate-methyl were described in the 2017 monograph JMPR, 2021).

> "In a carcinogenicity study in CD-1 mice, thiophanate-methyl was administered at dietary concentrations of 0, 150, 640, 3000 or 7000 ppm (equal to 0, 24, 99, 468 and 1079 mg/kg bw per day for males and 0, 29, 123, 558 and 1329 mg/kg bw per day for females, respectively). The NOAEL was 150 ppm (equal to 29 mg/kg bw per day) based on the hepatocellular centrilobular hypertrophy at nine months and hepatocellular adenomas in females observed at 640 ppm (equal to 123 mg/kg bw per day) (Tompkins, 1992).
>
> In a two-year combined chronic toxicity/carcinogenicity study in Fisher 344 rats administered dietary thiophanate-methyl concentrations of 0, 75, 200, 1200 or 6000 ppm (equal to 0, 3.3, 8.8, 54 and 281 mg/kg bw per day for males and 0, 3.8, 10.2, 64 and 335 mg/kg bw per day for females, respectively), the NOAEL was 200 ppm (equal to 8.8 mg/kg bw per day) based on reduced body weight gain in both sexes; increased total cholesterol and total protein in both sexes; decreased albumin to globulin ratio in both sexes at 12 and/or 18 months; decreased levels of chloride and potassium; decreased T4 and T3 and increased TSH in males at 24 months; increased urinary protein and granular kidneys in males; follicular cell hyperplasia and hypertrophy in the thyroid in both sexes at 12 and 24 months; a possible increase

in the incidence of thyroid follicular cell adenoma in males; centrilobular hepatocellular hypertrophy and occurrence of lipofuscin pigment in both sexes at 12 and 24 months; lipidosis of the adrenal cortex in females at 12 months; and increased severity of nephropathy in both sexes at 24 months observed at 1200 ppm (equal to 54 mg/kg bw per day). In males, the incidence of thyroid follicular cell adenoma was increased at 1200 ppm and above, but reached statistical significance only at 6000 ppm (Takaori, 1993).

A mechanistic study in rats showed that thiophanate-methyl induced cytochromes CYP450 (not further specified) and cytochrome b5 as well as uridine diphosphate glucuronosyltransferase (UDPGT), an enzyme that plays an important role in the clearance of T4 in the liver. Thiophanate-methyl also inhibited porcine thyroid microsomal peroxidase, an enzyme involved in thyroid hormone synthesis. T4 supplementation counteracted the hypertrophy of the thyroid and the TSH response, indicating that thiophanate-methyl caused the hypertrophy by negative feedback mechanism. The mechanistic study demonstrates that the thyroid effects resulting from thiophanate-methyl are likely to be the result of a reduction in thyroid hormones. In another study, thiophanate-methyl induced increases in CYP3A and CYP2B1 in the liver of rats (Takaori, 1993; Paolini et al., 1999)."

The 2017 Meeting concluded that thiophanate-methyl is carcinogenic in mice and rats. The Meeting considered the different modes of action (MOA) that might underlie the observed tumour induction. The effects on the thyroid, including the induction of thyroid follicular cell adenoma, may be secondary effects resulting from liver enzyme induction that enhances thyroid hormone excretion and leads to perturbations in systemic thyroid hormone levels (that is, decreased thyroid T4 and T3 hormone values and, as a consequence, an increase in TSH concentration). The continuous stimulation of the thyroid gland by TSH is known to result in follicular cell hypertrophy/hyperplasia and, depending on dose and time, in follicular cell adenomas/adenocarcinomas. Rodents are particularly sensitive to decreases in thyroxine (T4) and triiodothyronine (T3) levels resulting from liver enzyme induction; these reductions eventually lead to thyroid tumour formation. This is a well established adverse outcome pathway without relevance to humans. The TM-induced increase in hepatocellular adenomas may be a consequence of activation of nuclear receptors involved in the induction of the cytochrome P450 drug metabolizing system. Another possible MOA for the carcinogenic effect may be the interference of the thiophanate-methyl metabolite carbendazim with mitotic spindle proteins leading to aneuploidy (see below).

No new carcinogenicity studies were submitted for the purposes of this evaluation, but further in vitro thyroid mechanistic assays have been performed; for these see Section 2.6 below).

## 2.4 Genotoxicity

### (a) In vitro and in vivo studies

The genotoxicity of thiophanate-methyl was described in the 2017 monograph (JMPR, 2021). However, as four additional in vivo genotoxicity studies have been provided by the sponsor for the current evaluation, more extensive details of the original studies considered by the 2017 Meeting have been presented below in Table 5.

***Table 5. Overview of original genotoxicity studies with thiophanate-methyl from the 2017 monograph***

| End-point | Test system | Concentration/doses tested | Purity | Results | Reference |
|---|---|---|---|---|---|
| **In vitro** | | | | | |
| Bacterial reverse mutation | *Salmonella typhimurium* TA98, TA100, TA1535, TA1537<br>*Escherichia coli* WP2 *uvrA* | 39.1–5000 µg/plate (± S9)<br>0, 16, 50, 158, 500 and 1581;<br>5000 µg/tube in DMSO (± S9, pre-incubation) | 96.6% | Negative | Nishibe, 1990a |
| Mammalian cell gene mutation, HPRT locus | V79 Chinese hamster lung cell | 6.25–100 µg/mL in DMSO (± S9) | 96.4% | Negative | McSheehy et al., 1984 |

| End-point | Test system | Concentration/doses tested | Purity | Results | Reference |
|---|---|---|---|---|---|
| Chromosomal aberration | Chinese hamster ovary (CHO) cells | 100–400 µg/mL in DMSO (–S9, 20 h)<br>250–1000 µg/mL in DMSO (+S9, 20 h) | 95.0% | Negative | Murli, 1988 |
| Unscheduled DNA synthesis | Primary rat hepatocytes | 5–1000 µg/mL (–S9, 18 h)<br>250–1000 µg/mL (+S9, 18 h) | NR | Negative | Myhr & Brusick, 1981 |
| Micronucleus | Human peripheral lymphocytes | 47.5–200 µg/mL in DMSO (–S9, 18 h)<br>150–700 µg/mL in DMSO (+S9, 18 h) | 98.2% | Positive | Marshall, 1996a |
| Micronucleus (examination of four chromosomes) | Human peripheral lymphocytes | 8.5–200 µg/mL in DMSO (range-finding study)<br>0.05–39.0 µg/mL in DMSO (–S9, 18 h) | 96.4% | Positive Threshold for aneugenicity 6 µg/mL (–S9 mix) | Marshall, 1996b |
| **In vivo** | | | | | |
| Micronucleus with FISH analysis for centromere | B6D2F1 mouse, bone marrow | 500, 1000 and 2000 mg/kg bw in 1% aqueous carboxymethyl cellulose by gavage, as single doses | 97.3% | Weakly positive (numerical chromosome aberrations) | Proudlock, 1999 |
| Micronucleus | Swiss albino mouse, bone marrow | 1000 mg/kg bw in 70% kaolin by gavage as a single dose | 95% | Weakly positive | Barale et al., 1993 |
| Chromosomal aberrations | Male Wistar rats, bone marrow and spermatogonia | 62.5–1000 mg/kg bw in 5% gum arabic (administered intraperitoneally for 5 days) | 94% | Negative | Makita et al., 1972 |
| Dominant lethal | Male ICR mice | 8–500 mg/kg bw in 1% gum arabic (administered intraperitoneally) | 94% | Negative | Makita et al., 1972 |

bw: Body weight; NR: Not reported; DMSO: Dimethyl sulfoxide; DNA: Deoxyribonucleic acid; HPRT: Hypoxanthine–guanine phosphoribosyltransferase; FISH: Fluorescence in-situ hybridization

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*

The 2017 monograph continues as follows (JMPR. 2021).

"Thiophanate-methyl was tested in an adequate range of in vitro and in vivo assays for genotoxicity. In an Ames test, no increase in the number of revertant colonies was recorded for any of the strains of bacteria used up to the limit concentration for this assay, neither with nor without metabolic activation (Nishibe, 1990a).

In a HPRT test in V79 Chinese hamster lung cells, there were no reproducible increases in mutant numbers or mutation frequency observed at any concentration of the test substance, neither in the absence nor in the presence of metabolic activation up to cytotoxicity. Occasional increases were not dose related. The positive control substances gave the expected response (McSheehy et al. 1984).

In a chromosome aberration test in Chinese hamster ovary cells, no significant increase in chromosomal aberrations was observed with or without metabolic activation at any concentration up to cytotoxicity and/or precipitation. The positive control substances induced a marked increase in chromosomal aberration rate (Murli, 1988).

In an unscheduled DNA synthesis (UDS) assay in primary rat hepatocytes, there was no indication that thiophanate-methyl increased UDS activity up to cytotoxicity. The positive control 2-acetyl aminofluorene

demonstrated the sensitivity of the test system by apparent induction of UDS (Myhr & Brusick, 1981).

In an in vitro micronucleus test in peripheral human lymphocytes, treatment of cultures with the thiophanate-methyl in the absence of metabolic activation resulted in micronucleus frequencies which were statistically significantly greater than those noted in concurrent negative controls at all concentrations and which fell outside the historical negative control range. Although not all cells with micronuclei were evaluated, FISH (fluorescence in-situ hybridization) analysis of the positive samples showed that the majority of the micronuclei induced contained one or more centromeres and were thus formed from whole chromosomes rather than from chromosomal fragments. The increase in the number of cells with centromere-negative micronuclei reaching statistical significance at the lowest concentration level analysed was very small. A clear dose-response was lacking. Thus, this finding was not considered to provide evidence of an additional clastogenic effect. Treatment in the presence of metabolic activation resulted in micronucleus frequencies similar to those seen in concurrent negative controls. A statistically significant increase in cells with micronuclei was apparent at the highest concentration analysed, but the effect was attributable to an increase in a single replicate. FISH analysis of positive samples indicated that the majority of the micronuclei induced contained one or more centromeres, and were thus formed from whole chromosomes rather than from chromosomal fragments. The positive control substances gave the expected increase in the occurrence of micronuclei confirming the sensitivity of the test system. For colchicine (–S9 mix), the increase was only due to the induction of aneuploidy since there were no binucleated cells with micronuclei lacking centromeres. For cyclophosphamide, the respective analysis was not performed. It was concluded that thiophanate-methyl induced a significant increase in micronuclei in cultured human peripheral blood lymphocytes at least without metabolic activation system and that there was evidence for aneuploidy induction (Marshall, 1996a).

A second in vitro micronucleus test was conducted in human peripheral lymphocytes with the aim to identify the threshold for the aneugenic activity of thiophanate-methyl. The distribution of chromosomes among the nuclei of binucleated cells was investigated by FISH technique (simultaneous detection of centromeres). Centromeres were investigated in the chromosome pairs 1/11 and X/17. Abnormalities were classified under the headings of chromosome loss, chromosome gain, and aneuploidy or polyploidy. A number of closely spaced concentrations were used in order to determine a threshold level and a reliable no-observed-effect concentration (NOEC). For all four chromosomes evaluated, threshold levels and NOECs could be obtained in the first trial. Since there was evidence of an effect (non-disjunction) on chromosomes X and 17 at lower concentrations yet, a second trial was performed which however revealed even higher threshold concentrations and NOECs for these chromosomes. Thus, the results of the first trial were considered reliable. The results obtained for the chromosomes 1, 11, 17 and X showed that the lowest threshold for aneuploidy in cultured human peripheral blood lymphocytes was 6 µg/mL in two independent trials, and the corresponding NOEC was 4 µg/mL. It was concluded that thiophanate-methyl induces aneuploidy in cultured human peripheral blood lymphocytes (Marshall, 1996b).

In an in vivo bone marrow micronucleus test, male and female B6D2F1 mice were treated by single gavage doses of thiophanate-methyl at 500, 1000 or 2000 mg/kg bw. A concurrent negative control group received the vehicle only, while a positive control (PC) group was treated with carbendazim at 1000 mg/kg bw. Bone marrow smears were obtained each from 5 mice/sex per dose group at 24 and at 48 hours after dosing with the exception that mice in the PC group were sampled at the 24-hour time point only. No unscheduled deaths and no clinical signs of toxicity were seen in any of the groups. At all three doses, thiophanate-methyl caused a statistically significant increase in the incidence of micronucleated immature erythrocytes. However, this increase was much smaller than that induced by carbendazim. Carbendazim induced the expected high proportion (68%) of micronuclei containing centromeres, while Mitomycin C caused a low proportion of centromere-positive micronuclei (24%). Thiophanate-methyl appeared to cause an intermediate proportion of centromere-positive micronuclei (34%). It was concluded that thiophanate-methyl has a weak potential to induce the formation of micronuclei in immature mouse erythrocytes. It can be assumed that the mechanism is the same as for carbendazim. The micronucleus incidence was more than six-fold lower in thiophanate-methyl-exposed mice as compared with carbendazim-exposed mice at 1000 mg/kg bw (Proudlock, 1999). The current Meeting however concludes that this study provides a clear positive result as thiophanate-methyl caused up to a 5-fold increase in the incidence of micronuclei at both the 24-hr and 48-hr sampling times, with a clear dose–response relationship seen at 48 hours.

In a second in vivo bone marrow micronucleus study, the aneugenic potential of thiophanate-methyl (purity: 95%; dispersed in 70% kaolin) was tested in male Swiss albino mice given oral doses of 1000 mg/kg bw. Bone marrow cells were analysed 16, 24, 36, and 48 h after treatment for micronuclei, chromosomal aberrations, hyperdiploidy, and polyploidy. Large micronuclei were significantly induced, but the response was relatively weak, and thiophanate-methyl was less effective than benomyl

or carbendazim. There was no increase in the frequency of chromosomal aberrations. At 24 and 36 h, a possibly treatment-related increase in the frequency of polyploid and hyperdiploid cells was observed, which was of borderline significance in view of the very low frequency of changes in ploidy (Barale et al., 1993).

In an in vivo bone marrow and spermatogonial chromosomal aberration study, 30 male Wistar rats per dose group received intraperitoneal administrations of thiophanate-methyl once daily at doses 0, 62.5, 125, 250, 500 and 1000 mg/kg bw for five consecutive days. Colchicine was administered at 1 mg/kg bw at 4 to 5 hours prior to termination and necropsy. Following necropsy, metaphases of bone marrow cells and of spermatogonial cells were processed and examined microscopically. For each dose, 100 well-spread metaphase chromosomes from the bone marrow and spermatogonial cells were analysed. Signs of toxicity were not reported in this study. According to the study authors, no abnormal chromosome configurations such as breakage, suspected chromatid breakage, chromosome translocation, fragmentation or formation of chromosome bridges were observed in either bone marrow or spermatogonial cells (Makita et al. 1972). It was noted that this study had several reporting and design deficiencies.

In a dominant lethal assay, groups of 10 male ICR mice per dose level were treated with a single intraperitoneal injection of thiophanate-methyl at 0, 8, 40, 200, 400 and 500 mg/kg bw. Following treatment, each male was housed for one week with three primiparous females. The females were replaced after one week with new primiparous ones. This procedure was repeated for a total of eight weeks. Each female was scored for pregnancy, for total number of uterine implants as well as for pre- and post-implantation losses on day 13 after the confirmation of vaginal plugs. Thiophanate-methyl was clearly toxic to the male mice at the higher doses. There were four deaths in the 500 mg/kg bw group and one death in the 400 mg/kg bw group. The incidence of pregnancies in the treated groups did not differ consistently. Total implant number including living fetuses, early fetal deaths and late fetal deaths did not indicate any consistent dose-related effect although a considerable degree of intergroup variation was observed. There was no indication of induction of dominant lethal mutations in male mice by thiophanate-methyl (Makita et al. 1972). It was noted that this study had several reporting and design deficiencies.

Overall, thiophanate-methyl did not cause gene mutations or structural chromosomal aberrations; however, it caused changes in chromosome number (aneuploidy) both in vitro and in vivo. Induction of micronucleus formation in B6D2F1 mice was seen after single doses (500 mg/kg bw and above), but the response was about six times lower when compared with that for the metabolite of thiophanate-methyl, carbendazim.

Carbendazim caused changes in chromosome number (aneuploidy) both in vitro and in vivo (in somatic cells and germ cells) as a result of its interference with mitotic spindle proteins. The mechanism by which aneuploidy is induced by carbendazim is well understood and consists of inhibition of the polymerization of tubulin, the protein that is essential for the segregation of the chromosomes during cell division. The nature of the mechanism is thus consistent with the identification of a threshold dose below which no toxicological effect would occur. Like thiophanate-methyl, carbendazim did not cause gene mutations or structural chromosomal aberrations."

The 2017 Meeting concluded that the genotoxic effect of thiophanate-methyl is a threshold phenomenon and is probably related to the production of carbendazim.

*New genotoxicity studies*

Following the 2017 monograph, four in vivo mutagenicity studies (two in somatic cells and two in germ cells) were submitted by the sponsor for the purposes of the current evaluation.

In a GLP- and OECD (474)-compliant in vivo bone marrow micronucleus test, male Crl:CD-1 (ICR) mice (five per group) were dosed by oral gavage with thiophanate-methyl (purity 97.9%, Batch no. TBC-035G) in aqueous 1% methylcellulose . Mice were dosed twice at an interval of approximately 24 hours with 0, 500, 1000 or 2000 mg/kg bw. The positive control group received mitomycin C once intraperitoneally at 1 mg/kg bw. About 24 hours after the second administration, bone marrow smears were prepared and evaluated to assess the incidence of micronuclei and signs of toxicity. No deaths or clinical signs of toxicity were observed in any group. No statistically significant or dose-dependent increases were observed in micronucleated polychromatic erythrocytes per 4000 polychromatic erythrocytes, in any of the treated groups compared with the negative control group. No statistically significant increase was observed in the incidence of polychromatic erythrocytes in a total of 500 erythrocytes compared to the negative control group, indicating a lack of toxicity towards the bone marrow. The positive control substance gave the expected response. Overall, thiophanate-methyl

was negative in this micronucleus study in ICR mice up to the limit dose (Ishii, 2017a). However, it is possible that this result was a false negative, as for a substance like thiophanate-methyl which interferes with the progression of the cell cycle, one single administration followed by two samplings at 24 and 48 hours after administration would have been more appropriate.

In a GLP- and OECD (475)-compliant in vivo chromosomal aberration test, male B6D2F1/Crl mice (six per group) were dosed once by oral gavage with thiophanate-methyl (purity 97.9%, Batch no. TBC-035G) in aqueous 1% methylcellulose at 0, 500, 1000 or 2000 mg/kg bw. The positive control group received mitomycin C at 4 mg/kg bw once by intraperitoneal injection. The bone marrow cells of groups treated with thiophanate-methyl and the negative and positive control substances were prepared 24 hours or 48 hours (negative controls and 2000 mg/kg bw groups only) after administration. No clinical signs of toxicity or mortality were observed during the course of the study. No changes in body weight compared to the negative control group were observed. A dose-related increase in the mitotic index was seen at 24 hours (2.68%, 2.93% and 3.42% at 500, 1000 and 2000 mg/kg bw respectively, compared with 2.21% for the controls) and a slight increase was seen at the top dose at 48 hours (4.18% compared with 3.57% for the controls). These results indicate that thiophanate-methyl interferes with the progression of the cell cycle. Thiophanate-methyl did not induce any structural chromosomal aberrations in mouse bone marrow. The positive control MMC showed a clear statistically significant clastogenic effect. It was concluded that thiophanate-methyl did not demonstrate clastogenic potential in male B6D2F1/Crl mice (Kuboki, 2018). These negative findings are consistent with the lack of clastogenic changes induced by thiophanate-methyl as demonstrated in the older studies.

In a GLP- and OECD (474)-compliant spermatid micronucleus test, male Crl:CD-1 (ICR) mice (five per group) were administered a single dose of thiophanate-methyl (purity 97.9%, Batch no. TBC-035G) in aqueous 1% methylcellulose by oral gavage. Dose levels were 0 (negative control group), 500, 1000 or 2000 mg/kg bw. Mitomycin C at 2 mg/kg bw or colchicine at 1 mg/kg bw, administered by intraperitoneal injection served as positive controls. Testes were removed 24, 48 and 72 hours, and then 14 days after administration. Spermatids were isolated and slides prepared and stained. The frequency of micronucleated spermatids was determined by means of fluorescence microscopy in 4000 cells per dose group and time point. There was no mortality, clinical signs of toxicity, or relevant changes in body or testis weights. There was no statistically significant increase in the frequency of micronucleated spermatids at any of the exposure times. The positive control substances gave the expected response. Overall, thiophanate-methyl did not cause increases in micronuclei in spermatids of ICR mice up to the limit dose (Kuboki, 2017a). Although no proof of target tissue exposure was provided in the current mutagenicity study, in a kinetic study in the same strain of mice dosed orally with 2000 mg/kg bw of thiophanate-methyl, levels of thiophanate-methyl were similar in plasma and testes (Kuroiwa, 2017; see Section 1.1 above). However, the levels of carbendazim, the metabolite responsible for the aneugenicity when thiophanate-methyl is administered, were significantly lower in the testes than in plasma. In view of this, and considering that a threshold dose exists for this effect, it is possible that the levels of carbendazim in germ cells in the study were insufficient to trigger aneuploidy to any detectable degree.

In a GLP- and OECD (483)-compliant spermatogonial chromosomal aberrations test, male Crl:CD-1(ICR) mice (six per group) were dosed once by oral gavage with thiophanate-methyl (purity 97.9%, Batch no. TBC-035G) in aqueous 1% methylcellulose at 0 (negative control group), 500, 1000 or 2000 mg/kg bw. Mitomycin C served as a positive control and was administered once at 2 mg/kg bw by intraperitoneal injection. Testes were removed 24 hours (for the 500 and 1000 mg/kg bw groups and positive controls) or 48 hours (for the 0 and 2000 mg/kg bw groups) after administration. Five hours before sacrifice, animals were administered colchicine at 5 mg/kg bw intraperitoneally for cell division arrest. Spermatogonia were collected after collagenase treatment and slides were prepared and stained for analysis. No clinical signs of toxicity or mortality were observed during the course of the study. A dose-related increase in the mitotic index was seen at 24 hours (0.58%, 0.76% and 1.02% at 500, 1000 and 2000 mg/kg bw respectively, compared with 0.62% for controls). These results confirm once again that thiophanate-methyl interferes with the progression of the cell cycle. There was no increase in spermatogonial cells with structural chromosomal aberrations up to the top dose. The positive control substance gave the expected response. Overall, thiophanate-methyl was negative in this spermatogonial chromosomal aberration test in ICR mice up to the limit dose (Kuboki, 2017b). These negative findings are consistent with the lack of clastogenic changes induced by thiophanate-methyl as demonstrated in the older studies.

In summary, the four new in vivo mutagenicity studies showed no evidence of clastogenicity or aneugenicity in the bone marrow or testes of mice treated with thiophanate-methyl up to the limit dose. However, the results of the two cytogenetic studies (Kuboki, 2017b and 2018) are consistent with the lack of clastogenicity induced by thiophanate-methyl, as shown by the older studies. The negative result obtained in the recent bone marrow micronucleus study (Ishii, 2017a) is possibly a false negative, as for a substance like thiophanate-methyl which interferes with the progression of the cell cycle, two administrations, 24 hours apart, followed by one single sampling time 24 hours after sacrifice would have not been sufficient to detect micronuclei. In addition, the negative result obtained in the recent micronucleus study in spermatids (Kuboki, 2017a) can be explained by the fact that the levels of carbendazim (the metabolite responsible for the aneugenicity of thiophanate-methyl) found in a kinetic study in the same strain of mice dosed orally with 2000 mg/kg bw thiophanate-methyl (Kuroiwa, 2017) were significantly lower in the testes than the in the plasma. It is therefore possible that the levels of carbendazim in germ cells were insufficient to trigger aneuploidy to a detectable degree.

Overall, the new evidence provided by the sponsor on the genotoxicity of thiophanate-methyl is not relevant, or suffers from limitations, and the previous conclusion from the 2017 monograph that thiophanate-methyl is aneugenic in vitro and in vivo is still valid.

One study evaluating the genotoxic effects of thiophanate-methyl was found in the public literature. In an in vitro study (not GLP-compliant) the cytotoxicity and genotoxicity of a commercial fungicide product (Cercobin VR-M) containing thiophanate-methyl (concentration not specified) was evaluated in human skin fibroblasts (BJ) ATCCTM CRL-2522 at concentrations of 100, 150, 200, 250, 300, 400, 500, 800 and 1000 µM. Thiophanate-methyl generated DNA damage (double-strand breaks) in the treated cells by activating ataxia telangiectasia-mutated protein kinase (ATM) and histone H2A.X sensor proteins. After a 24-hour exposure a significant reduction in cell viability was observed, indicating that fungicides based on thiophanate-methyl possess cytotoxic activity at all studied concentrations (Moo-Muñoz et al., 2021). The findings from this paper suggest that thiophanate-methyl causes secondary genotoxicity in vitro as a consequence of severe cytotoxicity. Overall, the results from this publication are of little relevance to the evaluation of the mutagenicity of thiophanate-methyl which is caused by interference with the mitotic spindle assembly, resulting in aneuploidy.

## 2.5 Reproductive and developmental toxicity

The reproductive and developmental toxicity of thiophanate-methyl was described in the 2017 monograph (JMPR, 2021). No new studies were submitted for the purposes of the current evaluation.

> "In a two-generation dietary reproductive toxicity study in rats administered thiophanate-methyl at dietary doses of 0, 200, 630 or 2000 ppm (equal to premating doses of 0, 14.6, 46.0 and 147.1 mg/kg bw per day for males and 16.8, 52.2 and 164.3 mg/kg bw per day for females, respectively), the NOAEL for parental toxicity was 200 ppm (equal to 14.6 mg/kg bw per day) based on thyroid hyperplasia in males and increased TSH levels in females at 630 ppm (equal to 46 mg/kg bw per day). The NOAEL for offspring toxicity was 200 ppm (equal to 16.8 mg/kg bw per day) based on decreased body weights (in F2b) during lactation at 630 ppm (equal to 52.2 mg/kg bw per day). The NOAEL for reproductive toxicity was 2000 ppm (equal to 147 mg/kg bw per day), the highest dose tested (Müller, 1993; Müller & Singer, 1995).
>
> In a developmental toxicity study in rats administered gavage doses of thiophanate-methyl of 0, 100, 300 or 1000 mg/kg bw per day, the NOAEL for maternal toxicity was 300 mg/kg bw per day based on a reduced body weight gain at 1000 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Rodwell, 1981a, b).
>
> In a developmental toxicity study in rabbits administered gavage doses of thiophanate-methyl of 0, 5, 10, 20 and 40 mg/kg bw per day, the NOAEL for maternal toxicity was 10 mg/kg bw per day based on a reduced body weight gain at 20 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 20 mg/kg bw per day based on supernumerary thoracic ribs at 40 mg/kg bw per day. This effect was considered unlikely to be an effect of a single dose (York, 1997a, b)."

The 2017 Meeting concluded that thiophanate-methyl was not teratogenic.

*New developmental toxicity studies*

One developmental toxicity study evaluating the effects of thiophanate-methyl was identified in a search of the open literature. In this study (not GLP-compliant), pregnant Wistar rats (10 per group) received daily, by gavage, a commercial TM formulation, Cerconil WP®. This formulation composed of 200 g of thiophanate-methyl per kg, plus 500 g of chlorothalonil per kg (purities not given) was administered at 0, 400, 800 or 1200 mg/kg bw per day, from gestational day (GD) 6 until GD 15. The rats were killed on day 21 post coitum, and the fetuses were removed surgically by caesarean section and examined for developmental abnormalities.

Maternal liver weight was decreased in the 1200 mg/kg bw group and focal necrosis, microvesicular steatosis, inflammatory infiltrate and hepatocytes with pyknotic nuclei were observed at 800 mg/kg bw and above. Reproductive performance was similar among the groups, however, skeletal anomalies were increased at all three doses (no further information available). No NOAEL could be identified from the study (Silva et al., 2020). These findings appear inconsistent with those of the rat regulatory study, which identified a NOAEL for developmental toxicity of 1000 mg/kg bw per day. However, as the material tested was a formulation containing other chemicals and another active substance (chlorothalonil), it is not possible to attribute the reported findings to thiophanate-methyl. Overall, no meaningful conclusions can be drawn from this publication.

## 2.6 Special studies

### (a) Neurotoxicity, immunotoxicity

The relevant sections in the 2017 monograph (JMPR, 2021) conclude as follows.

> "In a study of acute neurotoxicity in rats administered gavage doses of thiophanate-methyl of 0, 50, 125, 500, 1000 or 2000 mg/kg bw, the NOAEL for general toxicity was 125 mg/kg bw based on transient reductions in body weight gains (including body weight losses) and feed consumption at 500 mg/kg bw. The NOAEL for neurotoxicity was 2000 mg/kg bw, the highest dose tested (Foss, 2005).
>
> In a 13-week study of neurotoxicity in rats administered thiophanate-methyl at dietary doses of 0, 100, 500 or 2500 ppm (equal to 0, 6.2, 30 and 150 mg/kg bw per day for males and 0, 6.8, 35 and 166 mg/kg bw per day for females, respectively), the NOAEL for general toxicity was 500 ppm (equal to 30 mg/kg bw per day) based on decreased body weights and feed consumption in females and increased liver and thyroid weights in both sexes at 2500 ppm (equal to 150 mg/kg bw per day). The NOAEL for neurotoxicity was 2500 ppm (equal to 150 mg/kg bw per day), the highest dose tested (Foss, 2007)."

The 2017 Meeting concluded that thiophanate-methyl was not neurotoxic.

No immunotoxicity tests with thiophanate-methyl were available. However, the data from the available toxicity studies did not indicate an immunotoxic potential of thiophanate-methyl.

In 2017, the Meeting concluded that thiophanate-methyl was unlikely to be immunotoxic.

*New studies on neurotoxicity and immunotoxicity*

No new special studies related to neurotoxicity or immunotoxicity were submitted by the sponsor for the purposes of the current evaluation, however, four in vitro thyroperoxidase activity inhibition assays of thiophanate methyl in different species were submitted, and have been presented here to explore further the potential underlying mechanism of the thyroid effects seen with thiophanate-methyl in rats.

### (b) Studies on the thyroid effects of thiophanate-methyl

In a thyroperoxidase activity inhibition assay (not GLP-compliant), microsomes were prepared from the thyroids of female Han Wistar rats. The known TPO inhibitor 6-propyl-2-thiouracil (PTU) was used as a positive control substance. Thiophanate-methyl (purity 97.9%, Batch no. TBC-035G) was tested at final concentrations of 0, 0.01, 0.1, 1, 2.5, 5, 10, 25, 50 and 100 µM. Under the conditions of this in vitro test, thiophanate-methyl was a weak inhibitor of TPO activity when tested up to its solubility limit. The positive control substance PTU induced the expected inhibition of TPO, thus confirming the sensitivity of the test system used. An extrapolated virtual, half maximal inhibitory concentration ($IC_{50}$) value, (an

$IC_{50}$ exceeding the maximum solubility of TM), was estimated for comparison with the positive control substance. The extrapolated virtual $IC_{50}$ of thiophanate-methyl was estimated to be ca 260 µM with a 95% confidence interval (CI) of 30–750 µM, whilst for PTU an $IC_{50}$ of 2.2 µM (95% CI 1.7–2.8 µM) was calculated. Based on the results obtained in this study it was concluded that thiophanate-methyl is not a rat TPO inhibitor in vitro (Haines, 2018a).

In a thyroperoxidase activity inhibition assay (not GLP-compliant), microsomes were prepared from the thyroids of female Beagle dogs. The known TPO inhibitor PTU was used as a positive control substance. Thiophanate-methyl (purity 97.9%, Batch no. TBC-035G) was tested at final concentrations of 0, 0.01, 0.1, 1, 2.5, 5, 10, 25, 50 and 100 µM. Under the conditions of this in vitro test, thiophanate-methyl was a weak inhibitor of TPO activity when tested up to its solubility limit. The positive control substance PTU induced the expected inhibition of TPO, thus confirming the sensitivity of the test system used. An extrapolated virtual $IC_{50}$) value,(one that exceeded the maximum solubility of thiophanate-methyl), was estimated to be ca 880 µM (95% CI 124–3870 µM), whilst for PTU an $IC_{50}$ of 17.7 µM (95% CI 14.6–21.3 µM) was calculated. Based on the results obtained in this study it was concluded that thiophanate-methyl is not a dog TPO inhibitor in vitro (Haines, 2018b).

In a thyroperoxidase activity inhibition assay (not GLP-compliant), microsomes were prepared from the thyroids of female Yorkshire pigs. The known TPO inhibitor PTU was used as a positive control substance. Thiophanate-methyl (purity 97.9%, Batch no. TBC-035G) was tested at final concentrations of 0, 0.01, 0.1, 1, 2.5, 5, 10, 25, 50 and 100 µM. The positive control substance PTU induced the expected inhibition of TPO, thus confirming the sensitivity of the test system used. Thiophanate-methyl did not cause any appreciable inhibition of TPO activity at concentrations up to its solubility limit. Based on the results obtained in this study it was concluded that thiophanate-methyl is not a porcine TPO inhibitor in vitro (Haines, 2018c).

In a thyroperoxidase activity inhibition assay (not GLP-compliant), microsomes were prepared from female human thyroids. The known TPO inhibitor PTU was used as a positive control substance. Thiophanate-methyl (purity 97.9%, Batch no. TBC-035G) was tested at final concentrations of 0, 0.1, 1, 2.5, 5, 10, 25, 50, 100, 150, 200 and 500 µM. Thiophanate-methyl did not cause any appreciable inhibition of TPO activity at concentrations up to its solubility limit. Based on the results obtained in this study it was concluded that thiophanate-methyl is not a human TPO inhibitor in vitro (Haines, 2018d).

Overall, these recent thyroid endocrine activity in vitro tests showed that thiophanate-methyl does not inhibit thyroperoxidase.

**(c) Studies on metabolites**

The toxicological characterization of some plant/livestock metabolites of thiophanate-methyl was addressed in the 2017 monograph, which stated that the main residues in crops and livestock were thiophanate-methyl, carbendazim, 5-OH-MBC and 5-OH-MBC-S.

Carbendazim itself is used as a pesticide, for which JMPR established an ADI in 1995 and an ARfD in 2005. However, no toxicological information was submitted for its re-evaluation in 2017. As in 2017, insufficient toxicological information on carbendazim was submitted for the purposes of this evaluation to allow a re-evaluation of this substance/metabolite that might confirm or amend the reference values established in 1995 (ADI) and 2005 (ARfD). The mouse kinetic studies recently submitted (Kuroiwa, 2017, 2018) indicate that carbendazim is a major plasma metabolite of thiophanate-methyl, and hence, in principle it can be covered by the parent reference values. However, since it is well established that carbendazim is significantly more toxic than thiophanate-methyl, this approach was considered inappropriate. In view of this, and in line with the established practice of adopting the TTC approach for metabolites with no, or limited, test data, it was considered appropriate as a conservative approach to adopt the TTC Cramer Class III value (1.5 µg/kg bw per day) for both the chronic and acute dietary risk assessment of carbendazim. The genotoxicity (direct DNA reactivity) TTC value was considered inappropriate in this case because in the period since the last JMPR evaluation of carbendazim in 2005, no significant new information has emerged to challenge the conclusion reached then, that carbendazim is an aneugen with a threshold mechanism.

No specific toxicity studies were available on TM metabolites 5-OH-MBC or 5-OH-MBC-S, which are also metabolites of carbendazim. However, 5-OH-MBC-S was found in rats at more

than 40% of the absorbed dose in a toxicokinetic study with TM, and at 21%–43% of the absorbed dose in a toxicokinetic study with carbendazim. Metabolite 5-OH-MBC is an intermediate in the metabolic pathway leading to the formation of 5-OH-MBC-S. In the 2017 evaluation the toxicities of rat metabolites 5-OH-MBC and 5-OH-MBC-S were therefore considered to be covered by that of thiophanate-methyl. However, during the present evaluation this assertion that they were covered by the parent was regarded invalid. Given the close structural similarity of these two metabolites to carbendazim and an in silico analysis (DEREK Nexus) submitted for the purposes of this evaluation showing the same mutagenic (aneugenic) alerts as carbendazim, it was considered more appropriate to treat metabolites 5-OH-MBC and 5-OH-MBC-S in the same way as carbendazim.

Two new in vivo bone marrow micronucleus studies, one on metabolite 4-OH-TM and one on metabolite 5-OH-MBC were submitted by the sponsor for the purposes of the current evaluation. These studies are evaluated below.

*New studies on 5-OH-MBC and 4-OH-TM*

In a GLP- and OECD (474)-compliant in vivo bone marrow micronucleus test, male Crl:CDl(ICR) mice (five per group) were dosed by oral gavage with 4-OH-TM (purity: 98.2%, Batch no. 31-15247) at 0 (negative control group), 500, 1000 or 2000 mg/kg bw. The animals were treated twice at an interval of approximately 24 hours. The positive control group received mitomycin C once intraperitoneally at 1 mg/kg bw. At approximately 24 hours after the second administration, bone marrow smears were prepared and evaluated to assess the incidence of micronuclei and signs of toxicity. No deaths or clinical signs of toxicity were seen in any of the groups. No statistically significant increase was observed in the incidence of micronucleated polychromatic erythrocytes per 4000 polychromatic erythrocytes, in any of the treated groups compared to the negative controls. No statistically significant increase was observed in the incidence of polychromatic erythrocytes in a total of 500 erythrocytes compared to the negative control group, indicating no toxicity to the bone marrow. The positive control substance gave the expected response. Overall, 4-OH-TM was negative in this bone marrow micronucleus test in male ICR mice (Ishii, 2017b). However, it is possible that this result was a false negative, as for a substance such as 4-OH-TM (thiophanate-methyl with a hydroxy group) which interferes with the progression of the cell cycle, one single administration followed by two sampling times at 24 and 48 hours after sacrifice would have been more appropriate.

In a GLP- and OECD (474)-compliant in vivo bone marrow micronucleus test, male ICR mice (five per group) were dosed by oral gavage with 5-OH-MBC (purity: 99.6%, Batch no. 31-15247) at 0 (negative control group), 500, 1000 or 2000 mg/kg bw. The animals were treated twice at an interval of approximately 24 hours. The positive control group received mitomycin C once intraperitoneally at 1 mg/kg bw. At approximately 24 hours after the second administration, bone marrow smears were prepared and evaluated to assess the incidence of micronuclei and signs of toxicity. No deaths or clinical signs of toxicity were seen in any of the groups. No statistically significant increase was observed in the incidence of micronucleated polychromatic erythrocytes per 4000 polychromatic erythrocytes in any of the treated groups compared to the negative control group. No statistically significant increase was observed in the incidence of polychromatic erythrocytes in a total of 500 erythrocytes compared to the negative control group, indicating no toxicity to the bone marrow. The positive control substance gave the expected response. Overall, 5-OH-MBC was negative in this bone marrow micronucleus test in male Crl:CD1(ICR) mice (Ishii, 2017c). However, it is possible that this result was a false negative as for a substance such as 5-OH-MBC (carbendazim with a hydroxy group) which interferes with the progression of the cell cycle, one single administration followed by two sampling times at 24 and 48 hours after sacrifice would have been more appropriate.

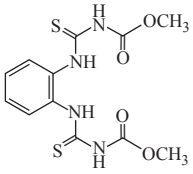
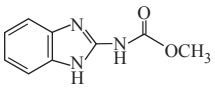
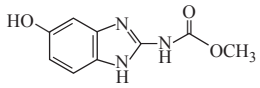
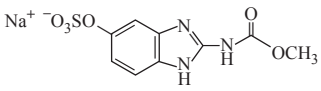
The current evaluation also identified additional plant/livestock metabolites; 4-OH-MBC, 4-OH-TM, 4-OH-TM-S, 3-OH-TM, 3-OH-TM-S, 5-OH-2-AB, 4-OH-2-AB and 4-OH-FH-432). A comparative (alongside thiophanate-methyl) in-silico analysis (using QSAR Toolbox, VEGA and DEREK Nexus) for genotoxicity and carcinogenicity was performed by the sponsor for 4-OH-TM, 4-OH-TM-S, 4-OH-2-AB and FH-432 (4-OH-FH-432 without the hydroxy group) for the purpose of the current evaluation. The analysis showed either no alerts for genotoxicity and carcinogenicity, or where some alerts were triggered, these were present also triggered for the parent substance, or were inconsistent between different models. Overall, therefore, metabolites 4-OH-TM, 4-OH-

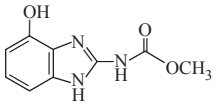
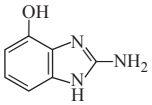
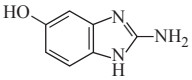
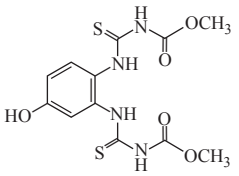
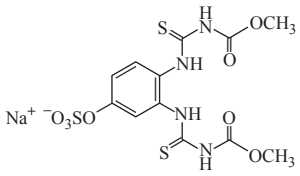
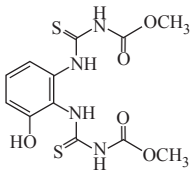
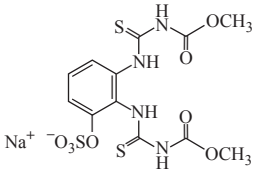
TM-S, 4-OH-2-AB and FH-432 should be regarded as being equally aneugenic as the parent substance, thiophanate-methyl. The negative bone marrow micronucleus study performed on 4-OH-TM (Ishii, 2017b) suffered from limitations and was considered to be a false negative. This result cannot therefore, be considered to override the in silico predictions obtained for 4-OH-TM. Given the very close structural similarity between thiophanate-methyl and both 4-OH-TM and 4-OH-TM-S, the parent reference values were assigned to these two metabolites at the current evaluation. Metabolite 4-OH-2-AB is structurally very similar to carbendazim and shares the benzimidazole moiety. Therefore, similarly to carbendazim, the TTC Cramer Class III value (1.5 µg/kg bw per day) should be used in any dietary risk assessment if required. Metabolite FH-432 (and by read-across metabolite 4-OH-FH-432) possesses different substituent groups (the two disulfide groups being replaced by carbonyls) compared to thiophanate-methyl. Therefore, despite the structural similarity to the parent substance, in the absence of any information on the general toxicity profile of this metabolite, the TTC Cramer Class III value (1.5 µg/kg bw per day) should be used in any dietary risk assessment if required.

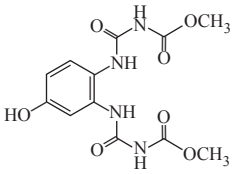
No information is available on metabolites 4-OH-MBC, 3-OH-TM, 3-OH-TM-S or 5-OH-2-AB. However, given the close structural similarity between carbendazim and both 4-OH-MBC and 5-OH-2-AB, the TTC Cramer Class III value (1.5 µg/kg bw per day) should be used in any dietary risk assessment if required. Similarly, given the close structural similarity between thiophanate-methyl and both 3-OH-TM and 3-OH-TM-S, the parent reference values should be assigned to these two metabolites.

The Table 6 below summarizes the available toxicological information on all the plant and livestock metabolites of interest and lists the associated toxicological reference values (TRVs)

***Table 6. Summary of toxicological information on plant/livestock metabolites of thiophanate -methyl***

| **Compound, codes and structure** | **Major rat metabolite** (> 10% AD) | **Genotoxicity assessment** (data, QSAR, RA) | **General toxicity** | **Toxicological reference values (TRVs)** |
|---|---|---|---|---|
| **Thiophanate-methyl (TM)**  | Parent | Aneugenic (data) | Full data set | ADI: 0–0.09 mg/kg bw<br>ARfD: 1 mg/kg bw |
| **Carbendazim (MBC)**  | Yes, but not covered by parent as well established that MBC is more potent than TM | Aneugenic (data) | Insufficient data to establish specific TRVs | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **5-OH-MBC** (5-hydroxy-carbendazim)  | Yes, but not covered by parent as similar to MBC, which is more potent than TM | Aneugenic (QSAR and RA from MBC) Negative in in vivo NM study; considered a potential false positive | No data but close structural similarity to MBC, hence MBC TRVs | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **5-OH-MBC-S** (5-hydroxy-carbendazim-sulfate)  | Yes, but not covered by parent as similar to MBC, which is more potent than TM | Aneugenic (QSAR and RA from MBC) | No data but close structural similarity to MBC, hence MBC TRVs | TTC Cramer class III value: 1.5 µg/kg bw per day |

| Compound, codes and structure | Major rat metabolite (> 10% AD) | Genotoxicity assessment (data, QSAR, RA) | General toxicity | Toxicological reference values (TRVs) |
|---|---|---|---|---|
| **4-OH-MBC**<br>(4-hydroxy-carbendazim)<br> | No | No data but close structural similarity to MBC, so aneugenic | No data but close structural similarity to MBC, hence MBC TRVs | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **4-OH-2-AB**<br>(2-Amino-1*H*-benzimidazol-4-ol<br> | No | Aneugenic (QSAR and RA from MBC) | No data but close structural similarity to MBC, hence MBC TRVs | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **5-OH-2-AB**<br>(2-Amino-1*H*-benzimidazol-5-ol<br> | No | No data but close structural similarity to MBC, so aneugenic | No data but close structural similarity to MBC, hence MBC TRVs | TTC Cramer class III value: 1.5 µg/kg bw per day |
| **4-OH-TM**<br>(4-hydroxy-thiophanate-methyl)<br> | No | No data but close structural similarity to TM, hence TM TRVs | No data but close structural similarity to TM, hence TM TRVs | Parental TRVs:<br>ADI: 0–0.09 mg/kg bw<br>ARfD: 1 mg/kg bw |
| **4-OH-TM-S**<br>(4-hydroxy-thiophanate-methyl-sulfate)<br> | No | No data but close structural similarity to TM, hence TM TRVs | No data but close structural similarity to TM, hence TM TRVs | Parental TRVs:<br>ADI: 0–0.09 mg/kg bw<br>ARfD: 1 mg/kg bw |
| **3-OH-TM**<br>(3-hydroxy-thiophanate-methyl)<br> | No | No data but close structural similarity to TM, hence TM TRVs | No data but close structural similarity to TM, hence TM TRVs | Parental TRVs:<br>ADI: 0–0.09 mg/kg bw<br>ARfD: 1 mg/kg bw |
| **3-OH-TM-S**<br>(3-hydroxy-thiophanate-methyl-sulfate)<br> | No | No data but close structural similarity to TM, hence TM TRVs | No data but close structural similarity to TM, hence TM TRVs | Parental TRVs:<br>ADI: 0–0.09 mg/kg bw<br>ARfD: 1 mg/kg bw |

| Compound, codes and structure | Major rat metabolite (> 10% AD) | Genotoxicity assessment (data, QSAR, RA) | General toxicity | Toxicological reference values (TRVs) |
|---|---|---|---|---|
| **4-OH-FH-432**<br>(Dimethyl 4,4′-(4-hydroxy-*O*-phenylene)bis(3-allophanate))<br> | No | Aneugenic (QSAR and RA from TM) | No data | TTC Cramer class III value: 1.5 µg/kg bw per day |

RA: Read-across; QSAR: Quantitative structure–activity relationship analysis;
ADI: Acceptable daily intake; ARfD: Acute reference dose; TTC: Threshold of toxicological concern;

## 3. Microbiological aspects

There was no information available in the public domain and no experimental data were submitted that addressed the possible impact of thiophanate-methyl residues on the human intestinal microbiome.

## 4. Observations in humans

No adverse effects reported in manufacturing plant personnel or from agricultural use of thiophanate-methyl.

# Comments

## Biochemical aspects

The absorption, distribution, metabolism and excretion (ADME) of thiophanate-methyl, as well as its toxicokinetics, were described in the 2017 monograph (JMPR, 2021) as follows

> "Thiophanate-methyl is rapidly and almost completely absorbed (88–89%) after oral administration of a dose of 14 mg/kg bw in mice (Noguchi, 1970; Noguchi & Kosaka, 1971). Thiophanate-methyl is rapidly excreted (approximately 47% in urine and approximately 40% in bile) within 48 hours of administration in rats (Bernard, 2011b). Plasma half-lives were 1.6–2.8 hours after a dose of 13 mg/kg bw and 2.4–7.8 hours after a dose of 140–170 mg/kg bw in rats (Tanoue, 1992a, b). Absorption and excretion patterns were similar in male and female rats (Bernard, 2011b). There is no potential for accumulation (Kosaka et al., 1975). Thiophanate-methyl is widely distributed in rats, with highest levels in liver and thyroid (Tanoue, 1992a, b). The major urinary metabolite in rats was 5-hydroxycarbendazim sulfate (5-OH-MBC-S at 8.7% of the administered dose). Minor metabolites were 5- and 4-hydroxy-thiophanate-methyl, each representing approximately 2% of the administered radiolabel. The major faecal metabolites were 4-hydroxy-thiophanate-methyl (6–10%), 5-OH-MBC-S (2–5%) and carbendazim (2–3%). Unchanged thiophanate-methyl accounted for approximately 20–24% and 50% of the administered radiolabel after repeated low and high doses, respectively, (Nabetani & Mori, 1993)."

Two oral, single-dose kinetic studies, one in ICR mice (Kuroiwa, 2017) and one in B6D2F1 mice (Kuroiwa, 2018) performed with unlabelled thiophanate-methyl and administered by gavage at 2000 mg/kg bw. These demonstrated systemic exposure and distribution to the testis of thiophanate-methyl and its major metabolites. The studies also demonstrated that carbendazim is a major plasma metabolite in the mouse, but levels were lower in the testes.

An in vitro comparative human/rat metabolism study showed that the metabolic pathways in both species were identical and no unique metabolite was detected in human microsome samples (Shimomura, 2017).

## Toxicological data

The acute toxicity of thiophanate-methyl was described in the JMPR 2017 monograph (JMPR, 2021). No new acute toxicity studies were submitted for the purposes of the current evaluation.

In rats, the oral $LD_{50}$ was greater than 5000 mg/kg bw (Nishibe, 1990b); in rabbits the dermal $LD_{50}$ was greater than 2000 mg/kg bw (Nishibe, 1990c); and in rats, the inhalation $LC_{50}$ was 1.7–1.98 mg/L (Nishibe, 1987). Thiophanate-methyl was not irritating to the skin or eyes of rabbits (Nishibe, 1986a, b, 1987). Thiophanate-methyl was a skin sensitizer in a Magnusson & Kligman test in Guinea pigs (Nishibe, 1989), but not in a Buehler test (Nishibe & Mochizuki, 1993).

The short-term toxicity of thiophanate-methyl was described in the JMPR 2017 monograph (JMPR, 2021). No new studies were submitted for the purposes of this evaluation.

"In repeated-dose oral toxicity studies with thiophanate-methyl in mice, rats and dogs, the most sensitive organs were the liver, thyroid and the haematological system.

In a pre-guideline six-month dietary toxicity study (with limited investigations) in mice administered dietary thiophanate-methyl concentrations of 0, 12.8, 64, 320, 1600 or 8000 ppm (equal to 0, 2, 10, 50, 250 and 1240 mg/kg bw per day for males and 0, 2, 11, 52, 231 and 1630 mg/kg bw per day for females, respectively), the NOAEL was 1600 ppm (equal to 231 mg/kg bw per day) based on decreased body weight gain and haematological changes indicative of slight anaemia in males and females at 8000 ppm (equal to 1240 mg/kg bw per day) (Nogushi & Hashimoto, 1970b).

In a 13-week dietary toxicity study in rats administered dietary thiophanate-methyl concentrations of 0, 200, 2200, 4200, 6200 or 8200 ppm (equal to 0, 13.9, 155, 293, 427 and 565 mg/kg bw per day for males and 0, 15.7, 173, 323, 479 and 647 mg/kg bw per day for females, respectively), the NOAEL was 200 ppm (equal to 13.9 mg/kg bw per day) based on haematological changes indicative of slight anaemia, increased thyroid and liver weights, follicular hyperplasia and hypertrophy of the thyroid and hepatocellular hypertrophy and increased lipofuscin pigment in both sexes and increased severity of glomerulonephrosis in males observed at 2200 ppm (equal to 155 mg/kg bw per day) (Nishibe & Takaori, 1990).

In a pre-guideline six-month dietary toxicity study (with limited investigations) in rats administered dietary thiophanate-methyl concentrations of 0, 12.8, 64, 320, 1600 or 8000 ppm (equal to 0, 1, 4, 20, 95 and 500 mg/kg bw per day for males and 0, 1, 5, 22, 110 and 660 mg/kg bw per day for females, respectively), the NOAEL was 1600 ppm (equal to 95 mg/kg bw per day) based on decreased body weight gain, haematological changes indicative of slight anaemia, decreased glucose levels and increased cholesterol levels, increased thyroid weights and histological changes in the thyroid in males and females at 8000 ppm (equal to 500 mg/kg bw per day) (Noguchi & Hashimoto, 1970b).

In a three-month toxicity study in dogs, administering thiophanate-methyl by gelatine capsule at doses of 0, 50, 200 and 800/400 mg/kg bw per day, the LOAEL was 50 mg/kg bw per day based on the hypertrophy of the follicular epithelial cells of the thyroid at this dose level (Auletta, 1991).

In a pre-guideline two-year toxicity study (with limited investigations) in dogs administered thiophanate-methyl by gelatine capsule at doses of 0, 2, 10, 50 or 250 mg/kg bw per day, the NOAEL was 10 mg/kg bw per day based on effects on thyroid weight and histopathology of the thyroid in both sexes at 50 mg/kg bw per day (Hashimoto et al., 1972).

The overall NOAEL for the studies in dogs was 10 mg/kg bw per day, and the overall LOAEL was 50 mg/kg bw per day."

The long-term toxicity and carcinogenicity of thiophanate-methyl were described in the JMPR 2017 monograph (JMPR, 2021). No new studies were submitted for the purposes of the current evaluation.

"In a 18-month carcinogenicity study in CD-1 mice, thiophanate-methyl was administered at dietary concentrations of 0, 150, 640, 3000 or 7000 ppm (equal to 0, 24, 99, 468 and 1079 mg/kg bw per day for males and 0, 29, 123, 558 and 1329 mg/kg bw per day for females, respectively). The NOAEL for chronic toxicity and carcinogenicity was 150 ppm (equal to 29 mg/kg bw per day) based on the hepatocellular centrilobular hypertrophy at nine months and hepatocellular adenomas in females observed at 640 ppm (equal to 123 mg/kg bw per day) (Tompkins, 1992).

In a two-year combined chronic toxicity/carcinogenicity study in Fisher 344 rats administered dietary thiophanate-methyl concentrations of 0, 75, 200, 1200 or 6000 ppm (equal to 0, 3.3, 8.8, 54 and 281 mg/kg bw per day for males and 0, 3.8, 10.2, 64 and 335 mg/kg bw per day for females, respectively), the NOAEL for chronic toxicity and carcinogenicity was 200 ppm (equal to 8.8 mg/kg bw per day) based

on reduced body weight gain in both sexes; increased total cholesterol and total protein in both sexes; decreased albumin to globulin ratio in both sexes at 12 and/or 18 months; decreased levels of chloride and potassium; decreased T4 and T3 and increased TSH in males at 24 months; increased urinary protein and granular kidneys in males; follicular cell hyperplasia and hypertrophy in the thyroid in both sexes at 12 and 24 months; a possible increase in the incidence of thyroid follicular cell adenoma in males; centrilobular hepatocellular hypertrophy and occurrence of lipofuscin pigment in both sexes at 12 and 24 months; lipidosis of the adrenal cortex in females at 12 months; and increased severity of nephropathy in both sexes at 24 months observed at 1200 ppm (equal to 54 mg/kg bw per day). In males, the incidence of thyroid follicular cell adenoma was increased at 1200 ppm and above, but reached statistical significance only at 6000 ppm (Takaori, 1993)."

A mechanistic study in rats showed that:

"... thiophanate-methyl induced cytochromes CYP450 (not further specified) and cytochrome B5 as well as uridine glucuronosyltransferase (UDP-GT), an enzyme that plays an important role in the clearance of T4 in the liver. Thiophanate-methyl also inhibited porcine thyroid microsomal peroxidase, an enzyme involved in thyroid hormone synthesis."

However, this was not confirmed in more recent studies. Four in vitro thyroperoxidase (TPO) activity inhibition assays of thiophanate methyl in different species (rat, dog, pig and human in Haines, 2018a, b, c, d respectively) were submitted to explore further the potential underlying mechanism of the thyroid effects seen with thiophanate-methyl in rats. These studies showed that thiophanate-methyl does not inhibit TPO.

Thyroxine (T4) supplementation counteracted the hypertrophy of the thyroid and the TSH response, indicating that thiophanate-methyl caused the hypertrophy by negative feedback mechanism. The mechanistic study demonstrates that the thyroid effects resulting from thiophanate-methyl are likely to be the result of a reduction in thyroid hormones. In further studies, thiophanate-methyl induced increases in CYP3A and CYP2B1 in the liver of rats (Takaori, 1993; Paolini et al., 1999).

The 2017 Meeting concluded that thiophanate-methyl was carcinogenic in mice and rats. That Meeting considered the different modes of action that might underlie the tumour induction observed. The effects on the thyroid, including the induction of thyroid follicular cell adenoma in rats, may be secondary effects resulting from liver enzyme induction that enhances thyroid hormone excretion and leads to perturbations in systemic thyroid hormone levels and an increase in thyroid-stimulating hormone (TSH) concentration. The continuous stimulation of the thyroid gland by TSH is known to cause follicular cell hypertrophy/hyperplasia and, depending on dose and time, to result in follicular cell adenomas/adenocarcinomas. Rodents are particularly sensitive to any decreases in T4 and T3 levels that might result from liver enzyme induction. This is a well-established adverse outcome pathway without relevance for humans. Another possible mode of action for the carcinogenic effect may be the interference of the thiophanate-methyl metabolite carbendazim with mitotic spindle proteins leading to aneuploidy (see below).

The genotoxicity of thiophanate-methyl was described in the JMPR 2017 monograph (JMPR, 2021).

"Thiophanate-methyl was tested in an adequate range of in vitro and in vivo assays for genotoxicity. Thiophanate-methyl did not cause gene mutations or structural chromosomal aberrations; however, it caused changes in chromosome number (aneuploidy) both in vitro and in vivo. Induction of micronucleus formation in [B6D2F1] mice was seen after single doses (500 mg/kg bw and above), but the response was weak (about six times lower) when compared with that for the metabolite of thiophanate-methyl, carbendazim (Myhr & Brusick, 1981; McSheehy et al., 1984; Murli, 1988; Nishibe, 1990; Barale et al, 1993; Marshall, 1996a,b; Proudlock, 1999).

Carbendazim causes changes in chromosome number (aneuploidy) both in vitro and in vivo (in somatic cells and germ cells) as a result of its interference with mitotic spindle proteins. The mechanism by which aneuploidy is induced by carbendazim is well understood and consists of inhibition of the polymerization of tubulin, the protein that is essential for the segregation of the chromosomes during cell division. The nature of the mechanism is thus consistent with the identification of a threshold dose below which no toxicological effect would occur. Like thiophanate-methyl, carbendazim does not cause gene mutations or structural chromosomal aberrations."

In 2017, the Meeting concluded that the genotoxic effect of thiophanate-methyl was a threshold phenomenon and was probably related to the production of carbendazim.

Two new micronucleus tests, one in bone marrow and one in spermatids were provided but were not considered useful for the assessment of aneuploidy.

The present Meeting confirmed that thiophanate-methyl causes aneuploidy, a threshold phenomenon.

The present Meeting also confirmed that thiophanate-methyl is unlikely to pose a carcinogenic risk to humans at dietary dose levels.

The reproductive and developmental toxicity of thiophanate-methyl was described in the JMPR 2017 monograph (JMPR, 2021). No new studies were submitted for the purposes of the current evaluation.

> "In a two-generation dietary reproductive toxicity study in rats administered thiophanate-methyl at dietary doses of 0, 200, 630 or 2000 ppm (equal to pre-mating doses of 0, 14.6, 46.0 and 147.1 mg/kg bw per day for males and 16.8, 52.2 and 164.3 mg/kg bw per day for females, respectively), the NOAEL for parental toxicity was 200 ppm (equal to 14.6 mg/kg bw per day) based on thyroid hyperplasia in males and increased TSH levels in females at 630 ppm (equal to 46 mg/kg bw per day). The NOAEL for offspring toxicity was 200 ppm (equal to 16.8 mg/kg bw per day) based on decreased body weights (in F2b) during lactation, at 630 ppm (equal to 52.2 mg/kg bw per day). The NOAEL for reproductive toxicity was 2000 ppm (equal to 147 mg/kg bw per day), the highest dose tested (Müller, 1993; Müller & Singer, 1995).
>
> In a developmental toxicity study in rats administered gavage doses of thiophanate-methyl of 0, 100, 300 or 1000 mg/kg bw per day, the NOAEL for maternal toxicity was 300 mg/kg bw per day based on a reduced body weight gain at 1000 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Rodwell, 1981a, b).
>
> In a developmental toxicity study in rabbits administered gavage doses of thiophanate-methyl of 0, 5, 10, 20 and 40 mg/kg bw per day, the NOAEL for maternal toxicity was 10 mg/kg bw per day based on a reduced body weight gain at 20 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 20 mg/kg bw per day based on supernumerary thoracic ribs at 40 mg/kg bw per day. This effect was considered unlikely to be an effect of a single dose (York, 1997a, b)."

The 2017 Meeting concluded that thiophanate-methyl was not teratogenic. In the absence of any new information, the present Meeting re-affirmed the same conclusion.

The neurotoxic and immunotoxic potentials of thiophanate-methyl were described in the JMPR 2017 monograph (JMPR, 2021). No new studies were submitted for the purposes of the current evaluation.

> "In a study of acute neurotoxicity in rats administered gavage doses of thiophanate-methyl of 0, 50, 125, 500, 1000 or 2000 mg/kg bw, the NOAEL for general toxicity was 125 mg/kg bw based on transient reductions in body weight gains (including body weight losses) and feed consumption at 500 mg/kg bw. The NOAEL for neurotoxicity was 2000 mg/kg bw, the highest dose tested (Foss, 2005).
>
> In a 13-week study of neurotoxicity in rats administered thiophanate-methyl at dietary doses of 0, 100, 500 or 2500 ppm (equal to 0, 6.2, 30 and 150 mg/kg bw per day for males and 0, 6.8, 35 and 166 mg/kg bw per day for females, respectively), the NOAEL for general toxicity was 500 ppm (equal to 30 mg/kg bw per day) based on decreased body weights and feed consumption in females and increased liver and thyroid weights in both sexes at 2500 ppm (equal to 150 mg/kg bw per day). The NOAEL for neurotoxicity was 2500 ppm (equal to 150 mg/kg bw per day), the highest dose tested (Foss, 2007)."

The 2017 Meeting concluded that thiophanate-methyl was not neurotoxic. In the absence of any new information the present Meeting re-affirmed the same conclusion.

No immunotoxicity tests with thiophanate-methyl were available. However, the data from the available toxicity studies did not indicate an immunotoxic potential for thiophanate-methyl.

The 2017 Meeting concluded that thiophanate-methyl was unlikely to be immunotoxic. In the absence of any new information, the present Meeting re-affirmed the same conclusion.

## Toxicological data on metabolites and/or degradates

The toxicological characterization of some plant/livestock metabolites of thiophanate-methyl was addressed in the JMPR 2017 monograph (JMPR, 2021). It is stated there that the main residues in crops and livestock were thiophanate-methyl, carbendazim, 5-OH-MBC and 5-OH-MBC-S.

As major rat metabolites of the parent, the toxicities of 5-OH-MBC and 5-OH-MBC-S were considered in the 2017 evaluation to be covered by that of thiophanate-methyl.

### New data on metabolites 4-OH-TM and 5-OH-MBC

Two new in vivo bone marrow micronucleus studies (Ishii, 2017b), one with 4-OH-TM, and one with 5-OH-MBC (Ishii, 2017c) were negative, however both studies had limitations and were considered to be potentially false negatives. Therefore, it cannot be excluded that both 4-OH-TM and 5-OH-MBC are aneugenic like their precursors (thiophanate-methyl and carbendazim, respectively).

### Assessment of metabolite carbendazim

Carbendazim itself is used as a pesticide. The JMPR 1995 Meeting established an ADI of 0–0.03 mg/kg bw per day, and the 2005 Meeting added ARfDs of 0.1 mg/kg bw for women of child-bearing age and 0.5 mg/kg bw for the general population. For the present Meeting insufficient toxicological information was submitted on carbendazim to allow a re-evaluation of this metabolite and thus confirm or amend the reference values established in 1995 (ADI) and 2005 (ARfD). The newly submitted mouse kinetic studies with thiophanate-methyl indicate that carbendazim is a major plasma metabolite of thiophanate-methyl (Kuroiwa, 2017, 2018). However, it is well established that carbendazim is significantly more toxic than thiophanate-methyl. In view of this, and in line with the established practice of adopting the threshold of toxicological concern (TTC) approach for metabolites with no (or limited) test data, the present Meeting agreed to use the TTC Cramer class III value of 1.5 µg/kg bw per day for both the chronic and acute dietary risk assessment of carbendazim as a metabolite of thiophanate methyl, as a conservative value. The genotoxicity (direct DNA reactivity) TTC value was considered inappropriate in this case because in the period since the last JMPR evaluation of carbendazim in 2005, no significant new information has emerged to challenge the conclusion reached then, that carbendazim is an aneugen with a threshold mechanism.

### New information on metabolites 5-OH-MBC and 5-OH-MBC-S

The present Meeting reconsidered the conclusion, previously reached, that the metabolites 5-OH-MBC and 5-OH-MBC-S, as major rat metabolites of thiophanate-methyl, are covered by the parent. Given their close structural similarity to carbendazim and an in silico analysis submitted for the purpose of this evaluation showing the same mutagenic (aneugenic) alerts as carbendazim, the Meeting agreed that both metabolites should be treated as for carbendazim. Therefore the TTC Cramer class III value of 1.5 µg/kg bw per day should be used in the dietary risk assessment of these metabolites if required.

The current evaluation has also identified additional plant/livestock metabolites (4-OH-MBC, 4-OH-TM, 4-OH-TM-S, 3-OH-TM, 3-OH-TM-S, 5-OH-2-AB, 4-OH-2-AB and 4-OH-FH-432). A toxicological characterization of these metabolites was performed using genotoxicity in silico analyses and structural similarity considerations.

Table 6 on p 672 summarizes the available toxicological information on all the plant and livestock metabolites of interest and shows the associated toxicological reference values (TRVs).

## Microbiological aspects

There was no information available in the public domain and no experimental data were submitted that addressed the possible impact of thiophanate-methyl residues on the human intestinal microbiome.

## Human data

Thiophanate-methyl has been commercially produced since 1969. No health effects related to thiophanate-methyl have been reported in manufacturing plant personnel or from agricultural use.

The present Meeting concluded that the existing database on thiophanate-methyl was adequate to characterize the potential hazards to the general population, including fetuses, infants and children

# Toxicological evaluation (addendum)

The Meeting re-affirmed the ADI of 0–0.09 mg/kg bw for thiophanate-methyl established by the 2017 Meeting.

The Meeting re-affirmed the ARfD of 1 mg/kg bw for thiophanate-methyl established by the 2017 Meeting.

***Levels relevant to risk assessment of thiophanate-methyl***

| Species | Study | Effect | NOAEL | LOAEL |
|---|---|---|---|---|
| **Mouse** | 78-week study of toxicity and carcinogenicity [a] | Toxicity | 150 ppm, equal to 29 mg/kg bw per day | 640 ppm, equal to 123 mg/kg bw per day |
| | | Carcinogenicity | 150 ppm, equal to 29 mg/kg bw per day | 640 ppm, equal to 123 mg/kg bw per day |
| **Rat** | Acute neurotoxicity study [b] | Neurotoxicity | 2000 mg/kg bw [c] | - |
| | Two-year studies of toxicity and carcinogenicity [a] | Toxicity | 200 ppm, equal to 8.8 mg/kg bw per day | 1200 ppm, equal to 54 mg/kg bw per day |
| | | Carcinogenicity | 200 ppm, equal to 8.8 mg/kg bw per day | 1200 ppm, equal to 54 mg/kg bw per day |
| | Two-generation study of reproductive toxicity [a] | Parental toxicity | 200 ppm, equal to 14.6 mg/kg bw per day | 630 ppm, equal to 46 mg/kg bw per day |
| | | Offspring toxicity | 200 ppm, equal to 16.8 mg/kg bw per day | 630 ppm, equal to 52.2 mg/kg bw per day |
| | | Reproductive toxicity | 2000 ppm, equal to 147 mg/kg bw per day [c] | - |
| | Developmental toxicity study [b] | Maternal toxicity | 300 mg/kg bw per day | 1000 mg/kg bw per day |
| | | Embryo/fetal toxicity | 1000 mg/kg bw per day [c] | - |
| **Rabbit** | Developmental toxicity study [b] | Maternal toxicity | 10 mg/kg bw per day | 20 mg/kg bw per day |
| | | Embryo/fetal toxicity | 20 mg/kg bw per day | 40 mg/kg bw per day |
| **Dog** | 13-week and one-year studies of toxicity [d,e] | Toxicity | 10 mg/kg bw per day | 50 mg/kg bw per day |

[a] Dietary administration; [b] Gavage administration; [c] Highest dose tested;
[d] Two or more studies combined; [e] Capsule administration;

*Acceptable daily intake (ADI)**

0–0.09 mg/kg bw

*Acute reference dose (ARfD)**

1 mg/kg bw

* Applies to thiophanate-methyl, and the following metabolites: 3-OH-TM, 3-OH-TM-S, 4-OH-TM and 4-OH-TM-S, expressed as thiophanate-methyl.

## Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure.

***Critical end-points for setting guidance values for exposure to thiophanate-methyl, (all re-affirmed information is italicized)***

| | |
|---|---|
| **Absorption, distribution, excretion and metabolism in mammals** | |
| Rate and extent of oral absorption | *Rapid and almost complete (up to 89% in rats)* |
| Dermal absorption | *≥ 53% at 0.3 mg/rat; ≥ 23% at 32 mg/rat* |
| Distribution | *Widespread distribution, highest concentrations found in liver and thyroid* |
| Potential for accumulation | *Low* |
| Rate and extent of excretion | *Rapid; 87% of AD in 48 hours* |
| Metabolism in animals | *Extensively metabolized*<br>*Major metabolites are 5-OH-MBC-S, 5-OH-MBC and MBC* |
| Toxicologically significant compounds in animals and plants | Thiophanate-methyl, carbendazim, 3-OH-TM, 3-OH-TM-S, 4-OH-TM and 4-OH-TM-S |
| **Acute toxicity** | |
| Rat, $LD_{50}$, oral | *> 5000 mg/kg bw* |
| Rat, $LD_{50}$, dermal | *> 2000 mg/kg bw* |
| Rat, $LC_{50}$, inhalation | *> 1.7 mg/L* |
| Rabbit, dermal irritation | *Not irritating* |
| Rabbit, ocular irritation | *Not irritating* |
| Guinea pig, dermal sensitization | *Sensitizing (Magnusson & Kligman)* |
| **Short-term studies of toxicity** | |
| Target/critical effect | *Liver, thyroid, haematological effects* |
| Lowest relevant oral NOAEL | *10 mg/kg bw per day (dog)* |
| Lowest relevant dermal NOAEL | *No data* |
| Lowest relevant inhalation NOAEC | *No data* |
| **Long-term studies of toxicity and carcinogenicity** | |
| Target/critical effect | *Body weight, clinical chemistry, urine analysis, histopathology of liver, thyroid, kidney, adrenal* |
| Lowest relevant NOAEL | *8.8 mg/kg bw per day (rat)* |
| Carcinogenicity | *Carcinogenic in mice (liver) and rats (thyroid)*[a]<br>*Tumours are not relevant for humans due to differences in thyroid homeostasis between rodents and humans* |
| **Genotoxicity** | Genotoxic in vivo, threshold phenomenon (aneuploidy) confirmed; new, but inadequate, negative in vivo studies |
| **Reproductive toxicity** | |
| Target/critical effect | *No reproductive effects* |
| Lowest relevant parental NOAEL | *14.6 mg/kg bw per day (rat)* |
| Lowest relevant offspring NOAEL | *16.8 mg/kg bw per day (rat)* |
| Lowest relevant reproductive NOAEL | *147 mg/kg bw per day (rat)* |

| | |
|---|---|
| **Developmental toxicity** | |
| Target/critical effect | *Supernumerary ribs* |
| Lowest relevant maternal NOAEL | *10 mg/kg bw per day (rabbit)* |
| Lowest relevant embryo/fetal NOAEL | *20 mg/kg bw per day (rabbit)* |
| **Neurotoxicity** | *Not neurotoxic* |
| Acute neurotoxicity NOAEL | *2000 mg/kg bw, highest dose tested (rat)* |
| Subchronic neurotoxicity NOAEL | *150 mg/kg bw per day, highest dose tested (rat)* |
| Developmental neurotoxicity NOAEL | *No data* |
| **Other toxicological studies** | |
| Immunotoxicity NOAEL | *No evidence from routine studies* |
| Mechanistic studies | Negative in vitro thyroid peroxidase assays in different species |

**Studies on toxicologically relevant metabolites**

| | |
|---|---|
| ***Carbendazim*** | No new information |
| ***4-OH-TM*** | Negative bone marrow micronucleus study, but potentially false |
| ***5-OH-MBC*** | Negative bone marrow micronucleus study, but potentially false |
| ***5-OH-MBC, 5-OH-MBC-S, 4-OH-2-AB, 4-OH-TM, 4-OH-TM-S, 4-OH-FH-432*** | Aneugenic based on genotoxicity in silico analysis and read-across from carbendazim and thiophanate-methyl |
| **Microbiological aspects** | No data was submitted |
| **Human data** | No clinical cases or poisoning incidents have been recorded |

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet

## Summary

| | Value | Study | Safety factor |
|---|---|---|---|
| ADI | 0–0.09 mg/kg bw [a] | Two-year study of toxicity and carcinogenicity (rat) | 100 |
| ARfD | 1 mg/kg bw [a] | Acute neurotoxicity study (rat) | 100 |

[a] Applies to thiophanate-methyl and metabolites 3-OH-TM, 3-OH-TM-S, 4-OH-TM and 4-OH-TM-S expressed as thiophanate-methyl

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# Tricyclazole

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## Explanation

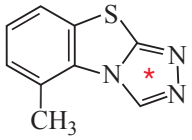
Tricyclazole is the International Organization for Standardization (ISO)-approved common name for 5-methyl[1,2,4]triazolo[3,4-b][1,3]benzothiazole (IUPAC), Chemical Abstracts Service number 41814-78-2. Tricyclazole is a systemic fungicide from the benzothiazole chemical group that is recommended for the control of the fungus *Pyricularia grisea*, popularly known as rice blast. As an antifungal agrochemical it acts by inhibiting melanin synthesis. It is a conjugate base of a 5-methyl[1,2,4]triazolo[3,4-b][1,3]benzothiazol-1-ium.

Tricyclazole has not previously been evaluated by the Joint FAO/WHO Meeting on Pesticides Residues (JMPR) and was reviewed by the present meeting at the request of the Codex Committee on Pesticides Residues (CCPR).

Some of the studies discussed for this evaluation do not comply with good laboratory practice (GLP) as the data were generated before the implementation of GLP guidelines. However, the critical studies were conducted in a similar manner to current test guidelines, unless otherwise specified. A limited amount of additional information from a literature search was identified that complemented

the toxicological information submitted for the current assessment. Overall, the Meeting considered that the database was adequate for the risk assessment.

***Figure 1. Chemical structure of tricyclazole***




* denotes uniform $^{14}C$ labelling of the triazole ring

# Evaluation for acceptable daily intake

## 1. Biochemical aspects

### 1.1 Absorption, distribution, excretion, and metabolism

#### (a) Oral route

A number of studies to investigate the biochemical aspects of tricyclazole have been conducted, and are supported by a recent review of the absorption, distribution, metabolism and excretion (ADME) properties of tricyclazole, alongside some in silico modelling of systemic exposure (Corvaro & Barteks, 2019).

*Study 1*

The ADME of tricyclazole labelled with $^{14}C$ in the triazole ring, [*triazole-U-*$^{14}C$]tricyclazole, was studied following oral gavage administration to Fischer 344 rats. Four male rats each received radiolabelled test material at either 2 mg/kg body weight (bw; the low-dose group) or 40 mg/kg bw (the high-dose group). An additional four male rats received 14 consecutive daily doses of unlabelled tricyc1azole at 2 mg/kg bw followed by the same dose level of [*triazole-U-*$^{14}C$]tricyclazole on day 15 (the multiple low-dose group). Rats were maintained in metabolism cages, with separate collection of urine (including cage washes), faeces and expired carbon dioxide. Analysis was conducted on pooled urine and faeces samples, with the weight of urine or faeces from each animal based on the total quantity excreted during the time period. All animals were euthanized 168 hours after receiving a labelled tricyclazole dose.

The mean total recoveries of radioactivity (total radioactivity found in volatiles/carbon dioxide traps, urine, faeces, cage rinse/cage wash, blood, organs, tissues, and carcass) ranged from 95.2% to 98.7% of administered radioactivity (AR). The collection of volatiles/carbon dioxide was discontinued at 96 hours since less than 1% of AR had been detected in the traps by that time. The mean total radioactivity found in urine and cage rinses in the single low- and high-dose groups and the multiple low-dose group was similar, representing 34.8%–39.3% of the administered dose (AD). The mean total radioactivity found in the faeces in the single low- and high-dose groups and the multiple low-dose group was similar at 57.2% 52.6% and 56.2% of AD respectively. Greater than 90% of the radioactivity found in the urine, cage rinses and faeces in all dose groups was eliminated by 72 hours after the last dose. Little radioactivity (less than 4% of AD) remained in the blood, organs, tissues and carcass of the animals 168 hours after the last dose, with the highest levels present in the blood and carcass. The relative distribution of radioactive equivalents was independent of dosing regimen. The values as a percentage of AD (and in particular the fat/blood radioactivity ratios) were not determined. Based on the low total radioactivity level retained at 168 hours (at most 4.1% of AD), even following repeated dosing, tricyclazole displayed a limited likelihood of bioaccumulating (Andre, 2004).

*Study 2*

In another study, male Fischer 344 rats were administered [*triazole-U-*$^{14}C$]tricyclazole once at a dose level of 2 mg/kg bw, by either an oral or intravenous (i.v.) route. Blood samples were collected for up to 96 hours after dosing. After oral dosing, absorption was very quick: plasmatic radioactivity was detected at 5 minutes post dosing,with a time to peak concentration ($T_{max}$) ranging from 10 to 30 minutes and displaying a plateau. This was followed by an efficient, biphasic depuration (no further toxicokinetic analysis was undertaken). The area under the concentration–time curve for the 96 hours following dosing ($AUC_{0–96h}$) was 11.7 µg.hours/mL. After intravenous injection, there was no plateauing, just an efficient, biphasic depuration (no further toxicokinetic analysis was undertaken). The $AUC_{0–96h}$ was 12.5 µg.hours/mL. The high bioavailability value determined for tricyclazole is indicative of rapid and extensive absorption. Evidence of secondary peaks in the i.v. and oral profiles support the existence

of biliary excretion and enterohepatic recirculation of tricyclazole. The absolute bioavailability value determined for tricyclazole in this study was 93.9% (Johnson, Veley & Monson, 2004).

*Study 3*

A study was conducted to determine ADME of [*triazole-U*-$^{14}$C]tricyclazole following oral and i.v. administration to groups of male and female F344/DuCrl rats. Tricyclazole was administered orally at a single, low dose (2 mg/kg bw) and single, high dose (40 mg/kg bw) to four animals of each sex per group, as well as by repeat dose administration, consisting of daily oral doses of unlabelled tricyclazole, followed by a single dose of radiolabelled tricyclazole at 2 mg/kg bw. Tricyclazole was administered at 2 mg/kg bw by i.v. administration. Blood, excreta and tissues were collected and analyzed for seven days post dosing following low- and high-level oral dosing, repeat oral dosing and i.v. dosing. An additional group of rats were used to assess the maximum concentrations achieved ($C_{max}$). Orally administered tricyclazole was rapidly absorbed without any apparent lag time. The total absorption of the orally administered dose in male rats was 89% of AR and 96% in females. Total recovery of radioactivity from all the animals averaged 96% ± 6% and 101% ± 11% in the oral and i.v. dose groups, respectively.

The orally absorbed dose was rapidly excreted in urine (31%–61% of AD) with slightly higher recoveries in the females. The majority of the urinary elimination (66%–86%) occurred within 24 hours of dosing. The test material administered by i.v. was also rapidly excreted in urine (35%–47% of AD in males and females respectively). The majority of the urinary elimination (68%–84%) occurred in the first 24 hours following dosing. A higher percentage (39%–65%) of the oral dose was eliminated in faeces, with the majority of the excretion (90% in males, 80% in females) occurring in the first 24 hours. Total faecal elimination of the i.v. dose was up to 59%, showing substantial biliary elimination of tricyclazole.

Only 0.8%–2% of the administered labelled tricyclazole remained in the tissues after 168 hours post dosing whatever the dosing regime. An average of 1.1% of the i.v. administered, labelled tricyclazole remained in the tissues of the animals sacrificed 168 hours post dosing. In all groups, residual concentration in blood at 168 hours post dosing (less than 0.5% of AD) was most likely due to the higher affinity of tricyclazole to red blood cells (RBCs). Test material-derived radioactivity was also present in RBCs, with $C_{max}$ concentrations ca 27%–48% higher than seen in plasma from the oral groups. Elimination from RBCs was very slow. After i.v. dosing, the radioactivity that did not bind to RBCs was rapidly eliminated. The residual level of bound radioactivity was eliminated slowly, similarly to what was observed after oral dosing.

In the rats dosed orally with [*triazole-U*-$^{14}$C]tricyclazole, elimination of the radioactivity from plasma was biphasic, with most of the elimination occurring during the rapid (α) elimination phase for which the half-life ($t_{½\alpha}$) was 0.08 hours or less. This was followed by a slower (β) elimination phase for which the half-life ($t_{½\beta}$) was 34–51 hours. The AUC for radioactivity in plasma after high-dose administration was 244–253 µg.hours/g, which was 29- to 37-fold higher than that observed at the low dose. Clearance was faster in the low-dose group than in the high-dose animals.

In summary, administered *triazole-U*-$^{14}$C]tricyclazole was rapidly absorbed, highly metabolized and eliminated fairly quickly from the rat (Hansen, et al., 2009).

*Study 4*

A study was performed to determine limited pharmacokinetics (in plasma), excretion, mass balance, and selected tissue distribution of radioactivity following administration of a single, oral dose of the test article, [*triazole-U*-$^{14}$C]tricyclazole, administered to jugular vein-cannulated (JVC) Crl:WI Wistar Han rats. Multiple doses were used to identify ranges of linearity/non-linearity (if any) in terms of total radioactivity.

Four treatment groups, each composed of one female rat, were administered a single, oral dose of the test article via gavage at 2, 7, 28 and 50 mg/kg bw for groups 1 to 4 respectively, An additional group was administered 2 mg/kg bw for analysis of additional tissues. The test article was administered in 0.5% Methocel A15C in water as a vehicle.

Blood samples were collected from groups 1–4 at designated time points (5, 10, 15, 20, 30 and 45 minutes, and 1, 2, 4, 8, 12, 24, 48, 72, 96, 120, 144 and 168 hours) for plasma radioactivity analysis. Urine, faeces, and cage residue were collected at 24 hour intervals up to 168 hours post dose for radioactivity concentration analysis. At study termination, animals in groups 1–4 were euthanized and the carcasses stored frozen pending disposal. The additional animal was euthanized at six hours post

dose and selected tissues (bone marrow, stomach, stomach contents, liver, and kidney) and the carcass were collected and analyzed for total remaining radioactivity. Following radioanalysis, metabolite profiling was performed on pooled urine and faeces samples.

A single oral dose of [*triazole-U*-$^{14}$C]tricyclazole was rapidly absorbed in the rat. It was observed that $C_{max}$ was achieved anywhere from five minutes (at 7 mg/kg bw) to one hour (at 50 mg/kg bw) post dose across groups 1–4. The plasma concentration curves and AUC appeared to indicate that absorption became saturated between 28 and 50 mg/kg bw. This was supported by the changes seen in the rate of excretion (expressed as a percentage AR) across the four dose levels. Total radioactivity recovered from excretion ranged from 82.23% (at 7 mg/kg bw) to 104.94% (at 50 mg/kg bw). Total recovery in faeces ranged from 35.31% to 61.54%. Total recovery in urine ranged from 33.97% to 41.87%. The routes of excretion and total recoveries were similar across the four groups. However, the rate of excretion as a percentage of AR was slower for the groups receiving higher doses, with the majority of radioactivity excreted within 0–24 hours post dose at 2 and 7 mg/kg bw, whilst at 28 and 50 mg/kg bw significant fractions of the dose were excreted up to 72 hours post dose.

At six hours post dose, the majority of the radioactive dose was in the carcass, with the highest radioactivity concentrations in the liver and kidney, of the tissues evaluated. Lower concentrations of radioactivity were measured in the terminal plasma, stomach, stomach contents, and bone marrow. (Dubnicka, 2017a).

*Study 5*

A study was conducted to determine peak blood radioactivity and demonstrate that oral gavage administration of tricyclazole to mice resulted in systemic exposure, and so demonstrate adequate exposure in support of the outcome of a micronucleus assay. The test material, [*triazole-U*-$^{14}$C]tricyclazole, was administered via a single oral gavage dose of 300 mg/kg bw, to two male Crl:CD1(ICR) mice. Blood samples were taken at 0.25, 0.5, 1 and 2 hours post dosing, after which animals were euthanized. Levels of radioactivity in blood continued to rise from 0.25–2 hours post dosing. Blood concentrations reached 90 µg equiv./g on average, showing clear evidence of systemic exposure to tricyclazole after oral gavage administration. This level is approximately six-fold higher than the $C_{max}$ values (16 µg equiv./g on average) reported for male rats after an oral gavage dose of 40 mg/kg bw. These data show blood levels in male mice that are close to dose-proportional with the blood levels observed in male rats. Therefore, data from this study suggests that bioavailability of tricyclazole in male mice is similar to that in male rats (McCoy & Clark, 2014).

*Study 6*

The purpose of this study was to determine limited pharmacokinetics (in plasma), excretion, mass balance, and selected tissue distribution of radioactivity following administration of a single oral dose of the test article, [*triazole-U*-$^{14}$C]tricyclazole, administered to male mice. Multiple dose levels were used to identify ranges of linearity/non-linearity (if any) in terms of total radioactivity.

Five treatment groups, each composed of five, fasted male Crl:CD1 mice, were administered a single oral dose of the test article via gavage. Doses were 7, 28, 100, 300 and 300 mg/kg bw for groups 1–5 respectively, with the test article administered in 0.5% Methocel A15C in water as a vehicle.

In groups 1–4, blood samples were collected for plasma radioactivity analysis at designated time points: 15 minutes, 1.5, 4, 12, 24, 48, 96, 120, 144 and 168 hours post dose. Samples were taken from one mouse per group at each time point, with no animals used for more than two time points samplings. Urine, faeces, and cage residue were collected and pooled at 24-hour intervals up to 168 hours post dose, with samples from three mice pooled for each group and time point), and these samples used for radioactivity concentration analysis. At study termination, animals from groups 1–4 were euthanized and their carcasses stored frozen pending disposal. Animals of group 5 were euthanized at six hours post dose (which was the expected time to reach half maximum concentration, based on previous studies) and selected tissues (bone marrow, liver, kidney, stomach contents, stomach, whole blood) and carcasses were collected and analyzed for concentration of radioactivity.

A single oral dose of [*triazole-U*-$^{14}$C]tricyclazole was rapidly absorbed in mice. The $C_{max}$ in plasma was observed to occur at 4 hours, 15 minutes, 15 minutes and 12 hours post dose in groups 1 to 4, respectively. There did appear to be a more significant effect of enterohepatic recirculation

at the 300 mg/kg bw dose level based on the spike in plasma concentration seen at 12 hours post dose.

In terms of AUC values and their relationships to dose levels, there were no identifiable trends of non-linearity for total radioactivity in the range of doses examined in this study (single time point curves).

Total recovery of the radioactive dose in the excreta ranged from 81.78% to 91.71% across the four groups. The largest fraction of radioactivity was seen in the urine with recovery ranging from 49.52% to 64.06% across groups 1–4; the total percentage of radioactive dose recovered in faeces ranged from 22.23% to 28.86% across these groups. The routes of excretion and total recoveries were similar across the four groups. However, it does appear that the rate of excretion as a percentage of dose was slower as the dose level increased, which is consistent with increased enterohepatic recirculation observed in the plasma time course at this dose level.

Radioactivity analysis of selected tissues from group 5, harvested at six hours post dose, indicated that the bulk of the radioactivity was located in the stomach contents and the remaining carcass, which contained 52.68% and 20.06% respectively of the total radioactive dose. Concentrations of radioactivity were, in descending order, found in the stomach, liver, kidney and bone marrow (Dubnicka, 2017b).

*Study 7*

In a published article, data from regulatory studies investigating the oral ADME of tricyclazole in vivo, in silico and in vitro were reviewed with a particular focus on human health risk assessment. It was reported that tricyclazole is readily absorbed and highly bioavailable in rodents (greater than 86%) with indication of saturation of absorption at high doses. Enterohepatic recirculation was evident. Excretion occurred quickly, both via urinary (31%–64%) and faecal routes (39%–65%), with substantial biliary elimination in the rat (58% or greater) based on a comparison of excretion rates following oral and i.v. administration. The tricyclazole-derived radioactivity was distributed to major organs, including those investigated in in vivo genotoxicity studies (liver, kidney, gastrointestinal tract and bone marrow). No evidence of bioaccumulation was reported. (Corvaro & Bartels, 2019).

## 1.2 Biotransformation

A pre-GLP investigation of the metabolism of $^{14}$C-labelled tricyclazole in the rat was conducted. Methodology details were limited, and as the study was not conducted to GLP standards, limited conclusions can be drawn. Tricyclazole was administered as a single oral gavage dose to an unspecified number of male Wistar rats (body weight: about 250 g) at 100 mg/kg bw of tricyclazole, which was $^{14}$C labelled on the benzene ring (purity greater than 99%; Batch EL-29l, BEAM; , Specific activity: 0.5 µCi/mg). Test material was suspended in 10% acacia. Analysis was limited to urinary metabolites and faeces were discarded without analysis.

The radioactive content of all urine samples was determined by the direct counting of a sample aliquot in either a toluene or a dioxane/2-methoxyethanol-based scintillation.

In order to identify and quantify urinary metabolites, urine was extracted three times with ethyl ethanoate at pH 7.0 to separate the neutral fraction, then once at pH 1.5 to separate the acidic fraction, and finally hydrolyzed with HCl at pH 1.0 to separate the conjugated fraction from residual urine. Each fraction (except residual, unextracted urine) was first subjected to silica gel column chromatography, and then thin-layer chromatography (TLC) both of which were followed by quantification by liquid scintillation counting (LSC). These processes were followed by TLC followed by autoradiography and elution of radioactive zones with methanol), high-performance liquid chromatography (HPLC), followed by detection using absorbance at 232 nm, mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy.

The three-step solvent extraction procedure resulted in the partitioning of 11.7% of administered radioactivity into the neutral fraction, 4.8% into the acidic fraction, and 34.6% into the conjugated fraction. The remaining 8.9% remained in the spent urine. It was estimated that approximately 40 tricyclazole metabolites were present in urine, however these were not definitively identified. It was proposed that the free alcohol metabolite of tricyclazole made up approximately 2.1% of the total urine radioactivity, with the conjugated form of this metabolite making up 1.8% of the total radioactivity.

The major metabolite (identified as metabolite C) accounted for 31.4% of the total urine radioactivity. Its structure was not definitively confirmed, but was considered to include a glucuronic acid moiety. The next most prevalent metabolite (identified as metabolite B) represented 2.5% of total radioactivity, but was not identified. Metabolite A, proposed to be a phenolic derivative of tricyclazole with its hydroxy group ortho or para in relation to the methyl group, represented 0.6% of the total urine radioactivity; the identity of this metabolite was not definitively confirmed.

In the conclusion, tricyclazole represented less than 5% of the urinary radioactivity, but this was not expressed as a percentage of AD. There were therefore no data on the amount of radioactivity excreted, so the importance of metabolites cannot be expressed in relation to administered dose. A thioglucuronide metabolite represented the major urinary metabolite (31.4% of urinary radioactivity). A conjugated alcohol metabolite represented 1.8% of urinary radioactivity. A phenol metabolite represented 0.6% of urinary radioactivity (Magnussen & Rainey, 1979).

The metabolism of [*triazole-U*-$^{14}$C]tricyclazole was considered following oral gavage administration to Fischer 344 rats. Four male rats each received radiolabelled test material either at 2 mg/kg bw (low-dose group) or at 40 mg/kg bw (high-dose group). An additional four male rats received 14 consecutive daily doses of unlabelled tricyc1azole at 2 mg/kg followed by [*triazole-U*-$^{14}$C]tricyclazole at the same dose level on day 15 (multiple low-dose group). Rats were maintained in metabolism cages, with separate collection of urine (including cage washes), faeces and expired carbon dioxide. Analysis was conducted on pooled urine and faeces samples, with the weight of urine or faeces from each animal based on the total quantity excreted during the time period. All animals were euthanized 168 hours after receiving a labelled tricyclazole dose.

Pooled urine and pooled faecal extracts produced HPLC profiles of eluted $^{14}$C that consisted of one chromatographic peak, and the retention time was similar for both matrices. Comparative MS revealed that one of these peaks was tricyclazole alcohol (percentage of dose not given), and that tricyclazole acid was absent in urine and faeces. The occurrence of the parent compound in urine and faeces was not determined. The report concluded that both the urinary and faecal metabolites were tricyclazole alcohol (Andre, 2004).

A study was conducted to determine metabolism of [*triazole-U*-$^{14}$C]tricyclazole following oral and i.v. administration to groups of male and female F344/DuCrl rats. Tricyclazole was administered orally as a single, low dose (2 mg/kg bw) and a single, high dose (40 mg/kg bw) to four animals of each sex per group. There was also a repeat-dose group (daily oral doses of unlabelled tricyclazole, followed by a single dose of labelled tricyclazole at 2 mg/kg bw), and a further group that received a single, low dose of labelled tricyclazole by i.v. administration. Blood, excreta and tissues were collected and analyzed for seven days following the low and high oral doses, the final dose of the repeat dosing, and the rats receiving the i.v. dose. An additional group of rats were used to assess the $C_{max}$.

The absorbed test material was highly metabolized in both male and female rats. A total of 29 metabolites were found in urine and faecal samples. Nine of the metabolites were found to represent above 5% of AD. Tentative identification was made for seven of these nine metabolites, with conclusive or tentative identifications of:

- 4-[2-(hydroxymethyl)-6-(methylthio)phenyl]-2,4-dihydro-3*H*-1,2,4-triazole-3-thione),
- four isomers of an *S*-glucuronide conjugate of 3-methyl-2-[3-(methylthio)-4*H*-1,2,4-triazol-4-yl]benzenethiol,
- the *N*-acetyl cysteine conjugate of 4-(2-methylphenyl)-5-(methylthio)-4,5-dihydro-1*H*-1,2,4-triazole, and
- the *O*-acetylated product of peak 12 (3-(methylthio)-2-(5-thioxo-1,5-dihydro-4*H*-1,2,4-triazol-4-yl)benzyl acetate).

Of these, the four isomers of the *S*-glucuronide conjugate. of 3-methyl-2-[3-(methylthio)-4*H*-1,2,4-triazol-4-yl]benzenethiol were present in total at more than 10% of AD. A number of other metabolites, present at greater than 5% of AD were also tentatively identified.

Regarding the unidentified peak 1, (found in all faecal extracts at approximately 3.8%–6.9% of AD), this metabolite would appear to be of low polarity (rather than very polar, as stated by study

author) and should have been identified on a common C18 RP column, rather than a hydrophilic phase column (Hansen, et al., 2009).

A single oral dose of [*triazole-U*-$^{14}$C]tricyclazole of 2, 7, 28 or 50 mg/kg bw was extensively metabolized by the rat. In urine, the two major metabolites, corresponding to peaks 14 and 15, were seen at concentrations greater than 10% of the total radioactive dose. Several other metabolites were seen in the urine, all of which represented less than 5% of total radioactive dose. Unchanged tricyclazole and the metabolites of interest (peaks 11, 12, 13, and 27, MW 239; all derived from the metabolite tricyclazole alcohol, peak 13) were seen in the radiochromatograms of the pooled urine, but none of these were found to comprise a significant fraction of the total dose, all being present at less than 5% of AR, with the exception of the putative metabolite MW 239, which was not observed. It was seen from the faecal radiochromatograms, that unchanged tricyclazole was present, but none of the metabolites of interest mentioned above were detected (Dubnicka 2017a)

In vitro metabolism data was derived for tricyclazole using liver microsomes from F344 rat, beagle dog, and human donors to evaluate whether metabolites unique to humans were formed upon exposure to human liver microsomes. After confirmation of the microsomal cytochrome (CYP) activity (as the rate of 7-ethoxycoumarin-*O*-dealkylation), and a pilot experiment to verify suitability of methods (Stage I), all three types of liver microsome at a concentration of 1 mg/mL were incubated with [*triazole-U*-$^{14}$C]tricyclazole (three replicates per incubation) at a single substrate concentration of 1.89 µM (Stage II). Applicable cofactors for Phase I CYP-based metabolism and Phase II glucuronide formation were added to the incubation test systems to mimic potential in vivo metabolism. After incubation, metabolic profiles were generated by HPLC and eluent fractions collected, or by radioflow detection. Mass balances and radioactive recoveries were calculated by LSC. Metabolite identification was performed by HPLC with electrospray ionizations and quantitative time-of-flight mass spectrometry.

Using a similar method as detailed above for tricyclazole, rat and human liver microsomes were incubated with a $^{14}$C-labelled derivative of a major mammalian and environmental metabolite of interest, X355227. This was included at a target concentration of 2.06 µM under the same incubation conditions as used for tricyclazole, and the resulting final incubations also processed in the same manner.

Based on data from the final tricyclazole incubation supernatants (Stage II), the mass balance values for the complete [$^{14}$C]tricyclazole incubation mixtures (microsomes + cofactor + alamethicin) were 100.3%, 97.6% and 104.7% for rat, dog and human microsomes respectively. The control samples (without cofactor) had radioactive recoveries of 96.9%, 94.6% and 99.2% for rat, dog and human microsomes respectively. The control sample without enzymes (no microsomal protein) showed a recovery of 107.5% .

Across the three test species thirteen radiochemical peaks were detected in the incubations containing [$^{14}$C]tricyclazole, cofactors and microsomes. The metabolites that were present at 5% of the substrate concentration or more with human liver microsomes were also present in incubations with microsomes from one or both of the other species. Most metabolites present at less than 5% of the substrate concentration with human liver microsomes were also formed in the rat or dog incubations. Fewer metabolites were formed in rat liver microsomal incubations compared to other species. The four major metabolite peaks (Peaks 2, 5, 6 and 8) formed in the complete rat, dog and human incubations mixtures that comprised more than 5% of the substrate concentration, were identified as:

1. a metabolite formed by one CYP aromatic ring hydroxylation and one CYP hydroxylation of the methyl group in tricyclazole (accounting for an average of 39.37% of substrate concentration in dog, detected but unquantified in the rat, but not found in humans);
2. a metabolite formed by one CYP aromatic ring hydroxylation (accounting for an average of 24.48%–38.70%; Peak 5) found in all Stage II incubations;
3. two metabolites formed by two CYP aromatic ring hydroxylations (one on the aromatic ring and one on the methyl group of tricyclazole) followed by glucuronidation (accounting for an average of 6.78%–37.31% of the substrate concentration; Peak 6), and found in all three species of Stage II;
4. a metabolite formed by one CYP hydroxylation of the methyl group in tricyclazole

(accounting for an average of 2.91%–8.61% of the substrate concentration; Peak 8), confirmed to be metabolite X355227.

Based on data from the final (Stage III) [$^{14}$C] X355227 incubation supernatants, the average mass balance values for the complete [$^{14}$C] X355227 incubation mixtures (microsomes + cofactor + alamethicin) were 105.1% and 103.6% for rat and human preparations respectively. The control samples (without cofactor) had radioactive recoveries of 103.7% and 104.4% for rat and human microsomes, respectively. The control sample without enzymes (no microsomal protein) showed a recovery of 102.6%.

Across the two test species, four radiochemical peaks were detected in the incubations containing labelled X355227, cofactors and microsomes. The metabolites that were detected at 5% of the substrate concentration or more with human liver microsomes, were also present with rat liver microsomes. Aside from the parent metabolite X355227 (Peak 8), Peak 2 was formed in complete incubations at more than 5% of substrate concentration, and was identified as a metabolite formed by one CYP-mediated aromatic ring hydroxylation of X355227; this accounting for an average of 8.74% of substrate concentration but only detected with rat microsomes. Peak 6 was formed in human liver microsomal incubations and represented less than 5% of the substrate concentration. Peak 6 was identified as one of the metabolites formed by a single CYP-mediated aromatic ring hydroxylation of X322557, and was also detected in Stage II incubations.

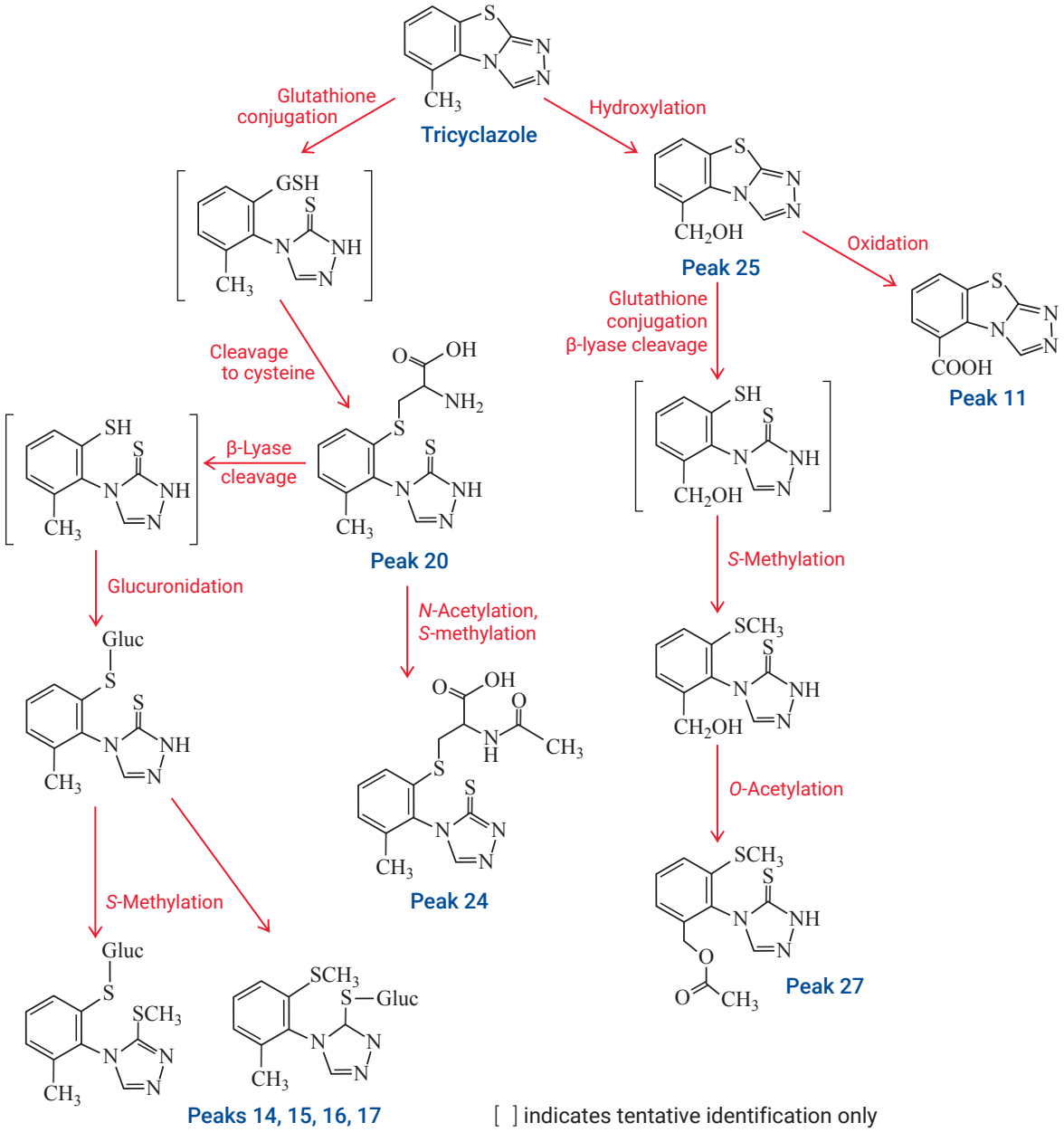
One major metabolite (Peak 1) was seen in human microsomal incubations in Stage II, and represented more than 5% of substrate concentration. This metabolite could not be identified and was also found in incubations with microsomes from rats (Stages II and III) and dogs (Stage II).

Overall, tricyclazole was extensively metabolized under in vitro incubation conditions with microsomes from F344 rats, beagle dogs or human donors. Metabolite X355227 was not metabolized as extensively as tricyclazole. Only two common metabolites were formed, tricyclazole acid and X355227, across species under *in vitro* conditions. No metabolites were detected at 5% of the substrate concentration or greater, that were present in the human microsomal incubations but not those with microsomes from other species; this was true for both tricyclazole and metabolite X355227, from which it was concluded no unique human metabolites were demonstrated (Zhang & Erskine, 2017).

In a published article, data from regulatory studies investigating the oral ADME of tricyclazole in vivo, in silico and in vitro were reviewed with a particular focus on human health risk assessment. It was reported that metabolism was extensive (approximately 30 metabolites), the liver being identified as the primary metabolism organ, with Phase I and II enzymes involved. Several metabolites are formed following an initial cleavage of the central thiazole ring, with no loss of free triazole from the remaining phenyl ring. A group of four metabolites derive from an initial oxidation step, with the formation of the tricyclazole alcohol, a relevant crop metabolite, and these account in total for up to 13% of AD. In vitro metabolism, investigated with liver microsomes, confirmed that humans do not produce unique metabolites. (Corvaro & Bartels, 2019).

A proposed scheme for the metabolism of tricyclazole in rats is shown in Fig. 2.

*Figure 2. Proposed metabolic scheme for tricyclazole in rats*




(note abbreviations used: Glucuronide, GSH = Glutathione)

Source: Hansen et al., 2009

# 2. Toxicological studies

## 2.1 Acute toxicity

### (a) Lethal doses, dermal and ocular irritation, and dermal sensitization

The results of acute oral, dermal and inhalation toxicity studies with tricyclazole, along with the results of dermal and eye irritation, and skin sensitization studies, are summarized in Table 1.

***Table 1. Acute toxicity of tricyclazole***

| Species | Strain | Sex | Route | Purity | Result | Reference |
|---|---|---|---|---|---|---|
| **GLP studies** | | | | | | |
| Rat | Fischer 344 | F | Oral/gavage (up & down protocol) | 99.3% | Estimated $LD_{50}$ was 237 mg/kg in females | Durando, 2005a |
| Rat | Fischer 344 | M/F | Dermal/topical (limit test) | 99.3% | $LD_{50}$: > 5000 mg/kg | Durando, 2005b |
| Rat | F344/DuCrl | M/F | Inhalation; nose only | 99.3% | $LC_{50}$: > 0.52 mg/L (HDA) | Hotchkiss, Radke & Krieger 2006 |
| Rat | Sprague-Dawley | M/F | Inhalation; nose only | NS | $LC_{50}$: > 2.58 mg/L (HDA) | Blagden, 1998 |
| Rabbit | Albino rabbits | M/F | Dermal/topical | 99.4% | Not a skin irritant | Durando, 2005c |
| Rabbit | New Zealand albino rabbits | M/F | Eye/instillation | 99.3% | Not an eye irritant | Durando, 2005d |
| Guinea pig | Dunkin Hartley albino (SPF) | F | Intradermal/topical (maximization test) | 96.7% | Not a skin sensitizer | Prinsen, 2003b |
| Mice | BALB/cAnNCrl | F | Dermal/topical (local lymph node assay) | 99.3% | Not a skin sensitizer | Woolhiser & Wiescinski, 2005 |
| **Pre-GLP studies** | | | | | | |
| Rat | Wistar | M/F | Oral/gavage | NS | $LD_{50}$: 337.5 mg/kg bw in males 289.7 mg/kg bw in females | Corteva Agriscience, 1973a |
| Rabbit | Albino rabbit | M/F | Dermal/topical | NS | $LD_{50:}$ > 2000 mg/kg bw | Corteva Agriscience, 1973b |
| Rabbit | New Zealand albino rabbits | M/F | Eye/instillation | NS | Eye irritant | Corteva Agriscience, 1973c |

M: Male; F: Female; GLP: Good laboratory practice; NS: Not stated in report;
$LD_{50}$: Median lethal dose; $LC_{50}$: Median lethal concentration; HDA: Highest technically achievable dose;

In pre-GLP studies tricyclazole is moderately toxic by oral exposure on an acute basis, with piloerection, hunched posture and hypoactivity observed in animals, which subsequently died, but of low acute toxicity via the dermal route, with no adverse clinical signs, dermal irritation or abnormal behaviour observed. The acute nose-only inhalation $LC_{50}$ is greater than 2.58 mg/L (the highest achievable concentration). At this dose death occurred in two out of five animals, with severe clinical signs, including hunched posture, lethargy, pilo-erecton and ptosis observed in all survivors. In a more recent study the $LC_{50}$ was greater than 0.52 mg/L (the highest achievable concentration) with some effects on body weight.

In rabbits tricyclazole caused very slight to well defined erythema and slight oedema of the skin, and mild conjunctivitis in the eye. Tricyclazole was not sensitizing in the Magnusson & Kligman maximization test, nor in a murine local lymph node assay.

## 2.2 Short-term studies of toxicity

### (a) Oral administration

***Mouse***

*Study 1*

A 90-day, pre-GLP oral toxicity study employed 4–5-week-old male and female ICR mice from Harlan Industries, with 15 of each sex per group (mean male body weight 12.3 ± 0.1 g, females 12.0 ± 0.1 g). Rats were fed a diet for 92 days containing tricyclazole (EL-291; purity 99.4%; Lot C53-C48-147) at concentrations of 0, 400, 1000, 2500 or 3600 ppm (equivalent to 0, 60, 150, 375 and 540 mg/kg bw per day). In reality there were 16 male mice and 14 female mice at 3600 ppm, since one mouse was mis-sexed at the beginning of the study. As a consequence of mis-sexing, two female high dose mice subsequently delivered progeny. Dietary admixture analyses were performed to investigate the stability (over four months at 25°C) and achieved concentrations of the test item. Twice a day the mice were monitored for death and once a week for clinical observations. Body weight and food consumption were registered each week. For all survivors the following haematological parameters were measured: erythrocyte and leukocyte counts, haemoglobin (Hb), haematocrit Ht), leukocyte differential count, platelets count and assessment of erythrocyte morphology. The clinical chemistry tests included the determination of glucose, blood urea nitrogen (BUN), creatinine, total bilirubin, alkaline phosphatase activity (ALP) and alanine transaminase activity (ALT; formerly glutamic–pyruvic transaminase activity or SGPT). During the study, necropsies were performed on all decedents. After three months all survivors were necropsied and a histopathological examination of the following organs performed: mammary gland, salivary glands, lungs, heart, thyroid, stomach, duodenum, jejunum, ileum, colon, mesenteric lymph node, liver, skeletal muscle, thymus, pancreas, spleen, kidney (with adrenals attached), urinary bladder, prostate, testis, uterus, ovaries, brain and sternal bone marrow.

No deaths occurred in the control or low-dose groups. Deaths occurred in the 1000 ppm group (2/15 males), in the 2500 ppm group (2/15 males and 1/15 females), and in the 3600 ppm (3/15 males and 1/14 females). Clinical signs related to tricyclazole administration included thinness and piloerection in animals from the 1000, 2500 and 3600 ppm groups. Other clinical signs observed included alopecia in one mouse at 3600 ppm. During the first week a number of mice (including control animals) died and many others lost weight, however these deaths were not counted in the overall mortality and, according to the protocol, the mice were replaced on study day 8. Average body weights were slightly decreased in males exposed to 2500 and 3600 ppm and in females at 2500 ppm. There were no differences in food consumption that were considered related to tricyclazole treatment. It was noted that there was a higher degree of spillage from food trays at 2500 and 3600 ppm. Some haematological parameters were altered. There was a decreased Hb concentration in males at 2500 ppm and in females at 400, 1000 and 2500 ppm. An increased platelet count was observed in males of the 2500 and 3600 ppm groups. In males in the 1000 and 3600 ppm groups an increased level of ALT activity was noted. Increased absolute and relative liver weights were recorded for males from the 2500 and 3600 ppm groups, and in females from the 1000, 2500 and 3600 ppm groups. There were no remarkable observations in gross pathology. A slight hyperplasia of small bile ducts in the liver of 7/16 males and of 8/14 females of the high-dose group (3600 ppm) were the only histopathological lesions noted in the microscopic examination. Target organs were identified as the liver (higher absolute and relative liver weight, proliferation of small bile ducts, higher ALT values) and the haematopoietic tissue in males (higher platelet counts). The no-observed-adverse-effect level (NOAEL) was 400 ppm for both sexes (equivalent to 60 mg/kg bw per day for both sexes) based on mortality at 1000 ppm (equivalent to 150 mg/kg bw per day) (Worth et al., 1978a).

*Study 2*

In a six-month pre-GLP toxicity study, tricyclazole (purity 99.4%, Batch no. C53-C58-147) was administered via the diet to ICR mice at intended concentrations of 0, 400, 1000, 2,00 or 3600 ppm for six months, followed by a four month non-treatment recovery period. There were 40 mice of each sex per group, with mean body weights 18.9 ± 0.3 g (males), and 17.2 ± 0.2 g (females), all 3–4 weeks old. Analysis of the treated feed indicated that the achieved dietary concentrations were lower than had been intended, with actual dietary concentrations of around 0, 310, 800, 1900 and 3000 ppm (equivalent to 0, 47, 120, 285 and 450 mg/kg bw per day). At the end of a six-month exposure period, 20 mice of each sex in each dosing group were sacrificed. The remaining 20 of each sex per group were put on a standard, untreated diet. At nine months after first dosing, 10 mice of each sex per group were sacrificed.

The remaining survivors were sacrificed one month later. Twice each day all cages were inspected for dead, dying or moribund mice. Once weekly each mouse was inspected for clinical observations. Each mouse was weighed at approximately weekly intervals. Necropsies were performed on all animals that died and at the end of 6, 9 and 10 months. Liver weight, microsomal proteins and in vitro *p*-nitroanisole metabolism were evaluated.

No treatment-related effects were reported regarding mortality, clinical signs, body weight, body weight gain, haematology or clinical chemistry. After six months of treatment, treatment-related effects on organ weights included increases in mean absolute (by 15%) and relative (by 12%) liver weights, observed at 800 ppm and above. Histopathological effects included proliferation of small bile ducts at 800 ppm and above, and an increased incidence of periportal lipocytes in all treatment groups. Significant increases in in-vitro *p*-nitroanisole demethylation were seen for males at 800 ppm and above and females at 3000 ppm and above. On this basis a NOAEL of 310 ppm (equivalent to 47 mg/kg bw per day) was identified. (Howard & Owen, 1979)

***Table 2. Key findings from the six-month dietary toxicity study in mice***

| | | Estimated concentration in diet (ppm)/sex | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | | 310 | | 803 | | 1900 | | 3017 | |
| **Parameter** | | **M** | **F** | **M** | **F** | **M** | **F** | **M** | **F** | **M** | **F** |
| Number in group | | 40 | 40 | 40 | 40 | 41 | 39 | 40 | 40 | 39 | 41 |
| Mortalities | | 2 | 4 | 3 | 2 | 1 | 2 | 3 | 0 | 3 | 1 |
| **Body and liver weights** | | | | | | | | | | | |
| **Initial and terminal mean body weights (g), [Standard deviation]** | | | | | | | | | | | |
| Initial body weight (g) | | 21.0 [3.6] | 19.1 [2.8] | 19.5 [3.3] | 18.0 [2.8] | 19.6 [3.3] | 17.3 [2.4]* | 17.5 [3.6]* | 16.0 [2.4]* | 16.9 [3.5]* | 15.5 [2.7]* |
| Terminal body weight (g) | | 44.8 [7.8] | 40.2 [5.6] | 41.0 [3.6] | 38.8 [4.6] | 42.1 [3.8] | 35.0 [7.5] | 41.7 [2.6] | 36.0 [5.5] | 42.3 [2.4] | 36.8 [8.1] |
| **Absolute and relative liver weights (at 6 month sacrifice)** | | | | | | | | | | | |
| Mean liver weight | Absolute (g) | 1.53 | 1.22 | 1.66 | 1.31 | **1.73**[#] | **1.40**[#] | **1.85**[#] | **1.54**[#] | **1.94v** | **1.58**[#] |
| | Relative (% of bw) | 3.70 | 3.71 | 3.97 | 3.90 | **4.13**[#] | **4.16**[#] | **4.05**[#] | **4.53**[#] | **4.63**[#] | **4.96**[#] |
| **Absolute and relative liver weights (after 3 months of untreated diet)** | | | | | | | | | | | |
| Mean liver weight | Absolute (g) | 2.27 | 1.69 | 2.28 | 1.84 | 2.46 | 1.67 | 2.28 | 1.75 | 2.20 | 1.82 |
| | Relative (% of bw) | 5.44 | 4.70 | 5.48 | 4.85 | 5.86 | 4.74 | 5.74 | 4.99 | 5.58 | 4.94 |
| **Absolute and relative liver weights (after 4 months of untreated diet)** | | | | | | | | | | | |
| Mean liver weight | Absolute (g) | 1.85 | 1.54 | 1.67 | 1.63 | 1.72 | 1.38 | 1.91 | 1.45 | 1.86 | 1.50 |
| | Relative (% of bw) | 4.14 | 3.84 | 4.07 | 4.21 | 4.09 | 3.97 | 4.59 | 4.05 | 4.39 | 4.11 |
| **Histopathological findings in the liver (mice affected/mice examined)** | | | | | | | | | | | |
| **At 6 months** | | | | | | | | | | | |
| Slight proliferation of small bile ducts | | 0/20 | 1/19 | 0/17 | 0/19 | 7/20 | 3/18 | 13/18 | 5/20 | 14/19 | 15/19 |
| Lipocytes around portal spaces | | 1/20 | 0/19 | 3/17 | 2/19 | 7/20 | 7/18 | 13/18 | 10/20 | 9/19 | 15/19 |
| **At 9 months** | | | | | | | | | | | |
| Slight proliferation of small bile ducts | | 0/8 | 2/9 | 0/10 | 3/9 | 1/10 | 1/10 | 0/9 | 2/10 | 5/8 | 3/10 |
| Lipocytes around portal spaces | | 0/8 | 0/9 | 1/10 | 4/9 | 3/10 | 6/10 | 4/9 | 7/10 | 5/8 | 8/10 |
| **At 10 months** | | | | | | | | | | | |
| Slight proliferation of small bile ducts | | 0/10 | 1/8 | 0/10 | 0/10 | 1/9 | 0/10 | 1/10 | 1/10 | 1/10 | 2/10 |

| | Estimated concentration in diet (ppm)/sex | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | 310 | | 803 | | 1900 | | 3017 | |
| **Parameter** | **M** | **F** | **M** | **F** | **M** | **F** | **M** | **F** | **M** | **F** |
| Lipocytes around portal spaces | 0/10 | 0/8 | 0/10 | 1/10 | 0/9 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 |
| **Hepatic in vitro *p*-anisole metabolism and microsomal protein content** | | | | | | | | | | |
| Mean *p*-nitrophenol formed (μM/g liver/hour) | 3.26 | 3.52 | 3.87 | 3.42 | 4.38¶ | 3.94 | 5.38¶ | 3.93 | 6.12¶ | 4.36¶ |
| Mean microsomal protein in liver (mg/g) | 12.7 | 14.9 | 14.8 | 17.0 | 14.9 | 17.2 | 17.2 | 15.2 | 13.2 | 16.3 |

M: Male; F: Female; bw: Body weight; Source: Howard & Owen, 1979

* significant decrease ($p < 0.05$ Dunnett's test)

\# Significant increase ($p \leq 0.05$) by analysis of variance, comparison of means by Dunnett's procedure, two tailed

¶ Significant increase ($p < 0.05$) by analysis of variance, comparison of means by Dunnett's procedure, two tailed

**bold** at least 10% higher liver weight

### *Rat*

In a 90-day pre-GLP toxicity study, an initial dose range finding study was conducted with three groups of five Wistar rats of each sex, fed diets containing 500, 1000 or 2000 ppm tricyclazole for 14 days (no control group). Body weights were determined twice weekly. Dose-related decreases in body weight were observed for both male and female rats.

The main study employed 65 male rats (weighing 128 ± 1.4 g) and 65 females (weighing 124 ± 1. 2 g. All the rats, from Harlan Industries, Cumberland, Indiana, were 28–35 days old. Groups of 15 rats of each sex per treatment group and 20of each sex for the control group, were administered tricyclazole (purity 99.4%, Batch no. C53-C21-147) for 90 days at dietary concentrations of 0, 282, 635 or 1640 ppm (equal to 0, 21, 47 and 180 mg/kg bw per day for males, 0, 23, 54 and 190 mg/kg bw per day for females). Cages were inspected for dead, dying or moribund rats twice daily, and rats were examined once each week. Food consumption and body weight were measured approximately weekly. Haematological parameters (erythrocyte and leukocyte counts, Hb, Ht, leukocyte differential count and erythrocyte morphology) and clinical chemistry (glucose, BUN and ALT activities) were determined for all of the survivors, and prothrombin time (PT) for approximately half of the survivors. In vitro hepatic *p*-nitroanisole-*O*-demethylase activity (for five rats of each sex per group) was also measured. During the study, necropsies were performed on all animals that died. Rats surviving the three-month testing period were necropsied after carbon dioxide asphyxiation. Gross examination along with histopathological evaluations were performed. Organs were weighed and the organ to body weight ratios calculated.

Mortality was observed in one male receiving 635 ppm and three females, one from each dose level. Decreased food consumption was observed in males at 635 and 1640 ppm for the majority of the study, and in females at 635 and 1640 ppm for the first two weeks. Also observed were decreased mean body weights (by up to 19% and 31% in males, and up to 13% and 28% in females at 635 and 1640 ppm respectively), and decreased body weight gains (at the end of the study suppressed by 10%, 24% and 44% in males at 282, 635 and 1640 ppm respectively, and by 12% and 50% in females at 635 and 1640 ppm respectively compared to controls). Changes in haematologic and clinical chemistry parameters were limited to the high-dose group for both males and females, although increased activity of hepatic *p*-nitroanisole demethylase and ALT activity were seen at 635 ppm. Decreases in absolute organ weights, and increases in relative organ weights were observed in both males and females for several organs at 635 and 1640 ppm, however no correlated changes were observed on histopathological examination. Slight centrilobular hepatocyte hypertrophy was seen in males at high doses, and this was consistent with the induction of hepatic metabolizing enzymes at the same doses.

The results of this study indicate that the liver is the target organ. Because of the effects on mean body weight and relative liver weight, as well as the increased activity of hepatic *p*-nitroanisole demethylase and ALT at 635 ppm (equal to 47 and 54 mg/kg bw per day respectively for males and females), the NOAEL was identified as 282 ppm (equal to 21 and 23 mg/kg bw per day, respectively, for males and females) (Worth et al., 1978b).

*Table 3. Key findings of the 90-day dietary toxicity study in rats; mean body weights, weight gain and mean food consumption*

| | | Nominal dietary concentrations (ppm)/sex | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | | 282 | | 635 | | 1640 | |
| **Parameter** | | **M** | **F** | **M** | **F** | **M** | **F** | **M** | **F** |
| Dose level (mg/kg bw/day) | | 0 | 0 | 20.4 | 22.5 | 46.7 | 54.2 | 135.3 | 186.3 |
| Mortality | | 0/20 | 0/15 | 0/16 | 1/15 day 19 | 1/15 day 8 | 1/15 day 7 | 0/15 | 1/15 day 64 |
| **Body weight (g)** | | | | | | | | | |
| Initial | | 129.1 | 126.5 | 129.5 | 123.1 | 124.6 | 121.5 | 131.7 | 127.1 |
| Week 1 | | 162.9 | 146.1 | 162.5 | 123.1 | 135.4* | 127.3* | 125.5* | 116.2* |
| Week 2 | | 211.2 | 179.1 | 205.4 | 145.9 | 178.8* | 156.4* | 159.4* | 140.6* |
| Week 3 | | 257.2 | 202.4 | 252.7 | 171.4 | 220.9* | 181.1* | 199.1* | 156.7* |
| Week 6 | | 363.8 | 251.3 | 358.7 | 195.4 | 310.2* | 227.9* | 285.8* | 188.2* |
| Week 9 | | 425.0 | 272.9 | 404.9 | 263.9 | 353.4* | 245.0* | 307.5* | 203.1* |
| Week 12 | | 456.9 | 285.4 | 425.4 | 275.6 | 374.1* | 258.6* | 317.0* | 208.7* |
| Week 13 | | 472.0 | 292.6 | 438.1 | 280.5 | 383.7* | 267.1* | 324.7* | 210.7* |
| Body weight gain (g) | | 342.9 | 166.1 | 308.6 | 157.4 | 259.1 | 145.6 | 193.0 | 83.6 |
| **Food consumption (g/rat per week)** | | | | | | | | | |
| Week 1 | | 102.7 | 96.7 | 95.7 | 86.9 | 76.9* | 79.0 | 67.7* | 59.5* |
| Week 2 | | 142.8 | 132.3 | 140.7 | 122.3 | 119.6* | 104.4* | 105.0* | 106.7* |
| Week 3 | | 170.5 | 140.0 | 164.6 | 124.1 | 145.4* | 119.8 | 128.2* | 154.3 |
| Week 6 | | 182.1 | 153.4 | 180.3 | 136.8 | 160.1 | 134.9 | 150.2* | 139.9 |
| Week 9 | | 175.7 | 146.0 | 170.0 | 127.6 | 147.9* | 126.8 | 151.1* | 155.8 |
| Week 12 | | 164.1 | 151.5 | 168.7 | 125.2 | 152.9 | 133.9 | 176.5 | 173.7 |
| Week 13 | | 180.9 | 155.4 | 169.0 | 125.5 | 155.2 | 142.0 | 172.8 | 161.5 |
| **Selected organ weights** | | | | | | | | | |
| Terminal body weight (g) | | 472.0 | 292.6 | 438.1 | 280.5 | 383.7* | 267.1* | 324.7* | 210.7* |
| Liver | Abs (mg) | 11.52 | 7.68 | 11.73 | 7.68 | 11.62 | 7.54 | 11.57 | 7.33 |
| | Rel (%) | 2.44 | 2.73* | 2.67* | 2.73* | 3.03* | 2.83* | 3.55* | 3.47* |
| Thyroid | Abs (mg) | 23.50 | 17.9 | 21.93 | 17.9 | 21.36 | 16.8 | 22.73 | 16.4 |
| | Rel (%) | 5.0 | 6.4 | 5.0 | 6.4 | 5.7 | 6.3 | 7.1* | 7.7* |
| Kidney | Abs (mg) | 2.64 | 1.72 | 2.54 | 1.72 | 2.48 | 1.73 | 2.21* | 1.53 |
| | Rel (%) | 0.56 | 0.61 | 0.58 | 0.61 | 0.65* | 0.65* | 0.68* | 0.73* |
| Heart | Abs (mg) | 1.37 | 0.99 | 1.36 | 0.99 | 1.21* | 0.95 | 1.07* | 0.78 |
| | Rel (%) | 0.29 | 0.36 | 0.31* | 0.36 | 0.31* | 0.36 | 0.33* | 0.37 |
| Spleen | Abs (mg) | 0.81 | 0.56 | 0.76 | 0.56 | 0.69* | 0.52 | 0.58* | 0.49 |
| | Rel (%) | 0.17 | 0.19 | 0.18 | 0.20 | 0.18 | 0.20 | 0.18 | 0.23* |
| Adrenal | Abs (mg) | 52.85 | 60.9 | 54.20 | 62.4 | 50.36 | 60.9 | 49.93 | 51.0 |
| | Rel (%) | 11.2 | 21.0 | 12.5 | 22.6 | 13.3* | 22.8 | 15.5* | 24.3 |
| Prostrate | Abs (mg) | 0.93 | - | 0.66* | - | 0.83 | - | 0.38* | - |
| | Rel (%) | 0.20 | - | 0.15 | - | 0.22 | - | 0.12 | - |
| Testis | Abs (mg) | 3.53 | - | 3.40 | - | 3.27 | - | 2.71* | - |
| | Rel (%) | 0.75 | - | 0.79 | - | 0.87 | - | 0.85 | - |

| Parameter | | Nominal dietary concentrations (ppm)/sex | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | | 282 | | 635 | | 1640 | |
| | | M | F | M | F | M | F | M | F |
| Uterus | Abs (mg) | - | 0.49 | - | 0.45 | - | 0.42 | - | 0.23* |
| | Rel (%) | - | 0.17 | - | 0.16 | - | 0.16 | - | 0.11* |
| Ovary | Abs (mg) | - | 109.2 | - | 99.1 | - | 101.6 | - | 78.8* |
| | Rel (%) | - | 37.7 | - | 35.3 | - | 38.1 | - | 37.3 |
| **In vitro *p*-nitrosoanisole metabolism; mean ± standard deviation** | | | | | | | | | |
| Activity (µmole/g liver per hour) | | 1.86 ± 0.15 | 1.29 ± 0.12 | 1.97 ± 0.12 | 1.70 ± 0.30 | 2.40 ± 0.11 | 2.07 ± 0.200** | 3.09 ± 0.20** | 2.33 ± 0.23** |

***Italics*** indicate weight loss; M: Male; F: Female Source: Worth et al., 1978b

Abs: Absolute weight; Rel: Organ weight as a percentage relative to body weight;

* Significant decrease at $p < 0.05$

### *Dog*

In a GLP-compliant, one-year toxicity study, four beagle dogs of each sex per group were orally treated for one year with tricyclazole (purity 97.0%, Batch no. P68931) via gelatin capsules at doses of 0, 5, 15 or 45 mg/kg bw per day. Animals were observed daily for clinical signs and mortalities. Body weights were recorded prior to treatment and then weekly until study termination. Ophthalmological and physical examinations were performed at the start of treatment and at study termination. Blood samples were collected for haematology and blood biochemistry investigations prior to study initiation, at week 1, 2 and 4, and monthly thereafter. The haematologic parameters examined were: packed cell volume (PCV), Hb, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), erythrocyte count and morphology, total and differential leukocyte counts, thrombocyte count, and reticulocyte count. A cytological evaluation of bone marrow smears including an estimate of ratio of myeloid to erythroid precursors (M:E ratio) was performed at termination of the study. The clinical chemistry parameters determined were: glucose, BUN, creatinine, and total bilirubin levels; activities of the ALP, ALT, lactate dehydrogenase (LDH), and aspartate transaminase (AST); calcium, cholesterol, total protein, albumin, and phosphorus levels. Urinalysis was performed at the same times as haematological examinations. The $t_{½}$ of antipyrine metabolism in serum was investigated prior to treatment and at 1, 3, 6 and 12 months of treatment to evaluate enzyme induction effects A portion of the liver was collected during necropsy from all animals for measurement of in vitro hepatic 2-nitroanisole *O*-demethylase activity and hepatic microsoma1 P-450 content. At study termination all animals were sacrificed and subjected to necropsy examinations. The weights of the following organs were recorded: brain, pituitary, liver, kidneys, heart, thyroids, adrenals, testes, prostate, ovaries, and uterus. Organ to body weight ratios were calculated. A systematic gross examination of each animal and a histopathological examination were performed (tissue samples collected: aorta, cerebrum, cerebellum, brain stem, heart, colon, duodenum, jejunum, skeletal muscle, sciatic nerve, lymph nodes, adrenals, testes, thymus, thyroids, trachea, stomach, bone, bone marrow, liver, mammary glands, salivary glands, pituitary, ileum, oesophagus, ovaries, pancreas, lungs, prostate, spleen, kidneys, uterus, gall bladder, urinary bladder, skin and eyes). Serum concentrations of tricyclazole were determined by HPLC at 0, 1, 2, 3, 4 and 6 hours after dosing on test days 1, 42, 105, 196 and 365.

A statistically significant decrease in body weight gain was seen in females receiving 45 mg/kg bw per day, resulting in a final body weight lower by 14% than for controls, which was not statistically significant. This finding was also driven by low body weight in one animal (animal 15182: 7.5 kg). Increased absolute and relative liver weight (by ca 35% in males and by 59% and 83% for absolute and relative, respectively, in females), and increased absolute and relative kidney weight (by 24% in males) and relative kidney weight (by 43% in females) were also observed at 45 mg/kg bw per day. There were no treatment-related changes in thyroid weight. At the lower dose levels the liver weight increases were not considered adverse since they were in general by less than 15%, and it was agreed that the 20%–26% increase in relative liver weight in females should be considered secondary to lowered body weights. When compared with controls, males exhibited a lowered hepatic *p*-nitroanisole *O*-demethylase

activity (by 10%, 19% and 32% at 5, 15 and 45 mg/kg bw per day respectively) and a lowered hepatic cytochrome P-450 content (by 21%, 30% and 30% at 5, 15 and 45 mg/kg bw per day respectively), with no consistent effect in females. This effect in males is most probably an adaptive change and was not considered adverse.

No histopathological findings and no clinical chemistry changes were noted at any dose. The NOAEL was identified as 15 mg/kg bw per day based on body and liver weight effects at 45 mg/kg bw per day. (Holzhausen, 1986)

***Table 4. Key findings of the one-year dietary toxicity study in dogs; mean body weights, weight gains and food consumption***

| | | Nominal dietary concentrations (ppm)/sex | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | | 5 | | 15 | | 45 | |
| **Parameter** | | **M** | **F** | **M** | **F** | **M** | **F** | **M** | **F** |
| Dose level (mg/kg bw/day) | | 0 | 0 | 20.5 | 22.5 | 46.7 | 54.2 | 135.3 | 186.8 |
| **Mean body weights and weight gains (kg)** | | | | | | | | | |
| Week 0 | | 6.5 | 6.3 | 7.0 | 5.5 | 6.0 | 6.4 | 6.6 | 6.1 |
| Week 52 | | 10.0 | 11.0 | 11.3 | 9.3 | 10.2 | 10.2 | 10.1 | 9.5 |
| Weight gain (weeks 0–52) | | 3.5 | 4.7 | 4.3 | 3.8 | 4.2 | 3.8 | 3.5 | 3.4* |
| **Absolute and relative liver, kidney and thyroid weights** | | | | | | | | | |
| Liver | Abs (mg) | 252.00 | 238.25 | 284.00 | 237.00 | 286.75 | 276.75 | 340.25* | 377.75* |
| | Rel (%) | 2.515 | 2.173 | 2.531 | 2.611 | 2.822 | 2.728 | 3.376* | 3.973* |
| Kidney | Abs (mg) | 49.0 | 44.3 | 51.8 | 42.5 | 52.8 | 49.8 | 60.8* | 54.0 |
| | Rel (%) | 0.49 | 0.40 | 0.46 | 0.47 | 0.52 | 0.49 | 0.61* | 0.57* |
| Thyroid | Abs (mg) | 801.25 | 696.75 | 842.25 | 670.75 | 812.00 | 664.75 | 951.00 | 873.75 |
| | Rel (%) | 8.12 | 6.50 | 7.48 | 7.48 | 7.99 | 6.53 | 9.18 | 9.59 |
| Uterus | Abs (mg) | - | 7.87 | - | 8.40 | - | 4.92 | - | 5.72 |
| | Rel (%) | - | 0.074 | - | 0.082 | - | 0.048 | - | 0.060 |
| Ovaries | Abs (mg) | - | 1046.00 | - | 1610.00 | - | 820.00 | - | 837.50 |
| | Rel (%) | - | 9.74 | - | 15.68 | - | 7.92 | - | 9.25 |
| **Hepatic enzyme activity** | | | | | | | | | |
| Hepatic ***p***-nitroanisole *O*-demethylase activity (nmol *p*-nitrophenol produced/mg protein/hour) | | | | | | | | | |
| | | 17.71 | 14.97 | 15.99 | 15.41 | 14.40* | 10.90 | 11.98* | 14.28 |
| Hepatic cytochrome P-450 content (nmol P-450/mg protein). | | | | | | | | | |
| | | 0.523 | 0.371 | 0.415 | 0.382 | 0.368* | 0.319 | 0.365* | 0.336 |

M: Male; F: Female Source: Holzhausen, 1986

Abs: Absolute weight; Rel: Organ weight as a percentage relative to body weight;

* Different from controls with statistical probability $p < 0.05$

### (b) Dermal application

In a 28-day GLP-compliant dermal toxicity study, tricyclazole (purity 95.2%, Batch No. 2002090020) in carboxymethyl cellulose (CMC) was applied to the clipped back skin of and flanks of five Wistar rats of each sex per treatment group at levels of 100, 300 and 1000 mg/kg bw, for at least six hours per day, five days per week. The rats were nine weeks old with body weights 234.0–247.7 g for males and 168.8–173.3 g for females).

Dosing sites were covered with a semi-occlusive dressing, with a gauze patch and self-adhesive tape. After removal, sites were washed with water. Observations included skin reactions, clinical signs and mortality, body weight, food consumption, haematology and clinical chemistry, gross necropsy, organ weight. Also histopathology of samples from liver, kidney, ovaries, spleen, treated and untreated skin, adrenals, testes, heart, uterus and prostate.

No mortality or treatment-related clinical signs were observed during the study. There were no signs of skin irritation at the application site. Body weight and food consumption were not affected.

No treatment-related effects were observed on haematological examination. Increases in total bilirubin were seen in all groups of treated males, however these only achieved statistical significant at the top dose. Decreases in LDH activity were seen in the mid- and high-dose males, achieving statistical significance at the high dose. A statistically significant increase (by 12.6% and 18.3% in males and females respectively) in mean relative liver weight (only) was noted at the top dose. At necropsy, no treatment-related macroscopic changes were observed. Microscopical examination did not reveal any treatment-related histopathological changes.

Repeated topical skin application of tricyclazole for 28 days was associated with only minor findings when compared to the control animals. The only meaningful systemic effect was a slightly increased bilirubin plasma level (ten times controls in males) associated with a limited but statistically significant increase in just the relative liver weight. Consequently, the NOAEL for systemic toxicity was 300 mg/kg bw per day (Prinsen, 2003a).

***Table 5. Key findings of the 28-day dermal toxicity study in rats***

| | Dose level (mg/kg/day)/sex | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | | 100 | | 300 | | 1000 | |
| **Parameter** | **M** | **F** | **M** | **F** | **M** | **F** | **M** | **F** |
| Absolute liver weight (g) | 11.14 | 6.80 | 11.19 | 7.24 | 11.01 | 7.31 | **12.27** | **7.98** |
| Relative liver weight (g/kg bw) | 37.4 | 35.4 | 37.8 | 37.1 | 39.6 | 38.2 | **42.2**** | **41.9**** |

M: Male; F: Female; **bold** indicates at least 10% higher than controls; Source: Prinsen, 2003a

** Different from controls with statistical probability $p < 0.01$

## 2.3 Long-term studies of toxicity and carcinogenicity

The long-term toxicity and carcinogenicity potential of tricyclazole was evaluated in rats and in mice and the results are summarized in Table 6 below.

***Table 6. Tricyclazole – summary of long-term toxicity and carcinogenicity studies***

| Study | Species/ strain | Dosages (ppm) | NOAEL | LOAEL | Target organ/ principal effects at LOAEL | Reference | GLP |
|---|---|---|---|---|---|---|---|
| Two-year dietary chronic toxicity/ carcinogenicity study | Rat/ Wistar Han male and female rats | 40, 126 and 400 | Neoplastic NOAEL: 400 ppm (18/22 mg/kg bw/day in M/F).<br>Non-neoplastic NOAEL: 126 ppm (5.9/7.1 mg/kg bw/day in M/F) | Neoplastic LOAEL: not applicable.<br>Non-neoplastic LOAEL: 400 ppm (18 mg/kg bw/day for males, 22 mg/kg bw/day for females). | Neoplastic LOAEL: not applicable<br>Non-neoplastic LOAEL: Body weight gain effects (> 10%) in both sexes | Heward, 2020 | Y |
| 22-Month chronic oral and oncogenicity | Mouse/ ICR | 25, 75, 250 and 1000 | NOAEL = 75 ppm (equivalent to 7.98 mg/kg/day for males and 6.67 mg/kg/day for females | LOAEL: 250 ppm (equivalent to 24.9 mg/kg/day for males, 21.8 mg/kg/day for females) | Not carcinogenic; histopathological liver findings observed at 250 and 1000 ppm | Harada, 1985 | N[a] |
| **Pre-GLP studies** | | | | | | | |
| One-year toxicity, dietary | Mouse/ ICR | 50, 140, 400 or 620 | NOAEL: 620 ppm (equivalent to approximately 93 mg/kg bw/day) | NA | Not carcinogenic. No systemic toxicity at highest administered concentration | Howard, & Owen, 1979b | Pre |

| Study | Species/ strain | Dosages (ppm) | NOAEL | LOAEL | Target organ/ principal effects at LOAEL | Reference | GLP |
|---|---|---|---|---|---|---|---|
| Two-year combined toxicity and carcinogenicity dietary | Mouse/ ICR | 50, 140 and 400 | NOAEL: 400 ppm (estimated to be 29 mg/kg bw/day for males, 30 mg/kg bw/day for females) | NA | Not carcinogenic. No systemic toxicity at highest administered concentration | Howard et al., 1978 | Pre |
| Two-year combined toxicity and carcinogenicity dietary | Rat/ Wistar-derived male & female rats | 100, 275, 620 and 1600 | NOAEL for chronic toxicity: 275 ppm (11–16 mg/kg bw/day)<br>NOEL for carcinogenicity > 620 ppm (equivalent to 25.8 mg/kg bw/day for males, 40.1 mg/kg bw/day for females) | LOAEL: 620 ppm (equivalent to 25.8 mg/kg bw/day for males, 40.1 mg/kg bw/day for females) | Not carcinogenic<br>Body weight, liver weight (> 20%) | Howard, et al., 1977, revised in Stebbins, Golden & McFadden, 2016) | Pre |

M: Male; F: Female; NA: Not applicable; GLP: Good laboratory practice

[a] Not GLP, but independent quality assurance provided

### *Mouse*

A one-year pre-GLP toxicity study employed 4–5-week-old ICR mice (Harlan Industries), males weighing 26.2 ± 0.5 g, females 22.4 ± 0.3 g. Tricyclazole (purities 99.4% and 98.4%, for the batches C53-C48-147 and B07-C98-21, respectively) was administered in the diet to 15 mice of each sex per group at 0, 50, 140, 400 or 620 ppm (equivalent to 0, 7.5, 21, 60 and 93 mg/kg bw per day) for one year. During the initial five months of the study, each mouse was weighed at approximately weekly intervals. Thereafter, body weights were measured at 2–3 week intervals. All mice were inspected twice daily for mortality. Once a week all animals were observed for clinical signs, postural and locomotor abilities and external lesions or growths. Haematologic evaluation included the determination of erythrocyte and leukocyte counts, Hb, Ht and leukocyte differential counts and erythrocyte morphology on all of the survivors, and platelet count on three mice of each sex per dose. Clinical chemistry evaluation was performed on fresh serum collected on the day of necropsy from all survivors except those used for determination of platelet counts, and included the determination of glucose, BUN, creatinine, total bilirubin, ALP and ALT activities. At study termination all surviving animals were subjected to necropsy, as were any that died prior to study termination. Gross pathological examinations was performed, including evaluation of the general condition of the animal, external and internal lesions and body orifices, with special attention being directed to treatment-related changes. Histopathological examination was performed on the following collected organs and tissues: mammary gland, salivary gland, lungs, heart, thyroids, stomach, duodenum, jejunum, ileum, colon, brain, liver, pituitary, skeletal muscle, thymus, pancreas, spleen, kidneys (with adrenals attached), urinary bladder, prostate, testes, ovaries (with uterus attached), mesenteric lymph node and bone marrow. Stomach, duodenum, jejunum, ileum and colon were not collected from animals which died prior the study termination due to onset of autolysis. The weights of the following organs were recorded: heart, brain, liver, spleen, kidney (with adrenals attached), testes and ovaries (with uterus attached). Organ to body weight ratios was calculated.

There were no treatment-related effects on mortality during the study. The results of this study show that dietary administration of tricyclazole to male and female mice at concentration of up to 620 ppm for one year did not induce any abnormal effect in any organ. Slight, and not dose-related, variations were seen in certain organs (heart and ovary for instance), however these were considered to be within the range of normal biological variation. It was noted that the group size was very small, reducing the power of the study to detect effects, and there were other variations from the OECD TG 452 guidelines. A NOAEL of 620 ppm (equivalent to approximately 93 mg/kg bw per day) was identified, the highest dose tested (Howard & Owen, 1979b).

A pre-GLP and pre-guideline carcinogenic study employed ICR mice (Harlan Industries) with initial body weights for males of 24.7 ± 0.2 g, and females of 21.4 ± 0.1 g. In duplicate two-year chronic toxicity/oncogenic studies (M-9045 and M-9055) 40 mice of each sex per dose group (treated) and 60 of each sex (controls) were exposed orally to tricyclazole (purity: 99.4% and 98.4% for batches C53-C48-147 and B07-C98-20 respectively) at dose levels in the diet of 0, 50, 140 or 400 ppm (estimated as daily intakes of 0, 3.5, 10 and 29 mg/kg bw for males, 0, 3.6, 10.4 and 30 mg/kg bw for females). The procedure was the same in both studies except where otherwise stated. Twice each day all cages were inspected for dead, dying or moribund mice. Dead mice were submitted for necropsy. An examination was performed, including observations of muscle tone, condition of pelage, colour and appearance of eyes, condition of teeth, respiration, postural and locomotor abilities and any external lesions or growths. The size of external masses was measured and recorded. Body weights were recorded at weekly intervals for the initial five months. Thereafter, body weights were measured at intervals of 2–3-weeks. Water consumption was measured at various intervals during study M-9045; 24-hour water consumption was determined for five groups of three mice of each sex per treatment group. For all of the survivors haematological samples were collected on the morning of necropsy and their analysis included the determination of erythrocyte and leukocyte counts, Hb, Ht, leukocyte differential count, platelet count, MCV, MCH, MCHC and erythrocyte morphology. Clinical chemistry tests were performed on fresh serum from 11 survivors (except for those used in determination of platelet counts) and included the determination of glucose, BUN, creatinine, total bilirubin, ALP and ALT activities. Aspartate transaminase activity determinations were performed on surviving mice from study M-9045 where sufficient amounts of serum allowed. During the test, necropsies were performed on animals that died or were killed because of poor physical condition and/or the presence of large or ulcerated tissue masses, and mice surviving the two-year testing period. Gross pathology evaluation was performed on all mice that died prior to the termination of the study and on all surviving animals. Necropsy consisted of an examination of the general condition of the animal, external and internal lesions, body orifices, with special attention being directed to chronic lesions, treatment-related changes, tissue masses, and any metastatic lesions. After gross examination histopathological evaluation was conducted on the following tissues and organs: gross lesions, tissue masses, mammary gland, salivary gland, lungs, heart thyroids, stomach, duodenum, liver, skeletal muscle, thymus, jejunum, ileum, colon, mesenteric lymph node, pancreas, spleen, kidneys (with adrenals attached), urinary bladder, prostate, testes, ovaries (with uterus attached), brain, pituitary, eyes and sternal bone marrow. Stomach, duodenum, jejunum, ileum and colon were not collected from animals that died prior to study termination due to the onset of autolysis. The weights of the following organs were recorded: heart, brain, liver, spleen, kidneys, testes and ovaries.

Assays of the diets containing tricyclazole provided an indication that the pelleting process and/or aging had reduced the effective concentration of tricyclazole. The average effective percentage of the theoretical concentrations for aged pellet samples from the 50, 140 and 400 ppm diets was 77%, 79% and 77%, respectively.

Food consumption was not measured in this study and exposure to tricyclazole was estimated by the study authors based on food consumption data from previous studies (that did not involve tricyclazole), and took into account the loss of tricyclazole during the storage of the diet. The average percentage of effective tricyclazole using the aged pellet samples from the 50, 140 and 400 ppm diets was 77%, 79% and 77%, which resulted in corrected, estimated dietary levels of 39, 111 and 308 ppm. The average daily dose of tricyclazole was estimated from the corrected values to be 0, 3.5, 10 and 29 mg/kg bw per day for males, 0, 3.6, 10.4, and 30 mg/kg bw per day for females. It was noted that estimations based on food consumption data from previous studies, and taking into account the decrease in tricyclazole concentration with aging, resulted in lower estimated dose levels than obtained from general calculations of equivalence.

A range of clinical signs were observed throughout the study, however they were seen in both treated and control animals, or at a low incidence and in a pattern that was not related to dose, and so were not considered treatment-related.

At study termination mean body weights of males and females in all groups were unaffected by the treatment when compared to the controls. The only statistically significant ($p < 0.05$) alteration in the parameters evaluated in this study was a decrease in the MCHC of male mice in the 50 ppm group (study M-9045 only) which was not observed at higher concentrations and was not considered toxicologically significant. Statistically significant changes in clinical chemistry values were not observed in these

studies. No statistically significant differences in absolute or relative organ weights were observed. There was a trend towards increased liver weight for females exposed to tricyclazole. At necropsy no treatment-related lesions were found. The overall incidence of malignant neoplasms was decreased in the tricyclazole treatment groups compared to controls.

Mice (ICR) were treated for 24 months with dietary tricyclazole concentration up to 400 ppm (equivalent to 29 and 30 mg/kg bw per day in males and females, respectively). This dose level did not elicit any sign of toxicity in any of the investigated parameters. These studies was not considered a suitable basis from which to draw a conclusion on carcinogenicity as there were insufficient animals per group (40 of each sex, compared with 50 specified in OECD TG 453). Additionally, no toxicity was seen at any dose level tested (Howard et al., 1978).

In a dietary two-year chronic toxicity/oncogenicity test in mice, which included independent quality assurance procedures by the quality assurance unit, ICR-JCL mice were treated orally with tricyclazole (purity 98.85%, Batch no. 133AS1) for 95 weeks. An adjustment to the protocol was required to reduce treatment time from 104 weeks due to high mortality associated with the frequent incidence of spontaneous lesions such as dysuria. Dietary doses chosen were 0, 25, 75, 250 and 1000 ppm (males equal to 0, 2.59, 7.98, 24.9 and 101 mg/kg bw per day for males, 0, 2.2, 6.67, 21.8 and 91 mg/kg bw per day for females), administered to 64 mice of each sex per group. Ten mice of each sex per dose group were sacrificed at 52 weeks after dosing commenced.

All animals were observed daily for clinical signs and mortality. The body weight of each animal was recorded weekly during the first 26 weeks of treatment and every other week thereafter. Food consumption was measured twice a week during treatment. Mean daily food and water consumption per animal were calculated weekly during the first 26 weeks of treatment and every other week thereafter. Group mean chemical intake and food efficiency were calculated as group means. Stability and homogeneity of the test item at different storage time points was checked. Urinalysis was performed on 10 mice of each sex from each group at 52 weeks of treatment and on all surviving animals at 95 weeks. Blood samples and blood chemistry parameters were evaluated on the same animals sampled for urinalysis. Haematological parameters included: Hb, Ht, erythrocyte count, MCV, MCH, MCHC, platelet, leukocyte, differential leukocyte and reticulocyte counts. Blood chemistry parameters included: total protein, ALP, BUN, glucose, total cholesterol, AST, ALT and calcium. All animals were subjected to a complete necropsy, including gross pathology and histopathological examination of the following organs and tissues: cerebrum, brain stem, thymus, bone with marrow, lymph nodes (cervical, mesenteric), heart, salivary glands, stomach, pancreas, jejunum, caecum, trachea, kidneys, testes, ovaries, eyes and contiguous glands, skeletal muscle (M. triceps surae, unilateral), skin, mammary gland, cerebellum, pituitary, thyroids (with parathyroid), spleen, aorta, oesophagus, liver, duodenum, ileum, colon, lung, urinary bladder, prostate and uterus. Weights of the following organs were recorded for each animal subjected to interim or terminal kill: brain, thyroids (with parathyroid), heart, spleen, adrenals, pituitary, thymus, liver, kidney and testes/ovaries.

There were no treatment-related increases in mortality, clinical signs of toxicity or significant effects on body weight or body weight gain. Food consumption in the treated groups was comparable to the controls although significantly increased values were occasionally observed in some test groups of females during the first three weeks of treatment. There were no treatment-related abnormalities in urinalysis. Significant decreases compared to controls in erythrocyte count (by13%), Hb (by 14%) and Ht (by14%) were observed in the 250 and 1000 ppm male groups after 52 weeks of treatment, with no significant differences observed at 95 weeks. No abnormalities were observed in blood biochemistry. Macroscopic findings including distension of the urinary bladder, nodules and/or masses in the spleen in male mice as well as enlargement of the lymph nodes, thickening of the uterine wall and hair loss in females. These macroscopic findings were all considered incidental as corresponding histological lesions were not observed, no dose–response relationship was observed and/or the effects were within the background range for the strain of mice. Significant increases in absolute and relative liver weight were observed in male mice treated at 1000 ppm particularly at the 52 weeks interim time point. Though not attaining statistical significance, a trend towards increased liver weight was also observed in high-dose females at 52 weeks and in both sexes at 95 weeks. These effects correlated with histopathological findings in the liver, including increased periportal hepatocellular fatty change and periportal acidophilic degeneration of hepatocytes at 250 and 1000 ppm in both sexes (Table 7a).

There was no effect of treatment on the incidence, appearance or type of a tumours observed. All neoplastic lesions noted were determined to be spontaneous in nature.

An overall NOAEL of 75 ppm (equal to 7.98 mg/kg per day for males and 6.67 mg/kg per day for females) was identified, based on liver weight and histopathology and haematological changes observed at 250 ppm. The NOAEL for carcinogenicity was 1000 ppm (equal to 101 and 91 mg/kg per day in males and females respectively), the highest dose tested (Harada, 1985).

***Table 7a. Liver weights in a 95-week study in mice***

| Time point | Parameter | | Dietary dose (ppm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Males | | | | | Females | | | | |
| | | | 0 | 25 | 75 | 250 | 1000 | 0 | 25 | 75 | 250 | 1000 |
| Week 52 | Liver | Abs (g) | 2.20 | 2.52 | 2.24 | 2.66 | 2.86 | 1.97 | 2.05 | 2.25 | 2.07 | 2.42 |
| | | SD | ± 0.15 | ± 0.47 | ± 0.32 | ± 0.35 | ± 0.44 | ± 0.23 | ± 0.37 | ± 0.51 | ± 0.22 | ± 0.40 |
| | | Rel (%) | 4.64 | 5.11 | 4.90 | 5.45 | 5.78 | 4.10 | 4.11 | 4.53 | 4.24 | 5.16 |
| | | SD | ± 0.58 | ± 0.81 | ± 0.67 | ± 0.64* | ± 0.73 | ± 1.12 | ± 0.70 | ± 1.07 | ± 0.57 | ± 1.45 |
| Week 95 | Liver | Abs (g) | 2.37 | 2.42 | 2.69 | 2.56 | 3.29 | 2.44 | 2.38 | 2.50 | 2.54 | 2.80 |
| | | SD | ± 0.72 | ± 0.36 | ± 0.82 | ± 0.52 | ± 2.80 | ± 0.58 | ± 0.72 | ± 0.46 | ± 0.46 | ± 0.59 |
| | | Rel (%) | 5.47 | 5.47 | 5.86 | 5.75 | 7.19 | 6.04 | 5.42 | 5.75 | 5.84 | 6.49 |
| | | SD | ± 1.20 | ± 0.89 | ± 1.31 | ± 0.96 | ± 5.04 | ± 1.18 | ± 2.01 | ± 1.36 | ± 1.46 | ± 1.48 |

Abs: Absolute weight; Rel: Organ weight as a percentage relative to body weight; Source: Harada, 1985

Different from controls by Dunnett's test with statistical probability: * $p < 0.05$; ** $p < 0.01$

***Table 7b. Gross pathology findings in a 95-week study in mice***

| Parameter | Dietary dose (ppm) | | | | |
|---|---|---|---|---|---|
| | 0 | 25 | 75 | 250 | 1000 |
| | | | **Males** | | |
| Number of mice examined | 64 | 64 | 64 | 64 | 64 |
| Number of mice affected: | | | | | |
| spleen nodule/masses | 0 | 1 | 2 | 2 | 5* |
| liver spot(s) | 0 | 1 | 1 | 0 | 5* |
| distended urinary bladder | 8 | 19* | 17* | 16 | 20* |
| | | | **Females** | | |
| Number examined | 64 | 64 | 64 | 64 | 64 |
| Number of mice affected: | | | | | |
| lymph node enlargement (cervical) | 8 | 14 | 10 | 10 | 17* |
| lymph node enlargement (others) | 4 | 12* | 13* | 8 | 17** |
| uterine horn wall thickness | 1 | 7* | 11** | 2 | 8* |

Different from controls by Fisher's test with statistical probability: * $p < 0.05$; ** $p < 0.01$; Source: Harada, 1985

*Table 7c. Histopathology in a 95-week study in mice; (mice affected/number examined)*

| Time point | Parameter | Dietary dose (ppm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | | Females | | | | |
| | | 0 | 25 | 75 | 250 | 1000 | 0 | 25 | 75 | 250 | 1000 |
| Week 52 | Periportal acidophilic degeneration of hepatic cells | 0 | 0 | 0 | 0 | 5/10* | 0 | 0 | 0 | 0 | 3/10 |
| Week 95 | Periportal hepatocellular fatty change | 0 | 0 | 0 | 0 | 3/16 | 0 | 0 | 0 | 6/18* | 10/15*** |
| | Periportal acidophilic degeneration of hepatic cells | 0 | 0 | 0 | 3/24 | 5/16* | 0 | 0 | 0 | 3/18 | 8/15** |

Source: Harada, 1985

Different from controls by Fisher's test with statistical probability: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

***Rat***

In a combined long-term carcinogenicity study carried out before GLP or guidelines were in use, Wistar-derived rats (Harlan Industries) were treated orally with tricyclazole (purity: 99.4% and 98.4% for batches C53-C48-147 and B07-C98-20 respectively) in duplicate two-year chronic toxicity/oncogenicity studies (R-764 and R-774). The studies were performed by the toxicology division of Lilly Research Laboratories between 1974 and 1976. Previously, a single report was issued for both the R-764 and R-774 studies with statistical analyses reported separately for a majority of the study parameters. An additional assessment (Stebbins, Golden & McFadden, 2016) of the original tricyclazole two-year rat report was conducted to identify acceptability and deviations from the concurrent OECD 453 test guideline, as well as to conduct a more thorough statistical assessment of the data by combining the replicate datasets for various test parameters.

Combining data for the two studies, there were 120 Wistar-derived rats of each sex in the control group, and 80 rats of each sex in each of the treated groups at the initiation of the two-year studies. Test diets of 0, 100, 275 and 620 ppm in males corresponded to time-weighted average intakes of 0, 4.1, 11 and 26 mg/kg per day in males, and 0, 5.3, 16 and 40 mg/kg per day in females. Additionally, there was a fifth group (80 animals of each sex) that was given treated diet with 1600 ppm tricyclazole for three months, followed by control diet for 21 months. This corresponded to three-month, time-weighted averages of 109 mg/kg per day for males and 173 mg/kg per day for females.

A total of 880 animals (males weighing 140 ± 0.8 g, females 121 ± 0.7 g) were exposed to tricyclazole: for each of the duplicate studies, 40 of each sex in the five treatment groups and 60 of each sex as controls.

Twice each day cages were inspected for dead, dying or moribund rats. Dead rats were promptly submitted for necropsy. Each rat was weighed at approximately weekly intervals, and haematological samples were taken at study termination on the morning of necropsy from all surviving rats. Haematological tests included the determination of erythrocyte and leukocyte counts, Hb, Ht, MCV, MCH, MCHC, leukocyte differential count, platelet count and erythrocyte morphology for all of the survivors. Prothrombin time was determined on approximately half of the surviving rats. Clinical chemistry evaluation was performed on approximately half of the two-year survivors. This included the determination of glucose, BUN, creatinine, total bilirubin, ALP, ALT, AST and total protein. Urine samples from five rats of each sex per dose (for each study) were taken a few days prior the termination of the study, and their analysis included evaluation of specific gravity, sugar, pH, protein and occult blood, as well as a microscopic examination for leukocytes, erythrocytes, casts and sediment. Gross pathology evaluation was performed on all rats that died prior the termination of the study and on all surviving animals. The necropsy consisted of an examination of the animal's general condition, external and internal lesions, body orifices, with special attention being directed to chronic lesions, treatment-related changes, tissue masses and any metastatic lesions. After gross examination histopathological evaluation was conducted on the following tissue and organs: any gross lesions, tissue masses, mammary gland, salivary gland, lungs, heart thyroids (with parathyroid), stomach, duodenum, liver, skeletal muscle, thymus, jejunum, ileum, colon, mesenteric lymph node, pancreas, spleen, kidneys, adrenals, urinary

bladder, prostate, testes, ovaries, uterus, brain, pituitary, eyes, spinal cord, sciatic nerve and sternal bone marrow. Stomach, duodenum, jejunum, ileum and colon were not collected from animals that died prior to study termination due to the onset of autolysis. The weights of the following organs were recorded: heart, brain, liver, spleen, kidneys, testes and ovaries, prostate, thyroids and pituitary.

There were no treatment-related changes in mortality rates. Survival rates in the 0, 100, 275, 620 and 1600 ppm groups for both datasets combined at the end of the study were 30%, 31%, 32%, 25% and 32% for males and 32%, 32%, 38%, 30% and 34% for females, respectively. The number of surviving animals for each replicate was low, but because of the large combined group size the absolute number surviving was high and the percentage above the 25% threshold indicated in the OECD TG 453, (2009), the minimum threshold for terminating a study. In addition the combined number of surviving animals from the two studies was sufficient to achieve an appropriate level of confidence in the determining the presence or absence of treatment-related effects in a cancer bioassay, according to the criteria indicated in the OECD GD 116 (2012).

There were no treatment-related changes in clinical observations.

Males and females administered 620 ppm for 24 months suffered statistically significant depressions in body weight compared to controls and these were considered treatment-related. Group mean body weights in males and females treated at 620 ppm were consistently lower throughout the study and were lower (by 16% in males and 14% in females) than the controls at the end of 24 months of exposure. Additionally, males and females administered 1600 ppm for three months, followed by a 21-month recovery period, also showed a statistically significant, treatment-related depression in body weight compared to controls during the first 12 months of treatment. Body weights were depressed by 7%–26% for males in the period 3–12 months, and 7%–23% for females in the period 3–15 months, and these changes were considered treatment-related. Similarly to body weights, males and females administered 620 ppm or 1600 ppm showed a treatment-related suppression in body weight gain compared to controls. Reduction in body weight gain in the 620 ppm group ranged from 11%–20% for males, and 13%–20% for the females, and effects were present throughout the study. Reductions in body weight gain in the 1600 ppm group compared to controls were by 34% in males and 37% in females at the end of the three-month dosing phase. During the 21 month recovery period, the body weight gain in males returned to levels similar to controls by the 15-month time point. For females depressions in body weight gain ranged from 9%–14% throughout the 21 month recovery period.

Treatment-related decreases in food consumption and efficiency of feed utilization were limited to males and/or females administered 620 ppm or 1600 ppm, and occurred during the beginning of the study only.

Statistically identified changes in haematology and clinical chemistry parameters (increase in white blood cell count, glucose increase) were not considered related to treatment. No changes were observed from urinalysis.

There were treatment-related decreases in the mean final body weights of males and females administered 620 ppm for 24 months (by 16% and 14% respectively, compared to controls). Males and females administered 620 ppm and males administered 275 ppm had treatment-related, statistically significant raised mean relative liver weights. Compared to controls, the mean relative liver weights were greater by 19% and 30% in males administered 275 ppm and 620 ppm respectively, and greater by 24% in females treated at 620 ppm. The higher relative liver weights in males and females administered 620 ppm were interpreted to be adverse effects, and the higher relative liver weight of males at 275 ppm was interpreted to be non-adverse, as it was less than a 20% difference and was observed without accompanying histopathological changes.

There were no treatment-related histopathological changes in any of the organs/tissues examined after 24 months of treatment. There were no treatment-related increases in the incidence of neoplasms in any organs compared to controls, either in male or female rats at any dose level, indicating that tricyclazole was not carcinogenic to Wistar-derived rats under the conditions of this study.

The NOAEL was 275 ppm (equal to 11 mg/kg bw per day) based on body weight decreases and liver weight increases at 620 ppm. The NOAEL for carcinogenicity was 620 ppm (equal to 26 mg/kg bw per day), the highest dose tested (Howard et al., 1977, revised in Stebbins, Golden & McFadden, 2016).

***Table 8a. Key findings in the duplicated two-year combined toxicity and carcinogenicity dietary studies in rats; mortality (OECD 543 threshold for survival, 25%)***

| | Dose (ppm)/sex | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Males | | | | | Females | | | | |
| | **0** | **100** | **275** | **620** | **1600[b]** | **0** | **100** | **275** | **620** | **1600[a]** |
| *N* | 120 | 80 | 80 | 80 | 80 | 120 | 80 | 80 | 80 | 80 |
| Survivors | 36 | 25 | 26 | 20 | 26 | 39 | 26 | 30 | 24 | 27 |
| Survival | 30% | 31% | 32% | 25% | 32% | 32% | 32% | 38% | 30% | 34% |

*N*: Number of rats of per group; Source: Stebbings, Golden & McFadden, 2016

[a] Tricyclazole diet for 3 months, then control for 21 months;

***Table 8b. Key findings in the duplicated two-year combined toxicity and carcinogenicity dietary studies in rats; body weights***

| | Dietary dose (ppm)/sex | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Males | | | | | Females | | | | |
| | **0** | **100** | **275** | **620** | **1600[a]** | **0** | **100** | **275** | **620** | **1600[a]** |
| **Study 1 data (R-764)[b] ; mean ± standard deviation (g)** | | | | | | | | | | |
| Month 3 | 491 ± 5 | 482 ± 41 | 474 ± 42 | **447 ± 8^^** | **361 ± 7^^** | 299 ± 4 | 299 ± 29 | 295 ± 33 | **272 ± 4^^** | **234 ± 4^^** |
| Month 12 | 623 ± 80 | 592 ± 79 | 607 ± 59 | **557 ±70^^** | **566 ± 88^^** | 394 ± 56 | 387 ± 51 | 385 ± 63 | **339 ± 64^^** | 355 ± 47 |
| Month 24 | 567 ± 102 | 552 ± 77 | 527 ± 77 | 465 ± 74^^ | 548 ± 89 | 424 ± 74 | 424 ± 111 | 397 ± 76 | 359 ± 58 | 364 ± 83 |
| **Study 2 data (R-774)[b] ; mean ± standard deviation (g)** | | | | | | | | | | |
| Month 3 | 148 ± 20 | 140 ± 15 | 143 ± 15 | 141 ± 15 | 138 ±17^^ | 122 ± 14 | 121 ± 11 | 123 ± 13 | 122 ± 16 | 121 ±15 |
| Month 12 | 619 ± 86 | 618 ± 69 | 599 ± 81 | **548 ± 71^^** | 589 ± 85 | 388 ± 64 | 386 ± 61 | 378 ± 47 | **348 ± 42^^** | 372 ± 43 |
| Month 24 | 579 ± 130 | 552 ± 99 | 589 ± 102 | 497 ± 71 | 558 ± 117 | 411 ± 98 | 406 ± 132 | 403 ± 114 | 362 ± 81 | 382 ± 52 |
| **Combined study data; mean ± standard deviation (g) [percentage change compared to controls]** | | | | | | | | | | |
| Day 0 | 145 ± 17 | 142 ± 16 | 141 ± 16 | 140 ± 15 | 133* ± 16 [−8%] | 122 ± 13 | 122 ± 11 | 124 ± 13 | 121 ± 13 | 118 ± 15 |
| Month 3 | 499 ± 47 | 490 ± 40 | 484 ± 45 | **449* ± 43 [−10%]** | **368* ± 47 [−26%]** | 303 ± 27 | 301 ± 30 | 298 ± 28 | **276* ± 25 [−9%]** | **234* ± 29 [−23%]** |
| Month 6 | 576 ± 55 | 564 ± 50 | 560 ± 52 | **520* ± 48 [−10%]** | **509* ± 52 [−12%]** | 344 ± 36 | 344 ± 35 | 340 ± 31 | **313* ± 33 [−9%]** | **310* ± 30 [−10%]** |
| Month 9 | 596 ± 77 | 590 ± 67 | 588 ± 64 | **544* ± 65 [−8%]** | **549* ± 72 [−8%]** | 366 ± 49 | 369 ± 43 | 362 ± 41 | **331* ± 40 [−10%]** | **340* ± 41 [−7%]** |
| Month 12 | 621 ± 83 | 606 ± 74 | 603 ± 72 | **562* ± 71 [−10%]** | **577* ± 87 [−7%]** | 391 ± 60 | 386 ± 56 | 382 ± 55 | **343* ± 47 [−12%]** | **364* ± 46 [−7%]** |
| Month 15 | 619 ± 102 | 610 ± 85 | 600 ± 88 | **557* ± 85 [−10%]** | 597 ± 93 [↓4%] | 411 ± 69 | 405 ± 78 | 394 ± 75 | **353* ± 60 [−14%]** | **376* ± 64 [−9%]** |
| Month 18 | 637 ± 98 | 604 ± 97 | 627 ± 73 | **563* ± 80 [−12%]** | 601 ± 81 [↓6%] | 414 ± 81 | 426 ± 86 | 409 ± 76 | **360* ± 64 [−13%]** | 384 ± 62 [↓7%] |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Month 21 | 598 ± 121 | 582 ± 103 | 605 ± 94 | **517* ± 99 [−14%]** | 601 ± 116 - | 420 ± 87 | 424 ± 93 | 412 ± 92 | **359* ± 70 [−15%]** | 390 ± 75 [↓7%] |
| Month 24 | 572 ± 113 | 552 ± 87 | 559 ± 94 | **483§ ± 72 [−16%]** | 552 ± 100 | 418 ± 86 | 416 ± 118 | 400 ± 94 | **360 ± 68 [−14%]** | 372 ± 70 [↓11%] |

**Bold** values indicate effects interpreted to be treatment-related; Source: Stebbings et al., 2016

[a] Tricyclazole diet for 3 months, then control for 21 months;

[b] Statistics as described in the original report:
significantly different from control using Dunnett's t, two-tailed test at: ^ $p \leq 0.1$; ^^ $p \leq 0.05$; ^^^ $p \leq 0.01$

* Statistically different from control mean by Dunnett's test, alpha = 0.05;

§ Statistically different from control mean by Wilcoxon's test, alpha = 0.05

[Percentage of change were calculated from the concurrent control values and are shown if the difference is 5% or greater]

***Table 8c. Key findings in the duplicated two-year combined toxicity and carcinogenicity dietary studies in rats; body weights gains from combined datasets***

| | **Dietary dose (ppm)/sex** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Males** | | | | | **Females** | | | | |
| | **0** | **100** | **275** | **620** | **1600[a]** | **0** | **100** | **275** | **620** | **1600[a]** |
| **Combined study data; mean ± standard deviation (g) [percentage change compared to controls]** | | | | | | | | | | |
| Months 0–3 | 355 ± 41 | 347 ± 36 | 343 ± 38 | **309 ± 40 [–13%]** | **235 ± 42 [–34%]** | 182 ± 24 | 179 ± 25 | 173 ± 24 [–5%] | **156 ± 20 [–14%]** | **115 ± 24 [–37%]** |
| Months 0–6 | 431 ± 48 | 421 ± 45 | 420 ± 47 | **380 ± 45 [–12%]** | **376 ± 47 [–13%]** | 223 ± 34 | 222 ± 3 | 216 ± 27 | **193 ± 28 [–13%]** | **192 ± 26 [–14%]** |
| Months 0–9 | 451 ± 73 | 447 ± 64 | 449 ± 5 | **403 ± 64 [–11%]** | **416 ± 67 [–8%]** | 244 ± 48 | 247 ± 39 | 238 ± 38 | **210 ± 36 [–14%]** | **221 ± 36 [–9%]** |
| Months 0–12 | 476 ± 81 | 464 ± 73 | 463 ± 68 | **422 ± 71 [–11%]** | **445 ± 84 [–7%]** | 270 ± 58 | 264 ± 53 | 258 ± 51 | **223 ± 44 [–17%]** | **246 ± 42 [–9%]** |
| Months 0–15 | 475 ± 100 | 469 ± 82 | 460 ± 87 | **417 ± 84 [–12%]** | 464 ± 91 | 290 ± 69 | 283 ± 76 | 270 ± 70 [–7%] | **232 ± 59 [–20%]** | **258 ± 62 [–11%]** |
| Months 0–18 | 492 ± 93 | 465 ± 94 [−5%] | 488 ± 71 | **424 ± 78 [–14%]** | 470 ± 79 | 292 ± 80 | 304 ± 83 | 285 ± 72 | **240 ± 61 [–18%]** | **267 ± 59 [–9%]** |
| Months 0–21 | 454 ± 116 | 443 ± 101 | 466 ± 95 | **378 ± 95 [–17%]** | 470 ± 115 | 299 ± 86 | 302 ± 89 | 290 ± 89 | **241 ± 66 [–19%]** | **274 ± 73 [–8%]** |
| Months 0–24 | 427 ± 107 | 414 ± 85 | 423 ± 92 | **341 ± 69 [–20%]** | 422 ± 95 | 297 ± 84 | 295 ± 115 | 277 ± 96 [–7%] | **242 ± 64 [–19%]** | **258 ± 68 [–13%]** |

**Bold** values indicate effects interpreted to be treatment-related; Source: Stebbings et al., 2016

[a] Tricyclazole diet for 3 months, then control for 21 months;

[Percentage of change were calculated from the concurrent control values and are shown if the difference is 5% or greater]

*Table 8d. Key findings in the duplicated two-year combined toxicity and carcinogenicity dietary studies in rats; food consumption from combined datasets*

| | Dietary dose (ppm)/sex | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Males | | | | | Females | | | | |
| **Time point** | **0** | **100** | **275** | **620** | **1600 [a]** | **0** | **100** | **275** | **620** | **1600 [a]** |
| **Combined study data; mean ± standard deviation (g) [percentage change compared to controls]** | | | | | | | | | | |
| Week 1 | 126 ± 21 | 136[§] ± 21 | 127 ± 20 | **112[§] ± 30 [–11%]** | **72[§] ± 16 [–43%]** | 92 ± 28 | 109[§] ± 19 | 103 ± 24 | **94[§] ± 21** [+2.2%] | **67[§] ± 27 [–27%]** |
| Month 3 | 187 ± 23 | 183 ± 29 | 181 ± 31 | **169[§] ± 31 [–10%]** | **176[§] ± 56** [–6%] | 140 ± 27 | 151 ± 44 | 140 ± 37 | 150 ± 54 | 177 ± 88 |
| Month 6 | 177 ± 23 | 171 ± 23 | 171 ± 23 | **163[§] ± 20 [–8%]** | 170[§] ± 30 | 139 ± 38 | 138 ± 46 | 133 ± 27 | 138 ± 47 | 143 ± 46 |
| Month 9 | 161 ± 54 | 163 ± 42 | 164 ± 36 | 156 ± 47 | 149[§] ± 44 | 144 ± 49 | 140 ± 47 | 147 ± 50 | 145 ± 60 | 155 ± 60 |

**Bold** values indicate effects interpreted to be treatment-related; Source: Stebbings et al., 2016

[a] Tricyclazole diet for 3 months, then control for 21 months;

[§] Statistically different from control mean by Wilcoxon's test, alpha = 0.05

*Table 8e. Key findings in the duplicated two-year combined toxicity and carcinogenicity dietary studies in rats; test material intake (TMI) from combined datasets*

| | Dietary dose (ppm)/sex | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Males | | | | | Females | | | | |
| | **0** | **100** | **275** | **620** | **1600 [a]** | **0** | **100** | **275** | **620** | **1600 [a]** |
| Test material intake (TMI) (mg/kg bw) | 0 | 4.12 | 11.33 | 25.68 | 109.35 | 0 | 5.34 | 16.03 | 40.24 | 172.90 |

[a] Tricyclazole diet for 3 months, then control for 21 months; Source: Stebbings et al., 2016

*Table 8f. Key findings in the duplicated two-year combined toxicity and carcinogenicity dietary studies in rats; statistically identified haematology parameters (Stebbins et al., 2016)*

| | Dietary dose (ppm)/sex | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Males | | | | | Females | | | | |
| | **0** | **100** | **275** | **620** | **1600 [a]** | **0** | **100** | **275** | **620** | **1600 [a]** |
| **Study 1 data (R-764)[b]; mean ± standard deviation** | | | | | | | | | | |
| *N* | 20 | 12 | 12 | 9 | 15 | 20 | 15 | 15 | 12 | 15 |
| Platelets ($10^3/\mu L$) | 850.6 ± 257.0 | 814.3 ± 105.9 | 1066.8 ± 305.3^^ | 814.3 ± 105.9 | 1066.8 ± 305.3 | - | - | - | - | - |
| **Study 2 data (R-774)[b]; mean ± standard deviation** | | | | | | | | | | |
| *N* | 15 | 12 | 14 | 11 | 11 | 18 | 9 | 13 | 12 | 11 |
| WBC ($10^3/\mu L$) | 8.93 ± 2.26 | 9.23 ± 4.37 | 10.03 ±3.09 | 11.03 ± 2.79 | 12.24 ± 3.25^^ | 11.49 ± 8.01 | 8.99 ± 4.46 | 9.84 ± 4.56 | 6.09 ± 3.24^^ | 9.17 ± 2.33 |
| MCHC (%) | 34.26 ± 1.67 | 34.72 ± 1.46 | 34.60 ± 0.87 | 33.04 ± 0.96^ | 33.84 ± 0.74 | - | - | - | - | - |

| | Dietary dose (ppm)/sex | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Males | | | | | Females | | | | |
| | 0 | 100 | 275 | 620 | 1600 [a] | 0 | 100 | 275 | 620 | 1600 [a] |
| **Combined study data; mean ± standard deviation [percentage change compared to controls]** | | | | | | | | | | |
| *N* | 35 | 24 | 26 | 20 | 26 | 38 | 24 | 28 | 24 | 26 |
| WBC ($10^3/\mu L$) | 9.7 ± 3.2 | 10.4 ± 3.5 | 10.6 ± 3.0 | 10.5 ± 2.7 | 12.7* ± 4.5 [+30.9%] | 10.0 ± 7.0 | 8.9 ± 7.5 | 8.7 ± 4.7 | 6.7§ ± 5.0 | 8.9 ± 2.7 |
| RBC ($10^6/\mu L$) | NS | NS | NS | NS | NS | 6.63 ± 1.38 | 6.38 ± 1.38 | 6.73 ± 1.06 | 7.30 ± 1.52 | 7.49* ± 1.36 |
| Haemoglobin (g/dL) | NS | NS | NS | NS | NS | 13.2 ± 2.4 | 12.8 ± 2.6 | 12.9 ± 1.8 | 14.0 ± 3.0 | 14.9* ± 2.9 |
| Haematocrit (%) | NS | NS | NS | NS | NS | 37.5 ± 6.2 | 36.2 ± 6.7 | 37.0 ± 4.9 | 39.9 ± 8.4 | 42.1* ± 7.5 |

*N*: Number of animals in the group; WBC: White blood cells; Source: Stebbings et al., 2016

MCHC: Mean corpuscular haemoglobin concentration; RBC: Red blood cells; NS: Not significant, so not reported;

[a] Tricyclazole diet for 3 months, then control for 21 months;

[b] Statistics as described in the original report:
significantly different from control using Dunnett's t, two-tailed test at: ^ $p \leq 0.1$; ^^ $p \leq 0.05$;

* Statistically different from control mean by Dunnett's test, alpha = 0.05;

§ Statistically different from control mean by Wilcoxon's test, alpha = 0.05;

[Percentage of change were calculated from the concurrent control values and are shown if the difference is 5% or greater]

***Table 8g. Key findings in the duplicated two-year combined toxicity and carcinogenicity dietary studies in rats; statistically identified clinical chemistry parameters (Stebbins et al., 2016)***

| | Dietary dose (ppm)/sex | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Males | | | | | Females | | | | |
| | 0 | 100 | 275 | 620 | 1600 [a] | 0 | 100 | 275 | 620 | 1600 [a] |
| **Study 1 data (R-764)[b] ; mean ± standard deviation** | | | | | | | | | | |
| *N* | 11 | 7 | 7 | 5 | 8 | 10 | 8 | 8 | 6 | 8 |
| Glucose (mg/dL) | NS | NS | NS | NS | NS | 57.2 ±12.6 | 64.4 ± 17.4 | 77.8 ± 11.4^^ | 86.7 ± 19.1^^^ | 67.5 ± 22.3 |
| **Study 2 data (R-774)[b] ; mean ± standard deviation** | | | | | | | | | | |
| *N* | 8 | 3 | 7 | 6 | 7 | 9 | 6 | 7 | 6 | 6 |
| Creatinine (mg/dL) | NS | NS | NS | NS | NS | 1 ± 0.12 | **1.28** ± 0.27^^^ | 1.09 ± 0.12 | 1.03 ± 0.15 | 1.13 ± 0.10 |
| Total bilirubin (mg/dL) | 0.260 ± 0.051 | 0.343 ± 0.060 | 0.307 ± 0.079 | **0.340** ± 0.079^ | 0.241 ± 0.036 | NS | NS | NS | NS | NS |
| **Combined study data; mean ± standard deviation [percentage change compared to controls]** | | | | | | | | | | |
| *N* | 19 | 10 | 14 | 11 | 15 | 19 | 14 | 15 | 12 | 14 |
| Glucose (mg/dL) | NS | NS | NS | NS | NS | 57 ± 19 | 74 ± 24 | 80* ± 23 [–40.4%] | 81* ± 18 [–42.1%] | 74 ± 21 |

*N*: Number of animals in the group; NS: Not significant, so not reported; Source: Stebbings et al., 2016

[a] Tricyclazole diet for 3 months, then control for 21 months;

[b] Statistics as described in the original report:
significantly different from control using Dunnett's t, two-tailed test at: ^ $p \leq 0.1$; ^^ $p \leq 0.05$; ^^^ $p \leq 0.01$;

* Revised report; statistically different from control mean by Dunnett's test, alpha = 0.05;

***Table 8h. Key findings in the duplicated two-year combined toxicity and carcinogenicity dietary studies in rats; summary of final body and organ weights***

| | | Dietary dose (ppm)/sex | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | | Females | | | | |
| | | 0 | 100 | 275 | 620 | 1600 [a] | 0 | 100 | 275 | 620 | 1600 [a] |
| **Study 1 data (R-764)[b] ; mean ± standard deviation** | | | | | | | | | | | |
| Brain | Abs (g) | - | - | - | - | - | 2.01 ± 0.08 | 1.95 ± 0.07 | 1.95 ± 0.10 | 1.99 ± 0.11 | *1.93* ±0.1 ^^ |
| | Rel (%) | 0.41 ± 0.07 | 0.40 ± 0.04 | 0.42 ± 0.07 | *0.49* ±0.08^^ | 0.40 ± 0.06 | - | - | - | - | - |
| Liver | Rel (%) | 2.41 ± 0.41 | 2.60 ± 0.51 | 3.16 ± 1.73 | **3.27** ± 0.72^^ | 2.33 ± 0.46 | 2.65 ± 0.46 | 2.86 ± 0.36 | 3.01 ±0.42 | **3.42** ± 0.97^^^ | 2.65 ± 0.40 |
| **Study 2 data (R-774)[b] ; mean ± standard deviation** | | | | | | | | | | | |
| Brain | Abs (g) | 2.26 ±0.13 | 2.16 ± 0.13 | 2.17 ± 0.13 | 2.15 ± 0.15 | **2.10** ± 0.15^^ | - | - | - | - | - |
| Liver | Rel (%) | 2.32 ± 0.30 | 2.74 ± 0.66 | 2.52 ± 0.38 | **2.93** ± 0.40^^^ | 2.24 ± 0.28 | 2.63 ± 0.39 | 2.78 ± 0.55 | 2.62 ± 0.46 | **3.11** ± 0.59^^ | 2.46 ± 0.28 |
| Prostate | Rel (%) | 0.11 ± 0.05 | 0.14 ± 0.04 | 0.13 ± 0.05 | 0.21 ± 0.19^^ | 0.12 ± 0.03 | NA | NA | NA | NA | NA |
| Testis | Rel (%) | 0.55 ± 0.12 | 0.57 ± 0.15 | 0.57 ± 0.09 | 0.66 ± 0.10^^ | 0.62 ± 0.07 | NA | NA | NA | NA | NA |
| **Combined study data; mean ± standard deviation [percentage change compared to controls]** | | | | | | | | | | | |
| Final body weight (g) | | 572 ± 113 | 552 ± 87 [–3%] | 561 ± 96 [–2%] | **483* ± 72 [–16%]** | 552 ± 100 [–3%] | 418 ± 86 | 416 ± 118 0% | 400 ± 94 [–4%] | **360 ± 68 [–14%]** | 372 ± 70 [–11%] |
| Brain | Abs (g) | 2.25 ± 0.11 | 2.18 ± 0.15 [–3%] | 2.18 ± 0.12 [–3%] | 2.18 ± 0.13 [–3%] | 2.13* ± 0.13 [–5%] | - | - | - | - | - |
| | Rel (%) | 0.41 ± 0.08 | 0.40 ± 0.05 [–2%] | 0.40 ± 0.07 [–2%] | 0.46* ± 0.07 [+12%] | 0.40 ± 0.06 [–2%] | - | - | - | - | - |
| Liver | Rel (%) | 2.37 ± 0.36 | 2.67 ± 0.59 [+13%] | **2.81[$] ± 1.22 [+19%]** | **3.09[$] ± 0.58 [+30%]** | 2.29 ± 0.39 [–3%] | 2.64 ± 0.42 | 2.82 ± 0.44 [+7%] | 2.83 ± 0.47 [+7%] | **3.27[$] ± 0.80 [+24%]** | 2.57 ± 0.35 [–3%] |
| Heart | Rel (%) | 0.33 ± 0.06 | 0.36 ± 0.08 [+9% | 0.34 ± 0.06 [+3%] | 0.38* ± 0.07 [+15%] | 0.32 ± 0.04 [–3%] | - | - | - | - | - |
| Kidney | Abs (g) | - | - | - | - | - | 2.73 ± 0.55 | 2.81 ± 0.65 [+3%] | 2.74 ± 0.50 | 2.86 ± 1.03 [+5%] | 2.40[$] ± 0.47 [–12%] |
| Thyroid | Rel (%) | - | - | - | - | - | 8.17 ± 2.37 | 9.04 ± 4.66 [+11%] | 17.20 ± 48.43 [+111%] | 10.61[$] ± 4.61 [+30%] | 10.32 ± 3.71 [+26%] |
| Prostate | Rel (%) | 0.11 ± 0.04 | 0.14[§] ± 0.04 [+27%] | 0.14 ± 0.08 [+27%] | 0.16 ± 0.15 [+45%] | 0.12 ± 0.03 [+9%] | N/A | N/A | N/A | N/A | N/A |
| Testis | Rel (%) | 0.54 ± 0.12 | 0.59 ± 0.12 [+9%] | 0.57 ± 0.09 [+6%] | 0.63* ± 0.14 [+17%] | 0.60 ± 0.07 [+11%] | N/A | N/A | N/A | N/A | N/A |

Abs: Absolute weight; Rel: Organ weight as a percentage relative to body weight; Source: Stebbings et al., 2016

NS: Not significant, so not reported;

[a] Tricyclazole diet for 3 months, then control for 21 months;

[b] Statistics as described in the original report:
significantly different from control using Dunnett's t, two-tailed test at: ^ $p \leq 0.1$; ^^ $p \leq 0.05$ ^^^ $p \leq 0.01$;

* Statistically different from control mean by Dunnett's test, alpha = 0.05;

§ Statistically different from control mean by Wilcoxon's test, alpha = 0.05;

[Percentage of change were calculated from the concurrent control values and are shown if the difference is 5% or greater]

***Table 8i. Key findings in the duplicated two-year combined toxicity and carcinogenicity dietary studies in rats; statistically identified histopathology findings***

| | | Dietary dose (ppm)/sex | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Males | | | | | Females | | | | |
| | | 0 | 100 | 275 | 620 | 1600 [a] | 0 | 100 | 275 | 620 | 1600 [a] |
| **Study 2 data (R-774)[b] [percentage of examined rats affected]** | | | | | | | | | | | |
| Total number of rats examined | | 60 | 40 | 40 | 40 | 40 | 60 | 40 | 40 | 40 | 40 |
| Non-neoplastic lesions | Lungs: bronchiectasis and purulent (chronic) bronchitis | 23 [38%] | 12 [30%] | 19 [47%] | 25* [63%] | 22 [55%] | NS | NS | NS | NS | NS |
| | Kidneys: progressive glomerulonephrosis | NS | NS | NS | NS | NS | 14 | 18* | 10 | 16 | 5 |
| Neoplastic lesions | Mammary glands: fibroadenoma | NA | NA | NA | NA | NA | 28 | 9* | 8* | 5* | 18 |
| | Mammary glands: adenocarcinoma | NA | NA | NA | NA | NA | 9 | 4 | 2 | 0* | 0* |
| **Combined study data, [percentage of examined rats affected]** | | | | | | | | | | | |
| Total number of rats examined | | 120 | 80 | 80 | 80 | 80 | 120 | 80 | 80 | 80 | 80 |
| Non-neoplastic lesions | Lungs: bronchiectasis and purulent (chronic) bronchitis (males) | 47 [39%] | 29 [36%] | 37 [46%] | 47* [58%] | 41 [51%] | NS | NS | NS | NS | NS |
| | Skin and subcutaneous tissue: ulceration of heel(s) (males) | 39 | 23 | 22 | 12* | 16 | NS | NS | NS | NS | NS |
| Neoplastic lesions | Thyroid: parafollicular (light cell) cell adenoma (males) | 11 | 1* | 4 | 1* | 1* | NS | NS | NS | NS | NS |
| | Mammary glands: fibroadenoma (females) | N/A | N/A | N/A | N/A | N/A | 50 | 19* | 15* | 13* | 26 |
| | Mammary glands: adenocarcinoma (females) | N/A | N/A | N/A | N/A | N/A | 13 | 6 | 6 | 2* | 1 |

[a] Tricyclazole diet for 3 months, then control for 21 months; Source: Stebbings et al., 2016

NS: Not significant, so not reported;

* Statistically different from control value by Yates' Chi-square test, alpha = 0.05, two-sided

***Table 9. Consideration of whether 620 ppm achieves maximum tolerated dose models, compared with a number of models***

| Parameter | Males | Females | OECD, 2012 MTD criteria | EPA, 2005 MTD criteria | JMPR, 2015 Adversity criteria |
|---|---|---|---|---|---|
| Body weight | –16% | –14% | –10% or more | –10% or more | Outside ± 10% |
| Relative liver weight | +30% | +24% | - | +25% or more | Outside ± 15% |

MTD: Maximum tolerated dose;

Further to the Stebbins 2016 revision, a further innovative statistical approach was used which included a statistical assessment of whether the two replicate studies from the Howard, 1977 pre-guideline two-year dietary study of tricyclazole could be combined. In addition, an analysis of tumour incidence,

normalized for individual mortality (using poly-3 estimators), and a mixed-effect model for combining results from the replicate studies was investigated. Following these revised statistics, tricyclazole clearly showed no carcinogenic potential for individual or combined replicates and the original NOAEL of 275 ppm (equal to 11 mg/kg bw per day) was supported.

In a modern, guideline study tricyclazole was administered in the diet at dietary levels of 0, 40, 126 or 400 ppm (equal to 0, 1.8, 5.9 and 18 mg/kg bw per day for males, 0, 2.3, 7.1 and 22 mg/kg bw per day for females) to groups of 60 male and 60 female Wistar rats. A sentinel group of eight rats of each sex was also maintained to allow screening for a variety of infections which may affect the health of test animals: the sentinel animals received untreated diet. Ten animals of each sex per group were sacrificed after one year of treatment, with the remaining 50 rats of each sex per group maintained for assessment after up to two years of treatment.

Animals were observed twice daily for morbidity, mortality and injury. Detailed clinical observations were made weekly during the study. Body weights and food consumption were measured three times during week 1, weekly from weeks 2 to 13 and every four weeks thereafter. Ophthalmoscopic examinations were conducted on all animals pretest and on all surviving animals prior to the chronic-phase necropsy. Clinical pathology evaluations were conducted on all chronic-phase animals at 3, 6 and 12 months and on all surviving animals at 18 and 24 months. Blood samples were collected from 20 animals of each sex per group on days 15, 90, 91 and 187 for possible future hormone analysis. Postmortem examinations were performed on treated animals euthanized in extremis, animals found dead, and all surviving treated animals at 12 or 24 month scheduled necropsies. Sentinel animals were assessed weekly for body weight and food consumption for animal welfare purposes. Blood samples were collected from sentinel animals pretest and at 6, 12 and 18 months to detect the presence of pathogens. As the health surveillance reports were negative. they were not reported but records were maintained in the study file.

Mortalities observed during the study were infrequent, and were not dose-related. A sufficient number of animals survived until terminal sacrifice. There were no treatment-related clinical signs of toxicity, nor treatment-related abnormalities detected on ophthalmoscopic examination. It was noted that multiple animals across dose groups presented in bilateral retinal degeneration at week 52. Significant treatment-related decreases in body weight were observed in females at 400 ppm from day 343 (by up to 7.2%, $p < 0.01$), and in males at 400 ppm from day 539 (by up to 8.2%, $p < 0.01$). Body weight gain was affected in both males and females at 400 ppm, with maximum depressions compared to controls of 13.7% in males and 14.4% in females. Food consumption was not affected in males, but was statistically lower than control values in females at 400 ppm.

In clinical chemistry evaluations slight increases in triglyceride and cholesterol levels were observed in females at all dose levels from three months onwards, and cholesterol levels in males at 126 and 400 ppm. No treatment-related effects were observed on haematological parameters, coagulation or in urinalysis results.

At interim sacrifice, treatment-related differences were observed in liver weight at 400 ppm; relative weight increased by 13.9% and 14.4% in males and females respectively. There was a very slight increase in the incidence of bile duct hyperplasia in both males and females at 400 ppm. At the end of the study a slight increase in the incidence of lipomas was observed in males at 400 ppm, however this was consistent with historical control data, and the lipomas were not considered related to treatment.

Based on the slight suppression of body weight gain and food consumption in females, and slight decrease in body weight gain in males at 400 ppm, a NOAEL was identified at 126 ppm (equal to ca 5.9 mg/kg bw per day for males, 7.1 mg/kg bw per day for females). Tricyclazole was not considered to be carcinogenic under the condition of this study (Heward, 2020).

*Table 10a. Key findings of the two-year dietary study in rats; mortality*

| | Dietary dose (ppm)/sex | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Parameter** | **0** | **40** | **126** | **400** | **0** | **40** | **126** | **400** |
| Rats in chronic sub-study | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Mortality at one-year sacrifice (day 365) | 2 | 0 | 3 | 1 | 2 | 2 | 0 | 0 |
| Rats in carcinogenicity sub-study | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| Rats surviving to final sacrifice [survival as percentage of sub-group] | 39 [78%] | 37 [74%] | 33 [66%] | 35 [70%] | 31 [62%] | 28 [56%] | 29 [58%] | 33 [66%] |

Source: Heward, 2020

*Table 10b. Key findings of the two-year dietary study in rats; body weight and body weight change*

| | Dietary dose (ppm)/sex | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Test day** | **0** | **40** | **126** | **400** | **0** | **40** | **126** | **400** |
| | **Body weight; mean ± standard deviation (g) [% change compared to controls]** | | | | | | | |
| 91 | 415.0 ± 33.07 | 414.2 ± 28.08 | 412.5 ± 28.16 | 408.0 ± 31.89 [–0.6%] | 234.8 ± 13.70 | 230.4 ± 14.90 | 231.8 ± 14.84 | 228.4 ± 12.8 [–2.7%] |
| 175 | 466.6 ± 38.31 | 465.6 ± 33.27 | 462.1 ± 36.29 | 459.1 ± 38.40 [–1.6%] | 253.2 ± 13.47 | 249.5 ± 15.31 | 252.2 ± 16.26 | 246.6 ± 14.46 [–2.6%] |
| 343 | 524.3 ± 50.91 | 526.5 ± 42.04 | 526.8 ± 45.41 | 518.7 ± 49.62 [+0.5%] | 285.0 ± 25.57 | 281.1 ± 23.63 | 283.2 ± 24.44 | 270.4 ± 22.92[a] [–5.1%] |
| 539 | 584.2 ± 68.05 | 576.7 ± 57.91 | 581.3 ± 56.24 | 564.6 ± 64.20 [–3.4%] | 311.7 ± 34.25 | 305.4 ± 29.10 | 314.6 ± 38.22 | 293.8 ± 33.09[b] [–5.7%] |
| 707 | 608.8 ± 69.85 | 604.8 ± 64.09 | 601.2 ± 61.82 | 559.1 ± 64.21[a] [–8.2%] | 338.2 ± 36.45 | 324.3 ± 35.42 | 340.4 ± 48.81 | 314.0 ± 37.39[c] [–7.2%] |
| | **Body weight change ; mean ± standard deviation (g) [% change compared to controls]** | | | | | | | |
| 1–91 | 154.5 ± 25.99 | 153.3 ± 21.58 | 152.8 ± 20.97 | 147.2 ± 24.13 [–4.7%] | 59.2 ± 10.81 | 55.6 ± 11.34 | 56.4 ± 11.26 | 54.0 ± 10.41 [–8.8%] |
| 1–175 | 206.1 ± 31.45 | 204.8 ± 25.93 | 202.5 ± 28.77 | 198.3 ± 30.52 [–3.8%] | 77.6 ± 11.21 | 74.7 ± 12.79 | 76.8 ± 12.92 | 72.1 ± 12.68 [–7.1%] |
| 1–343 | 263.9 ± 44.43 | 265.6 ± 36.50 | 267.2 ± 38.26 | 257.7 ± 42.89 [–2.3%] | 109.5 ± 24.77 | 106.2 ± 21.47 | 107.8 ± 21.90 | 95.9 ± 21.74[d] [–12.4%] |
| 1–539 | 323.9 ± 61.38 | 317.6 ± 52.95 | 322.3 ± 51.90 | 304.0 ± 57.73 [–6.1%] | 135.6 ± 33.08 | 130.5 ± 26.41 | 140.3 ± 35.39 | 119.2 ± 31.40[e] [–12.1%] |
| 1–707 | 348.4 ± 62.87 | 345.1 ± 59.08 | 344.0 ± 56.34 | 300.8 ± 60.58[a] [–13.7%] | 161.7 ± 34.22 | 149.3 ± 33.12 | 166.6 ± 47.36 | 138.4 ± 36.09[e] [–14.4%] |

Statistical significance: Source: Heward, 2020

[a] $p < 0.01$ using Anova & Dunnett's, log; [b] $p < 0.05$ using Kruskal-Wallis & Dunn; [c] $p < 0.01$ using Anova & Dunnett's; [d] $p < 0.01$ using Kruskal-Wallis & Dunn; [e] $p < 0.05$ using Anova & Dunnett's, log;

Body weight data from pages 84–85, 89–93; body weight change data from pages 97–102, 105–110 of Heward, 2020

*Table 10c. Key findings of the two-year dietary study in rats; food consumption in females*

| Time period | Dose level (ppm) | | | |
|---|---|---|---|---|
| | 0 | 40 | 126 | 400 |
| | Food consumption; mean ± standard deviation (g/animal per day) | | | |
| Days 1–4 | 13.6 ± 1.16 | 13.2 ± 1.40 | 13.4 ± 1.60 | 11.1 ± 6.48 [a] |
| Days 4–7 | 13.8 ± 1.70 | 13.8 ± 1.67 | 13.6 ± 1.60 | 13.0 ± 1.52 [b] |
| Days 21–28 | 15.3 ± 2.22 | 15.0 ± 1.34 | 14.6 ± 3.00 | 14.3 ± 1.27 [a] |
| Day s84–91 | 13.0 ± 1.95 | 12.8 ± 1.15 | 11.6 ± 3.82 [c] | 12.0 ± 1.34 [a] |
| Days 336–343 | 13.4 ± 1.94 | 13.3 ± 2.52 | 13.5 ± 1.92 | 12.6 ± 1.41 [c] |
| Days 532–539 | 14.5 ± 2.02 | 14.2 ± 2.11 | 14.3 ± 1.84 | 13.6 ± 1.85 |
| Days 700–707 | 14.0 ± 2.03 | 13.6 ± 2.67 | 13.3 ± 2.47 | 13.2 ±1.50 |

Statistical significance: Data taken from pages 122–131 of Heward, 2020

[a] $p < 0.01$ using Kruskal-Wallis & Dunn;

[b] $p < 0.05$ using Anova & Dunnett's;

[c] $p < 0.05$ using Kruskal-Wallis & Dunn,

**Table 10d.** *Key findings of the two-year dietary study in rats; clinical chemistry findings*

| | Dietary dose (ppm)/sex | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| Time point | 0 | 40 | 126 | 400 | 0 | 40 | 126 | 400 |
| | Triglycerides (mg/dL); mean ± standard deviation (g) [% change compared to controls] | | | | | | | |
| Month 3 | 39.6 ± 10.28 | 49.2 ± 19.58 [+24.2%] | 36.6 ± 11.49 [–7.6%] | 50.4 ± 17/58 [+27.3%] | 26.1 ± 3.87 | 29.2 ± 4.59 [+11.9%] | **32.2** [a] **± 4.40** [+23.4%] | **38.3** [b] **± 6.89** [+46.7%] |
| Month 6 | 80.0 ± 25.67 | 79.2 ± 16.77 [–1.0%] | 67.1 ± 24.48 [–16.1%] | 73.3 ± 14.10 [–8.4%] | 33.9 ± 6.43 | 39.6 ± 11.20 [+16.8%] | 44.8 ± 22.32 [+32.2%] | 39.6 ± 6.66 [+16.8%] |
| Month 12 | 168.2 ± 94.96 | 133.4 ± 59.16 [–20.7%] | 159.4 ± 129.49 [–5.2%] | 145.9 ± 55.36 [–13.3%] | 61.7 ± 19.22 | 79.0 ± 37.34 [+28.0%] | 85.3 ± 34.79 [+38.2%] | 68.0 ± 16.11 [+10.2%] |
| Month 18 | 167.1 ± 49.08 | 183.7 ± 36.08 [+9.9%] | 186.6 ± 51.43 [+11.7%] | 176.7 ± 61.04 [+5.7%] | 87.6 ± 42.93 | 122.4 ± 57.73 [+39.7%] | 111.8 ± 48.24 [+27.6%] | 94.4 ± 46.34 [+7.8%] |
| Month 24 | 177.3 ± 52.64 | 140.6 ± 38.72 [–20.7%] | 207.1 ± 80.86 [+16.8%] | 164.9 ± 54.92 [–7.0%] | 92.9 ± 41.69 | 127.4 ± 81.17 [+37.1%] | 89.5 ± 31.87 [–3.7%] | 133.7 ± 81.16 [+43.9%] |
| | Cholesterol (mg/dL); mean ± standard deviation (g) [% change compared to controls] | | | | | | | |
| Month 3 | 78.3 ± 9.03 | 72.9 ± 7.36 [–6.9%] | 66.9 ± 15.55 [–14.6%] | 86.6 ± 17.31 [+10.6%] | 49.8 ± 18.07 | 51.1 ± 16.58 [+2.6%] | 62.1 ± 9.88 [+24.7%] | **73.9** [a] **± 15.83** [+48.4%] |
| Month 6 | 80.0 ± 13.56 | 76.1 ± 9.82 [–4.9%] | 72.2 ± 19.19 [–9.8%] | 94.3 ± 17.24 [+17.9%] | 52.1 ± 15.82 | 66.5 ± 28.59 [+27.6%] | 68.5 ± 18.67 [+31.5%] | 80.1 ± 17.98 [–53.7%] |
| Month 12 | 94.7 ± 16.28 | 80.9 ± 14.21 [–14.6%] | 81.7 ± 21.85 [–13.7%] | 106.9 ± 16.93 [+12.9%] | 61.2 ± 15.99 | 70.0 ± 20.59 [+14.4%] | 76.4 ± 22.32 [+24.8%] | 74.3 ± 24.66 [+21.4%] |
| Month 18 | 104.9 ± 25.61 | 109.3 ± 19.08 [+4.2%] | 123.9 ± 9.31 [+18.1%] | 124.7 ± 28.33 [+18.9%] | 82.3 ± 10.87 | 69.2 ± 8.14 [–14.6%] | 85.7 ± 19.86 [+24.8%] | **106.8** [a] **± 25.45** [+29.8%] |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Month 24 | 112.1<br>± 25.72 | 102.5<br>± 29.28<br>[–8.6%] | 142.6<br>± 34.11<br>[+27.2%] | 138.5<br>± 35.42<br>[+23.6%] | 94.8<br>± 26.60 | 83.5<br>± 20.03<br>[–11.9%] | 94.5<br>± 24.81<br>[–0.3%] | 111.8<br>± 19.90<br>[+17.9%] |

Data taken from pages 29, 1458, 1459 and 1471–1473 of Heward, 2020

[a] Significantly different from control at $p < 0.05$

[b] Significantly different from control at $p < 0.01$

**Bold** values indicate effects interpreted to be treatment-related.

***Table 10e. Key findings of the two-year dietary study in rats; body weight and test article-related changes in organ weights***

| | Dietary dose (ppm)/sex | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Parameter** | **0** | **40** | **126** | **400** | **0** | **40** | **126** | **400** |
| *N* | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| | **Mean ± standard deviation (g) [% change compared to controls]** | | | | | | | |
| Mean body weight (g) | 516.0<br>± 50.12 | 529.5<br>± 20.65<br>[+2.6%] | 527.0<br>± 56.58<br>[+2.1%] | 502.4<br>± 39.80<br>[–2.6%] | 279.3<br>± 22.16 | 271.6<br>± 23.99<br>[–2.8%] | 269.4<br>± 27.31<br>[–3.5%] | 259.7<br>± 13.57<br>[–7.0%] |
| Liver weight: absolute (g) | 15.5643<br>± 2.23452 | 14.5533<br>± 1.00493<br>[–6.5%] | 15.7591<br>± 2.22794<br>[+1.3%] | 17.2939<br>± 2.00654<br>[+11.1%] | 7.7732<br>± 0.54658 | 7.2773<br>± 0.78716<br>[–6.4%] | 7.9680<br>± 0.83525<br>[+2.5%] | 8.2800<br>± 0.80950<br>[+6.5%] |
| Liver weight: relative to body weight (%) | 3.01711<br>± 0.341245 | 2.75069<br>± 0.189403<br>[–8.8%] | 2.99190<br>± 0.305793<br>[–0.8%] | 3.43573<br>± 0.185501[a]<br>[+13.9%] | 2.78871<br>± 0.155773 | 2.68548<br>± 0.245736<br>[–3.7%] | 2.96873<br>± 0.287920<br>[+6.5%] | 3.19022<br>± 0.288356[a]<br>[+ 14.4%] |
| Liver weight: relative to brain weight (%) | 7.61713<br>± 1.428722 | 6.93407<br>± 0.618008<br>[–9.0%] | 7.57114<br>± 0.955308<br>[–0.6%] | 8.33032<br>± 0.614638<br>[+9.4%] | 4.09475<br>± 0.356549 | 3.84007<br>± 0.409125<br>[–6.2%] | 4.31467<br>± 0.478658<br>[+5.4%] | 4.38966<br>± 0.423216<br>[+7.2%] |

*N*: Number of rats in group; Source: Heward, 2020

[a] Significantly different from controls at $p < 0.01$

***Table 10f. Key findings of the two-year dietary study in rats; summary of tumour incidence in males and relevant historical control data***

| | Dietary dose (ppm) | | | | |
|---|---|---|---|---|---|
| | **0** | **40** | **126** | **400** | **Historical control data range** |
| Skin/skin, subcutis | | | | | |
| Lipoma[a] | 0/50<br>[0%] | 0/50<br>[0%] | 1/50<br>[2%] | 2/50*<br>[4%] | 0%[b]<br>0%–4%[c, d, e] |

[a] Includes morphological variant fibrolipoma;

[b] Heward, 2020; this contains details of historical control data supplied by CRL Mattawan, for Wistar Han rats (provided by CRL/Harlan-Envigo, 2-year studies, 2009–2019, 1.0)

[c] Poteracki & Walsh, 1998;

[d] Carlus et al., 2013;

[e] Walsh & Poteracki, 1994

## 2.4 Genotoxicity

### (a) In vitro and in vivo studies

Based on the results of the available genetic toxicology studies (Table 11) there is strong evidence that tricyclazole does not have potential for mutagenicity, clastogenicity and/or aneugenicity in humans.

***Table 11. Results of genotoxicity studies performed with tricyclazole***

| Type of study | Organism/cell | Dose range tested | Purity | Result | Reference |
|---|---|---|---|---|---|
| ***In vitro* genotoxicity tests** | | | | | |
| In vitro bacterial reverse mutation | *Escherichia coli* WP2 hcr$^-$ and *Salmonella typhimurium* strains TA1535, TA1537, TA1538, TA98 and TA100 | 10, 50, 100, 500, 1000 and 5000 µg/plate (± S9) | 99.4% | Negative [a] | Shirasu et al., 1978 |
| DNA damage repair | *Bacillus subtilis* H17 rec$^+$, M45 rec$^-$ | 20, 100, 200, 500, 1000 and 2000 µg/disk | 99.4% | Negative [b] | Shirasu, Moritani & Sugiyama, 1978 |
| In vitro bacterial reverse mutation (gradient plate assay) | *S. typhimurium* G46, TA1535, TA100, C3076, TA1537, D3052, TA1538, TA98 and *E. coli* WP2, WP2*uvr*A$^-$ | Four plates with gradient concentrations of 1000, 100, 10, 1 and 0.1 µg/mL (± S9) | 96.6% | Negative [b] | Thompson, 1981 |
| In vitro bacterial reverse mutation | *S. typhimurium* TA1537, TA1535, TA98, TA100 and TA102 | Trial I: tricyclazole technical 39.06, 78.13, 156.25, 312.5, 625 and 1250 µg/plate (± S9 mix 5% v/v)<br>Trial II: tricyclazole technical 12.8, 32, 80, 200, 500 and 1250 µg/plate (± S9 mix 10% v/v) | 98.8% | Negative [c] | Shukla, 2011 |
| In vitro bacterial reverse mutation | *S. typhimurium* TA1537, TA98, TA100 TA102 and TA1535, | Trial I: tricyclazole TGAI 31.25, 62.5, 125, 250, 500 and 1000 µg/plate (± S9 mix 5% v/v)<br>Trial II: tricyclazole TGAI 10.24, 25.6, 64, 160, 400 and 1000 µg/plate (± S9 mix 10% v/v) | 99.3% | Negative [c] | Patel, 2012 |
| In vitro bacterial mutation assay with Prival modification | *S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2 *uvr*A | 8 concentrations (2 plates/concentration), 1.50–5000 µg/plate, without or with either the rat S9 (oxidizing) or hamster S9 (reducing) metabolic activation system: | 96.9% | Negative [c] in both standard and Prival modification assays | Dakoulas, 2018 |
| Mammalian gene mutation assay (TK$^{+/-}$ locus) | Mouse lymphoma L5178Y TK$^{+/-}$ 3.7.2 cell line | Assay 1 (–S9): 2.1, 4.2, 8.3, 17, 33, 67, 95, 136, 194, 280, 400, 570 and 810 µg/mL<br>Assay 1 (+S9): 2.1, 4.2, 8.3, 17, 33, 67, 95, 136, 194, 280, 400, 570 and 810 µg/mL<br>Assay 2 (–S9): 6.4, 10, 15, 23, 36, 55, 85, 131, 164, 205, 256, 320 and 400 µg/mL<br>Assay 2 (+S9): S9: 0.15, 0.29, 0.59, 1.2, 2.3, 4.7, 9.4, 19, 38, 75, 150, 300 and 600 µg/mL | 95.2% | Positive [d] | Steenwinkel, 2003 |

| Type of study | Organism/cell | Dose range tested | Purity | Result | Reference |
|---|---|---|---|---|---|
| Mammalian gene mutation assay ($TK^{+/-}$ locus) | Mouse lymphoma L5178Y $TK^{+/-}$ 3.7.2C cell line | Assay 1 (–S9): 6.4, 10, 15, 23, 36, 55, 85, 131, 164, 205, 256, 320 and 400 µg/mL<br>Assay 1 (+S9): 0.15, 0.29, 0.59, 1.2, 2.3, 4.7, 9.4, 19, 38, 75, 150, 300 and 600 µg/mL | 99.3% | Positive [d] | Steenwinkel, 2004 |
| In vitro cytotoxicity assay | Mouse lymphoma L5178Y $TK^{+/-}$ and $TK^{-/-}$ 3.7.2C cell lines | 50, 100, 200, 400 and 800 µg/mL (–S9) | 99.3% | Not selectively cytotoxic [e] | Seidel et al., 2004a |
| Mammalian gene mutation assay (HGPRT locus) | Chinese Hamster Ovary CHO-K1-BH4 cell line | Assay 1 (–S9): 50, 100, 300, 500, 700 and 900 µg/mL<br>Assay 1 (+S9): 2.5, 5, 10, 20, 30, 40, 50, 80 and 100 µg/mL<br>Assay 2 (–S9): 100, 300, 500, 700 and 900 µg/mL<br>Assay 2 (+S9): 2.5, 5, 10, 20, 30, 40, 50, 80 and 100 µg/mL | 99.3% | Negative | Seidel et al., 2004b |
| DNA repair in mammalian cells (UDS) | Primary Fisher 344 male rat hepatocytes | 0.5, 1, 5, 10, 50, 100, 500 and 1000 nmol/mL | 96.3% | Negative [f] | Probst & Hill, 1981 |
| Induction of micronuclei | Human peripheral lymphocytes | Short treatment (4 h) without metabolic activation: 125, 250, 500, 750 and 1000 µg/mL<br>Short treatment (4 h) with metabolic activation: 125, 250, 500, 750 and 1000 µg/mL<br>Extended treatment (24 h) without metabolic activation: 125, 250, and 500 µg/mL | 97.4% | Negative [g] | Nagane, 2016 |
| **In vivo genotoxicity tests:** | | | | | |
| **Somatic cells** | | | | | |
| Sister chromatid exchange (SCE) | Female Chinese hamster | Doses administered: 21.25, 42.5, 85 and 170 mg/kg bw | 96.3% | Negative [h] | Neal, 1981 |
| Micronucleus induction | Mouse bone marrow polychromatic erythrocytes (CD-1 males and females) | Doses administered: 100, 200 and 300 mg/kg bw (equivalent to 12.5, 25 and 50% of the $LD_{50}$) | 96.3% | Negative [i] | Kehr, Parton & Garriott, 1988 |
| In vivo UDS | Fischer 344 rats hepatocytes (from males and females) | Rangefinder doses: 100, 250, 500 and 1000 mg/kg bw<br>Main doses: 100 or 200 mg/kg bw | 99.3% | Negative [j] | Cifone, 2004 |
| In vivo transgenic rodent assay – liver, glandular stomach, kidney | Muta™ Mouse | 200, 670 and 2000 ppm (corresponding to 28.6, 97.9 and 282.2 mg/kg bw/day) | 98.3% | Negative [k] | Masumori, 2017 |
| **Germ cells** | | | | | |
| In vivo dominant lethal in germ cells | Wistar-derived rats | Doses administered: 60 mg/kg bw | 99.4% | Negative [l] | Worth et al., 1977b |

[a] Poor reporting; nonstandard tester strains employed (TA1538 and E. coli WP2 *hcr*).

[b] Test currently considered obsolete.

[c] Study compliant with the current OECD TG 471.

[d] Study compliant with the current OECD TG 490.

[e] This study is not a genotoxicity study. It was conducted to determine whether a previously observed positive mutagenicity finding with tricyclazole in mouse lymphoma cells was an artifact of preferential cytotoxicity to $TK^{+/-}$ cells compared to $TK^{-/-}$ cells.

[f] The study was checked for compliance with the former OECD TG 482 which is not valid anymore. Deleted on 2 April 2014.

[g] Study compliant with the current OECD TG 487.

[h] This test does not measure mutations and although useful for research purposes, it is currently not included in the test batteries for risk assessment. It is presented here as supplementary information only.

[i] No shift in the ratios of polychromatic erythrocytes to normochromic erythrocytes (PCE:NCE ratios) in the tricyclazole-treated animal groups compared with the concurrent untreated controls and no indication of clinical signs post-dosing. Proof of bone marrow exposure was separately conducted (McCoy & Clark, 2014).

[j] According to EFSA (2017), OECD TG 486 is no longer valid as a follow-up for positive mutagenicity tests. The present study serves as supplementary information only.

[k] Study compliant with the OECD TG 488 (adopted 26 July 2013). The study can be considered an adequate in vivo follow-up study for induction of gene mutation in vitro.

[l] Deviations from the current OECD TG 478 include the number of animals used: 10 females/mating interval/dose (instead of at least 30–50); positive controls results are not shown in table The assay is considered to be of low sensitivity since detection of small increases in the frequency of mutations is generally limited because of the high spontaneous frequency of dominant lethal mutations.

In a battery of in vitro assays, tricyclazole did not induce gene mutations in bacteria (Ames test, including one with Prival modification) or at the *hprt* (hypoxanthine–guanine phosphoribosyl transferase) locus of Chinese hamster ovary of (CHO) cells. Tricyclazole induced a mutagenic response at the *Tk* locus of mouse lymphoma L5178Ycells in a mouse lymphoma assay (MLA), and this response occurred equally among small and large colony phenotypes. The possibility that this was due to the selection of pre-existing mutants, leading to a false-positive response in the MLA, was ruled out in follow-up experiments. Tricyclazole was also negative for the induction of micronuclei in human lymphocyte cultures and unscheduled DNA synthesis (UDS) in primary rat hepatocyte.

In vivo, tricyclazole was negative in the rat liver UDS assay, mouse bone micronucleus test (a proof of exposure was separately conducted by McCoy & Clark, 2014; see Section 1.1, p 686) and a transgenic rodent (MutaMouse™) gene mutation assay conducted analyzing glandular stomach (point of contact), liver (target organ of exposure/toxicity), and kidney (highly exposed organ with no apparent signs of toxicity in in vivo studies').

The combined evidence from the genotoxicity studies, together with the evidence from toxicokinetic, carcinogenicity, developmental and reproductive toxicity studies confirmed that mutagenicity does not occur in relevant in vivo systems. Data were also compared to potential animal and human exposure, mechanistic data on biological targets and data on analogues (particularly the structurally related metabolite tricyclazole alcohol, X355227), confirming adequacy of the available data for hazard identification and risk assessment (Corvaro, Gollapudi & Mehta, 2019).

## 2.5 Reproductive toxicity

*Table 12. The results of reproductive toxicity studies with tricyclazole*

| Study | Species/ strain | Dosages* | NOAEL | LOAEL | Target organ/ principal effects at LOAEL | Reference |
|---|---|---|---|---|---|---|
| **GLP-compliant** | | | | | | |
| 2-Generation | Rat; Wistar-derived | 30, 100 and 400 ppm | Reproductive NOAEL and offspring NOAEL: 400 ppm, equivalent in mg/kg bw/day to 27.7 (males), 34 (females) | Reproductive and offspring toxicity: Not applicable | Reproductive: Not applicable | Wolterbeek, 2004a |
| | | | Parental NOAEL (systemic toxicity): 100 ppm, equivalent in mg/kg bw/day to 7 (males), 9 (females) | Parental systemic toxicity: 400 ppm, equivalent in mg/kg bw/day to 27.7 (males), 34 (females) | Parental toxicity: Body weight and body weight gain decreased; Increased liver weight<br>Offspring: Not applicable | |
| Teratology | Rat; Wistar | 0, 5, 20 and 50 mg/kg bw per day | Maternal NOAEL (toxicity): 5 mg/kg bw per day | Maternal toxicity: 20 mg/kg bw per day, based on decreased body weight gain and food consumption. | Maternal toxicity: Body weight gain | Wolterbeek, 2004b |
| | | | Developmental NOAEL (toxocity): 20 mg/kg bw per day | Developmental toxicity: 50 mg/kg bw per day based on incomplete ossification | Fetal toxicity: developmental delay | |
| **Pre-GLP** | | | | | | |
| 4-Generation | Mouse; ICR | 50 and 275 ppm | Reproduction, offspring & parental NOAELs (toxicity): 275 ppm (estimated as 17–38 mg/kg bw/day) | Not applicable as NOAEL was highest dietary dose | Reproductive Not applicable<br>Parental toxicity: Not applicable<br>Offspring: Not applicable | Adams et al., 1977a |
| 4-Generation | Rat; Wistar-derived | 50 and 275 ppm | Reproduction, offspring & parental NOAELs (toxicity): 275 ppm equivalent in mg/kg bw/day to 15.1 (males), 20 (females) | Not applicable as NOAEL was highest dietary dose | Reproductive Not applicable<br>Parental toxicity: Not applicable<br>Offspring: Not applicable | Adams et al., 1977b |
| 'Teratology' (dietary administ-ration) | Mouse; ICR-derived | 50 and 275 ppm | Maternal NOAEL: 275 ppm (estimated as 17–38 mg/kg bw/day)<br>Developmental NOAEL 275 ppm (estimated as 17–38 mg/kg bw/day) | Not applicable as NOAEL/NOAEL were the highest dietary dose | Maternal and developmental: No effects in treated females<br>Not teratogenic or embryotoxic. | Markham, 1977a |

NOAEL: No-observed-adverse-effect level; LOAEL: Lowest-observed-adverse-effect level

### (a) Multigeneration studies

*Study 1*

In a multi-generation reproductive toxicity study carried out prior to GLP and other guidelines, weaned ICR mice (21 days old) were randomly allocated to three groups of 50 males and 50 females and fed tricyclazole (purity 99.4% for Batch no. C53-C48-147, and 98.4% for Batch no. B07-C48-20), at dietary concentrations of 0, 50 and 275 ppm. Treatment was maintained throughout three successive generations. The first 20 females of the F0, F1 and F2 generations for which copulatory plugs were found were separated from males and designated to be killed on gestation day (GD) 20 for teratogenicity evaluation (results presented in the developmental studies section below). For dietary admixtures the stability over three to four months was checked, as was the achieved concentrations in both mash diet and pellets. Homogeneity of the admixture was not checked. F0 males and females were treated from weaning, throughout growth period, mating and pregnancy, and lactation for females, until sacrifice at weaning of their pups. F1a pups from each dietary dose group were randomly selected (50 per group) to become F1 parents and were maintained under the same conditions as their parents. The growth period lasted for 56 days after weaning. These parents were sacrificed at weaning of F2a pups. F2a pups were randomly selected to become F2 parents (50 per group) and were maintained under the same conditions as previous generations. For F2 parents the growth period lasted for 63 days after weaning. They produced F3 pups and were sacrificed at weaning of these pups.

Each animal was checked for mortality and signs of morbidity. Body weights were recorded weekly during the pre-mating period. Reproductive and litter parameters were assessed, including date of parturition, fertility, number of live and stillborn fetuses in each litter, number and condition of live fetuses on lactation days (LDs) 1, 7, 14 and 21 and fetal weights (as litters) on LDs 1, 7 and 14 and individually at weaning. Two control pups and one treated pup of each sex per litter among the F1 and F2 pups were subjected to gross necropsy. All F3 offspring were subjected to gross necropsy; organs and tissues of one pup of each sex per litter were fixed and examined microscopically. All parents that died during either the growth or reproduction period of the study were subjected to gross necropsy. Pups that died were given a gross external examination.

Tricyclazole did not induce any remarkable mortality in the treated groups when compared to the controls. None of the reproductive parameters examined were modified compared to their respective control values. Abnormalities attributable to the treatment were seen in neither parents nor offspring. Under the conditions of this study, a NOEL for reproductive and systemic toxicity was identified at 275 ppm (the highest administered concentration), estimated to be equivalent to 17–38 mg/kg bw per day (Adams, et al., 1977b).

*Study 2*

As part of a modern, two-generation toxicity study, a dose-finding study was initially conducted. Wistar rats (9–10 weeks old) were randomly allocated to five groups of eight males and eight females and given tricyclazole (purity: 95.2%, Batch no. 2002090020) in their food at dietary concentrations of 0, 200, 625, 1250 and 1600 ppm during two weeks prior to mating, during mating, gestation and up to sacrifice at or shortly after postnatal day (PND) 7. Adverse effects observed at 625 ppm and above in this preliminary study informed the selection of doses in the main two-generation study.

The main two-generation rat reproduction study was conducted with tricyclazole (purity: 95.2%, Batch no. 2002090020) at dietary concentrations of 0, 30, 100 and 400 ppm (equal to 0, 2.1, 7.0 and 27.7 mg/kg bw per day for males, 0, 2.3, 7.0 and 30.6 mg/kg per day for females based on the 10-week premating feed intake in F0 generation animals). Wistar rats (five weeks old), were randomly allocated to four dose-groups of 28 rats of each sex. Treatment was maintained for a 10-week premating period, during mating, gestation and lactation, then throughout two successive generations. F0 adults were treated during 10 weeks before mating, during mating, and throughout gestation and lactation for females. Males were sacrificed shortly after mating, and females after weaning of the F1 pups. On PND 4, litters of more than eight pups were reduced to four males and four females where possible. On postpartum day (PPD) 21 pups were weaned and one male and one female per litter (28 rats of each sex per group) were randomly chosen to produce the F2 litter and were treated under the same experimental conditions as their parents from PND 22, during growth, mating, pregnancy and lactation until sacrifice at weaning of the F2 litter. F2 pups were culled at PND 4 and sacrificed at weaning (PND 22).

There were no treatment-related clinical signs observed during premating, mating, gestation or lactation periods in either sex or any generation. Statistically significant decreases in body weight, body weight gain and food consumption were seen in both sexes at the high dose in the F0 and F1 generations, however reductions were less than 10% on all occasions. No treatment-related differences were observed in estrus cycle parameters in the F0 or F1 generations, and the fertility and reproductive performance of males and females of the F0 and F1 generations were not affected. There were no treatment-related effects on the size of the litters, or the number of stillborn pups, or on pups found dead during the lactation period. Sex ratio was not affected. Decreased body weights (by 10% for F0 and 14% for F1 rats) and body weight gain (by 10%–14%) were observed at day 21 for pups in the high-dose groups of both generations. A statistically significant delay in the onset of preputial separation and vaginal opening for male and female rats in the high-dose groups, and the decreased anogenital distance in the high-dose male pups were observed. No treatment-related effects were observed on epididymal and testicular sperm counts or epididymal sperm mobility in males of either generation at any dose level. Pup organ weights were not affected in any generation at any dose level. Except for the limited increase in relative liver weight (by less than 20%), due in part to the decreased body weight, seen in the high-dose groups of both generations, and the decreased absolute and relative weight of the uterus in females of the F1 high-dose group (probably also related to some extent to decreased body weight), no treatment-related effects were observed on the organ weights of males or females in the F0 and F1 generations. No histopathological alterations were detected in any organ (most particularly in the livers) at any dose in either of the two generations.

Adverse effects in parental animals were demonstrated at 400 ppm, based upon slightly decreased mean body weight in males and females in the F0 and F1 generation, along with decreased mean body weight gain, decreased food consumption and increased liver weight (absolute and relative) in F0 and F1 parental animals. Offspring effects were considered secondary to the decreased body weight, body weight gain and food consumption in parental animals. No adverse effects on the fertility or reproductive parameters were reported. Based on these results the following NOAELs/NOELs were identified:

- NOAEL for systemic parental toxicity: 100 ppm (equivalent to 7.0 mg/kg bw per day for males, 7.8 mg/kg bw per day for females, specifically 7.8, 6.1 and 13.2 mg/kg bw per day for premating, gestation and lactation, periods respectively);
- NOEL for reproduction: 400 ppm (equivalent to 27.7 mg/kg bw per day for males, 30.6 mg/kg bw per day for females, specifically 30.6, 23.7 and 47.8 mg/kg bw per day for premating, gestation and lactation periods, respectively);
- NOAEL for offspring: 400 ppm (equivalent to 27.7 mg/kg bw per day for males, 34.0 mg/kg bw per day for females) the highest dose tested. (Wolterbeek, 2004a).

***Table 13a. Key findings in two-generation rat reproduction study (main study); body weight and body weight gain during the premating periods for F0 and F1 generations***

| | Dietary dose (ppm)/sex | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | | 30 | | 100 | | 400 | |
| **Parameter** | **M** | **F** | **M** | **F** | **M** | **F** | **M** | **F** |
| **F0 generation** | | | | | | | | |
| Dose level (mg/kg bw/day) | 0 | 0 | 2.06 | 2.34 | 6.93 | 7.99 | 26.7 | 30.7 |
| Body weight at day 0 (g) | 148.34 | 92.98 | 146.70 | 94.00 | 149.82 | 91.17 | 147.83 | 94.18 |
| Body weight at day 69 (g) | 390.51 | 219.46 | 383.14 | 221.20 | 392.33 | 218.16 | 355.79*** | 207.90** |
| Weight gain days 0–69 (g) | 242.17 | 126.48 | 236.44 | 127.2 | 242.51 | 126.99 | 207.96 | 113.72 |
| **F1 generation** | | | | | | | | |
| Dose level (mg/kg bw/day) | 0 | 0 | 2.14 | 2.82 | 7.11 | 7.64 | 28.7 | 30.46 |
| Body weight at day 0 (g) | 106.24 | 90.22 | 102.17 | 89.28 | 105.35 | 90.05 | 91.83*** | 81.38** |
| Body weight at day 69 (g) | 368.82 | 211.84 | 353.46 | 210.78 | 362.49 | 208.55 | 315.56*** | 194.10 |
| Weight gain days 0–69 (g) | 262.58 | 121.62 | 251.29 | 121.5 | 257.14 | 118.5 | 223.73 | 112.72 |

M: Males; F: Females; Source: Wolterbeek, 2004a

Different from controls with statistical probability: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

*Table 13b. Key findings in two-generation rat reproduction study (main study); body weight gain and food consumption during gestation and lactation periods for F0 and F1 generation females*

| Time period | | Dose (ppm) | | | |
|---|---|---|---|---|---|
| | | 0 | 30 | 100 | 400 |
| | | **F0** | | | |
| Gestation days 14–21 | Body weight gain (g) | 28.1 | 32.8 | 31.05 | 27.96 |
| Gestation days 14–21 | Food consumption (g/kg bw/day) | 47.51 | 47.83 | 47.37 | 48.39 |
| Lactation days 14–21 | Body weight gain (g) | 4.59 | 3.31 | 3.55 | 6.14 |
| Lactation days 7–14 | Food consumption (g/kg bw/day) | 166.97 | 166.93 | 168.35 | 147.70*** |
| | | **F1** | | | |
| Gestation days 14–21 | body weight gain (g) | 31.44 | 32.01 | 28.8 | 28 |
| Gestation days 14–21 | food consumption (g/kg bw/day) | 54.68 | 54.94 | 55.25 | 54.68 |
| Lactation days 14–21 | body weight gain (g) | 4.69 | 4.95 | 6.77 | 7.05 |
| Lactation days 7–14 | food consumption (g/kg bw/day) | 186 | 184 | 192 | 157.11*** |

Different from controls with statistical probability: *** $p < 0.001$ Source: Wolterbeek, 2004a

*Table 13c. Key findings in two-generation rat reproduction study (main study); organ weights of F0 and F1 generation males and females*

| | Dietary dose (ppm)/sex | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | | 30 | | 100 | | 400 | |
| | M | F | M | F | M | F | M | F |
| **F0 organ weights (g)** | | | | | | | | |
| Liver, absolute | 14.405 | 12.692 | 14.252 | 13.128 | 15.943# | 13.507* | 15.136 | 13.972*** |
| Liver, relative | 34.798 | 48.451 | 34.964 | 49.703 | 37.815# | 51.811** | 39.722*** | 57.851*** |
| Spleen, absolute | 0.775 | 0.487 | 0.743 | 0.501 | 0.778 | 0.5 | 0.708** | 0.489 |
| Spleen, relative | 1.875 | 1.857 | 1.826 | 1.897 | 1.844 | 1.917 | 1.865 | 2.023** |
| Brain, relative | 4.61 | 6.599 | 4.592 | 6.529 | 4.514 | 6.539 | 4.964*** | 7.063** |
| Testes, relative | 7.996 | - | 8.278 | - | 7.978 | - | 9.208*** | - |
| Epididymides, relative | 3.311 | - | 3.343 | - | 3.266 | - | 3.560* | - |
| Uterus, relative | - | 1.308 | | 1.354 | | 1.281 | | 1.093 |
| Ovary, relative | - | 0.285 | | 0.298 | | 0.307 | | 0.312 |
| **F1 organ weights (g)** | | | | | | | | |
| Liver, absolute | 14.562 | 14.466 | 14.065 | 14.631 | 15.273 | 15.669 | 14.102 | 14.904 |
| Liver, relative | 37.243 | 54.665 | 36.804 | 54.856 | 38.84 | 58.615* | 41.349*** | 62.393*** |
| Spleen, absolute | 0.713 | 0.53 | 0.715 | 0.539 | 0.73 | 0.553 | 0.659 | 0.49 |
| Spleen, relative | 1.83 | 2.004 | 1.873 | 2.036 | 1.856 | 2.068 | 1935 | 2.064 |
| Brain, relative | 4.926 | 6.479 | 4.979 | 6.477 | 4.886 | 6.422 | 5.447*** | 7.057*** |
| Testes, relative | 8.785 | - | 8.966 | - | 8.545 | - | 9.242 | - |
| Epididymides, relative | 3.513 | - | 3.612 | - | 3.445 | - | 3.715 | - |
| Uterus, relative | - | 1.421 | - | 1.511 | - | 1.343 | - | 1.035* |
| Ovary, relative | - | 0.328 | - | 0.33 | - | 0.32 | - | 0.304 |

M: Males; F: Females; Source: Wolterbeek, 2004a

Different from controls with statistical probability: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

***Table 13d. Key findings in two-generation rat reproduction study (main study); body weight and body weight gain of pups in the F0 and F1 generations***

| | Dietary dose (ppm)/sex | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | | 30 | | 100 | | 400 | |
| **Parameter** | **M** | **F** | **M** | **F** | **M** | **F** | **M** | **F** |
| **F0 generation** | | | | | | | | |
| Dam dose level (mg/kg bw/day) | 0 | 0 | 2.06 | 2.34 | 6.93 | 7.99 | 26.7 | 30.7 |
| Body weight at day1 (g) | 5.38 | 5.02 | 5.50 | 5.14 | 5.51 | 5.11 | 5.49 | 5.25 |
| Body weight at day 21 (g) | 44.89 | 42.95 | 44.71 | 42.93 | 44.16 | 42.37 | 40.14*** | 38.89*** |
| Weight gain days 1–4 (g) | 2.79 | 2.66 | 2.64 | 2.58 | 2.67 | 2.58 | 2.67 | 2.65 |
| Weight gain days 14–21 (g) | 18.03 | 17.12 | 17.78 | 17.14 | 17.67 | 16.89 | 15.88*** | 15.31** |
| **F1 generation** | | | | | | | | |
| Dam dose level (mg/kg bw/day) | 0 | 0 | 2.14 | 2.82 | 7.11 | 7.64 | 28.7 | 30.46 |
| Body weight at day1 (g) | 5.27 | 4.92 | 5.38 | 5.10 | 5.50 | 5.20 | 5.24 | 4.98 |
| Body weight at day 21 (g) | 45.45 | 43.26 | 45.73 | 43.68 | 45.81 | 44.24 | 39.14*** | 37.17*** |
| Weight gain days 1–4 (g) | 2.92 | 2.79 | 2.83 | 2.70 | 2.88 | 2.80 | 2.81 | 2.71 |
| Weight gain days 14–21 (g) | 17.39 | 16.46 | 17.40 | 16.53 | 17.67 | 16.98 | 14.92*** | 14.46*** |

M: Males; F: Females; Source: Wolterbeek, 2004a

Different from controls with statistical probability: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

*Study 3*

In a multigeneration reproduction study carried out prior to GLP and other guidelines, Wistar rats (21 days old, body weights not reported) were randomly allocated to three groups of 50 males and 50 females and fed tricyclazole (purity 99.5%, Batch no. C53-C48-147 and purity 98.4%, Batch no. B07-C48-20), at dietary concentrations of 0 (controls), 50 and 275 ppm. Treatment was maintained throughout three successive generations. The study was conducted as a parallel reproduction and teratology study. The first 20 females of each dose group in the F0 and F1 generations for which copulatory plugs were found were separated from males and designated to be killed on GD 20 for teratogenicity evaluation (results presented in the developmental toxicity section). One week's stability and concentration of dietary admixtures were checked before the start of dosing each generation. Homogeneity of the admixture was not checked. F0 males and females were treated from weaning, throughout an 87-day growth period after weaning, a three-week mating period and pregnancy and lactation for females, until sacrifice at weaning of their pups, designated F1a. These F1a pups from each dietary dose level were randomly selected to become F1 parents and were maintained under the same conditions as the F0 generation. The growth period lasted for 62 days. F1a dams reproduced twice in order to obtain F2a and F2b pups, and dams were sacrificed at weaning of the F2b pups. The F2a pups randomly selected to become F2 parents were maintained under the same conditions as their parents. The F2 parental growth period lasted for 53 days after weaning. They produced F3a pups and were sacrificed at weaning of these pups.

All animals were checked for mortality and signs of morbidity. Body weights and food consumption were recorded weekly during the premating period. Reproductive and litter parameters assessed included fertility, number of live and stillborn fetuses in each litter, number, weight and condition of live fetuses on LDs 1, 7, 14 and 21, fetal weight as litters on LDs 1, 7 and 14 and individually at weaning. For the F0 and F1 generations, gross necropsies were performed on two control pups of each sex per litter and one pup of each sex per litter for the tricyclazole test groups. A gross necropsy was performed on all F3 pups; organs and tissues (list not specified) of one pup of each sex per litter were examined microscopically. Any parents that died during either the growth or reproduction period of the study were subjected to gross necropsy. Pups that died during lactation were given a gross external examination. No treatment-related mortality or effects on body weight were observed. None of the reproductive parameters examined were modified compared to their respective control values. No abnormalities attributable to the treatment were seen in either parents or offspring.

Under the conditions of this test, a NOEL for reproduction and systemic toxicity was identified at 275 ppm, the highest dose tested (equivalent to 15.1 mg/kg bw per day in males and 20.0 mg/kg bw per day in females, taken as the means from three generations). (Adams et al., 1977a)

**(b) Developmental studies**

***Mouse***

Teratology studies were conducted in conjunction with a multi-generation (preGLP and preguideline) reproduction study. Twenty bred females (retained copulatory plugs identified) from each dietary group from the F0, F1 and F2 groups were killed on GD 18 and examined for relevant reproduction and fetal data.

Tricyclazole (purity 99.4%, Batch No. C53-C48-147, and purity 99.6%, Batch No. B07-C48-20) was administered at dietary concentrations of 50 and 275 ppm during growth, mating and gestation. Three to four month stability and achieved concentration of dietary admixtures were checked. Homogeneity of the admixtures was not checked. Mice were observed daily for clinical signs. Body weight was measured on GDs 1, 7, 14 and 18. Reproduction parameters included number of corpora lutea, number and distribution of fetuses, number of early and late resorptions, fertility index, gestation survival index, resorption index and implantation index. Fetal parameters included viability, weight, sex, and external visceral and skeletal examinations, with one-third of the fetuses fixed and prepared for visceral examination and the remainder processed for skeletal examination.

Compared to the controls tricyclazole administration did not induce any mortality or signs of toxicity, including effects on body weight gain, in the treated females assigned to the teratology phase. None of the reproductive parameters examined (fertility, litter size, implantations, fetal viability) were modified compared to the respective control values. Under the conditions of this test, the NOAEL for maternal toxicity and for developmental effects was the highest administered dose of 275 ppm (estimated to represent between 17 and 38 mg/kg bw per day). Fetal abnormalities observed were not considered treatment-related due to a lack of a dose–response relationship (Markham et al., 1977a).

***Rat***

In a GLP-compliant, prenatal developmental toxicity study, conducted according to the protocol entitled 'Oral prenatal developmental toxicity test with tricyclazole technical in rats' approved by the study director, female Wistar rats Crl (WI) WU BR (12 weeks old, body weights 177.6–227.2 g at start of the mating period) were randomly assigned to four dose groups of 28 mated females. Rats were dosed orally by gavage with tricyclazole (purity 95.2%, Batch No. 2002090020) suspended in 1% aqueous CMC, at dose levels of 0 (controls), 5, 20 or 50 mg/kg bw per day daily from fertilization (day 0) to GD 20, using a dosage volume of 5 mL/kg bw. Assays were performed to check seven-day stability, homogeneity and achieved gavage mixtures concentrations. Animals were observed at least once daily for mortality and overt clinical signs. Females were weighed on GDs 0, 3, 7, 10, 14, 17 and 21. Food consumption was recorded for the following intervals during gestation: GDs 0–3, 3–7, 7–10, 10–14, 14–17 and 17–21. On GD 21 dams were killed and a caesarean section was performed. Maternal tissues showing macroscopic abnormalities were removed and fixed in a neutral aqueous phosphate buffered 4% solution of formaldehyde for possible microscopic examination. Weights of uterus (both containing placenta and fetuses, and empty), ovaries and placenta were recorded. Ovaries and uterine horns were examined to determine numbers of corpora lutea, live and dead fetuses, early or late resorptions and implantation sites. Fetuses were examined for external malformations/variations, and were weighed and sexed. One half of the fetuses were fixed in Bouin's fluid and the remaining half in alcohol, to evaluate visceral and skeletal anomalies (by staining with Alizarin red-S) respectively. For each group the following parameters were calculated: female fecundity index, pre-implantation loss, post-implantation loss, gestation index, sex ratio.

Food consumption was decreased to a statistically significant extent at the mid dose between GD 0 and GD 10, and at the high dose from GD 0 to GD 17, with decreases at the mid dose of 21% for GDs 0–3, 14.5% from GDs 3–7 and 12% from GDs 7–10. At the high dose in the period on GDs 0–3 food consumption decreased by 65%, with decreases of 8% to 25% in the other periods up until GD 17. At the high dose, rats lost weight in the first three days of dosing, with recovery commencing between

day 7 and day 10. At the mid dose, decreased body weight gain compared with controls of 25% was seen in the period day 0–3, and depression by 43% in the period day 3–7. As this suppression of body weight gain was greater than the reduction in food consumption, it was considered to be related to systemic toxicity. Depressed body weight gain was observed particularly during GDs 0–10 at the high (by 89%) and mid (by 25%) doses, with increased body weight gain observed during GDs 10–21. There was a statistically significant decrease in gravid uterine weights at the high dose compared to controls, and this decrease was considered biologically significant. Carcass weight (terminal body weight minus gravid uterine weight) was unaffected by treatment. None of the reproductive parameters (fecundity index, gestation index, number of corpora lutea, implantation sites, live and dead fetuses, early and late resorptions, pre- and post-implantation losses, sex ratio, fetal viability) were affected by the treatment. The number of small fetuses (defined as body weight less than 75% of the mean control body weight) was statistically increased in the high-dose group, on both a fetal and litter incidence basis. In this group, as well as to a lesser extent the mid-dose group, skeletal variations were increased compared to controls. This finding was considered to be related to maternal toxicity, particularly the effects on food consumption early in the dosing period that was noted in these groups, and a direct toxic effect of the test material on fetuses. No visceral malformations or soft tissue abnormalities were recorded in any group. The NOAEL for maternal toxicity was 5 mg/kg bw per day based on decreased body weight gain and food consumption at 20 mg/kg bw per day, particularly in the initial dosing period, which was statistically significant and considered to be of biological significance. The NOAEL for developmental toxicity was 20 mg/kg bw per day on the basis of reduced weight gain of embryos, presence of accessory lumbar ribs and delayed ossification (including absent ossification) at 50 mg/kg bw per day (Wolterbeek, 2004b).

***Table 14a. Key findings in a prenatal developmental toxicity study; maternal body weight changes during gestation***

| | Dietary dose (mg/kg bw per day) | | | |
|---|---|---|---|---|
| **Parameter** | **0** | **5** | **20** | **50** |
| Number of pregnant females | 24 | 26 | 26 | 22 |
| Body weight at day 0 (g) | 199.47 | 199.06 | 201.24 | 200.72 |
| Body weight at day 3 (g)<br>% decrease compared to control | 211.01 | 209.73<br>99 | 208.92 | **188.45**<br>**11** |
| Body weight at day 7 (g)<br>% decrease compared to control | 219.44 | 216.75 | 213.75 | **198.52**<br>**10** |
| Body weight at day 10 (g)<br>% decrease compared to control | 228.11 | 225.83 | 222.13 | **203.86**<br>**11** |
| Body weight at day14 (g)<br>% decrease compared to control | 240.15 | 239.72 | 235.40 | **219.22**<br>**9** |
| Body weight at day17 (g)<br>% decrease compared to control | 260.19 | 258.95 | 254.90 | **233.06**<br>**10** |
| Body weight at day21 (g)<br>% decrease compared to control | 282.69 | 276.97 | 286.17 | **267.51**<br>**9** |
| Body weight gain, days 0–3 (g)<br>% decrease compared to control | 11.54 | 10.67 | 8.68<br>25 | **−12.27**<br>**206** |
| Body weight gain, days 3–7 (g)<br>% decrease compared to control | 8.43 | 7.02 | 4.83<br>43 | **−2.2**<br>**124** |
| Body weight gain, days 7–10 (g)<br>% decrease compared to control | 8.67 | 9.08 | 8.38<br>3 | **5.34**<br>**38** |
| Body weight gain, days 0–10 (g)<br>% decrease compared to control | 28.64 | 26.78 | 21.50<br>25 | **3.14**<br>**89** |
| Body weight gain, days 10–21 (g) | 54.58 | 51.12 | 64.05 | 63.65 |
| Net weight change from day 0 | 18.22 | 15.32 | 17.43 | 14.37 |
| Gravid uterine weight | 64.92 | 62.08 | 67.32 | 52.12** |

** Statistically significant at $p < 0.01$; **Bold:** Considered biologically significant; Source: Wolterbeek, 2004b

*Table 14b. Key findings in a prenatal developmental toxicity study; food consumption during gestation*

| | Dietary dose (mg/kg bw per day) | | | |
|---|---|---|---|---|
| **Time period** | **0** | **5** | **20** | **50** |
| Days 0–3 | 72.99 | 71.66 | 57.56*** | 25.70*** |
| Days 3–7 | 71.65 | 74.04 | 61.26*** | 56.91*** |
| Days 7–10 | 74.41 | 74.22 | 65.55*** | 52.7*** |
| Days 10–14 | 68.59 | 71.01 | 67.53 | 63.19* |
| Days 14–17 | 74.85 | 75.58 | 73.72 | 65.73*** |
| Days 17–21 | 50.14 | 50.12 | 49.82 | 60.57** |

Different from controls with statistical probability: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; Source: Wolterbeek, 2004b

*Table 14c. Key findings in a prenatal developmental toxicity study; number of small fetuses and mean fetal body weights*

| | | Dietary dose (mg/kg bw per day) | | | |
|---|---|---|---|---|---|
| **Parameter** | | **0** | **5** | **20** | **50** |
| Number of small fetuses: | fetal incidence | 2 | 4 | 0 | 20*** |
| | litter incidence | 1 | 4 | 0 | 10** |
| Fetal weight (g) | | 4.53 | 4.52 | 4.49 | 3.89*** |

Different from controls with statistical probability: ** $p < 0.01$; *** $p < 0.001$; Source: Wolterbeek, 2004b

*Table 14d. Key findings in a prenatal developmental toxicity study; skeletal and skeleton ossification variations in fetal incidence, individual values [litter values]*

| | Dietary dose (mg/kg bw per day) | | | |
|---|---|---|---|---|
| **Parameter** | **0** | **5** | **20** | **50** |
| Number of fetuses [litters] examined | 114 [20] | 110 [20] | 117 [20] | 105 [20] |
| Total skeletal variations | 13 [10] | 17 [11] | 13 [8] | 16 [10] |
| Accessory lumbar ribs | 0 [0] | 2 [1] | 2 [2] | 6* [6*] |
| Total variation in ossification of fetal skeletons | 114 [20] | 110 [20] | 117 [20] | 105 [20] |
| Nasal: incompletely ossified | 0 [0] | 1 [1] | 42*** [9**] | 58*** [15***] |
| Inter parietal: incompletely ossified | 20 [10] | 23 [12] | 46*** [17*] | 88*** [20***] |
| Supra occipital: incompletely ossified | 6 [5] | 6 [4] | 4 [3] | 37*** [15**] |
| Squamosal: incompletely ossified | 0 [0] | 0 [0] | 0 [0] | 5* [4] |
| Palatine: incompletely ossified | 0 [0] | 0 [0] | 2 [1] | 27*** [9**] |
| Presphenoid: incompletely ossified | 0 [0] | 0 [0] | 0 [0] | 9**[6*] |
| Hyoid: incompletely ossified | 1 [1] | 1 [1] | 4 [4] | 7*[4] |
| Sternebrae: 3 or more sternebrae incompletely ossified | 5 [4] | 4 [4] | 9 [9] | 36*** [16***] |
| Cervical bodies: 1 or 2 incompletely ossified bodies | 33 [10] | 28 [15] | 43 [14] | 62*** [17*] |
| Cervical bodies: 3 or more unossified bodies | 54 [17] | 36* [15] | 67 [19] | 100*** [20] |
| Caudal bodies: 1 or 2 incompletely ossified bodies | 69 [17] | 48*[13] | 45**[17] | 93***[19] |
| Caudal bodies: 1 or 2 unossified bodies | 4 [3] | 2 [2] | 2 [2] | 43***[16***] |
| Metacarpals: 1–2 metacarpals incompletely ossified | 1 [1] | 2 [2] | 8* [3] | 53*** [17***] |
| Phalanges front dist: 6–10 digits incompletely ossified | 4 [2] | 7 [5] | 19**[8] | 24***[10*] |
| Phalanges front prox: 3–6 digits unossified | 35 [13] | 18* [11] | 46 [13] | 73*** [17] |
| Phalanges front prox : 7–10 digits unossified | 2 [2] | 1 [1] | 2 [1] | 17*** [8] |
| Metatarsals : 1–2 metatarsals unossified | 10 [6] | 4 [4] | 18 [9] | 78*** [19***] |

| | Dietary dose (mg/kg bw per day) | | | |
|---|---|---|---|---|
| **Parameter** | **0** | **5** | **20** | **50** |
| Phalanges hind dist : 6–10 digits incompletely ossified | 2 [2] | 2 [2] | 7 [4] | 44*** [16***] |
| Phalanges hind prox: 7–10 digits unossified | 63 [17] | 42* [16] | 62 [16] | 98*** [20] |

Different from controls with statistical probability: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; Source: Wolterbeek, 2004b

Teratology studies were conducted in conjunction with a multi-generation reproduction study carried out prior to GLP and other guidelines. Twenty bred females (retained copulatory plugs identified) from each dietary group from the F0, F1 and F2 groups were killed on GD 18 and examined for relevant reproductive and fetal data.

The rats received tricyclazole (purity 99.4%, Batch no. C53-C48-147 and purity 99.6%, Batch no. B07-C48-20) in food, at dietary concentrations of 0, 50 and 275 ppm during growth, mating and gestation. Females were sacrificed on GD 20 and subjected to a caesarean section in order to study the teratogenic potential of tricyclazole. Dams were 3–4 months old at mating with body weights 248–368 g for F0 females, 200–360 g for F1 females and 225–360 g for F2 females.

Rats were observed daily for changes in appearance or behaviour, and body weight and food consumption were recorded weekly. Gross necropsy examinations were performed when dams were terminated. The reproductive status of each female was evaluated; ovaries were examined for the number of corpora lutea, and the uterus for the number and distribution of fetuses and resorptions. Resorptions were classified as early or late to indicate the relative time of mortality. The fetuses were examined for external abnormalities, viability and their sex determined; weights were recorded by litter. Approximately one-third of the fetuses of each litter were fixed in Bouin's solution for visceral examination (modified technique of Wilson), and the remainder were fixed in ethanol for Alizarin red-S staining, for skeletal examination. Mean group values were identified for reproductive parameters: fertility index, gestation survival index, resorption and implantation indices.

No mortality was observed in the teratology phase of this study. None of the reproductive parameters examined (fertility, litter size, implantations, fetal viability) were modified compared to the respective control values. While the incidence of bilateral hydronephrosis was well within the historical control range, the incidence of unilateral hydronephrosis at the top dose in the F3 pups was above historical controls, and a relationship with treatment could not be ruled out. The study was considered not to be acceptable since no toxicity effect has been evidenced in dams at the highest dose tested. Under the conditions of this test, the NOAEL for maternal toxicity and NOEL for developmental effects was the highest dose administered, 275 ppm (equal to 20.0 mg/kg bw per day) (Markham et al., 1977b).

***Table 15a. Key findings of teratogenicity study in rats; mean maternal body weights (g)***

| Dose group | Number of pregnant females | Gestation day | | | | Mean percentage weight gain ± SD |
|---|---|---|---|---|---|---|
| | | **0** | **7** | **14** | **20** | |
| **F0 parents (Study R-505)** | | | | | | |
| Control | 16 | 309 | 332 | 362 | 440 | 42.7 ± 8.1 |
| 50 ppm | 16 | 294 | 320 | 345 | 424 | 44.5 ± 8.2 |
| 275 ppm | 17 | 298 | 322 | 348 | 442 | 48.9 ± 9.3 |
| **F1 parents (Study R-1135)** | | | | | | |
| Control | 19 | 278 | 300 | 337 | 405 | 46.0 ± 9.9 |
| 50 ppm | 16 | 270 | 298 | 336 | 397 | 46.4 ± 11.4 |
| 275 ppm | 18 | 271 | 298 | 336 | 397 | 46.6 ± 7.6 |
| **F2 parents (Study R-36)** | | | | | | |
| Control | 18 | 281 | 304 | 336 | 417 | 49.2 ± 12.1 |
| 50 ppm | 19 | 276 | 298 | 331 | 407 | 47.4 ± 6.4 |
| 275 ppm | 19 | 277 | 297 | 333 | 408 | 48.1 ± 11.8 |

SD: Standard deviation Source: Markham et al., 1977b

*Table 15b. Key findings of teratogenicity study in rats; fertility indices, fetal mortality/survival, fetal weight per pregnant rat, litter size, resorption index and number of corpora lutea*

| Parameter/ generation | Dietary concentration (ppm) | | |
|---|---|---|---|
| | 0 (control) | 50 | 275 |
| **Fertility index[a] [pregnant/total]** | | | |
| F0 (Study R-505) | 0.80 [16/20] | 0.80 [16/20] | 0.85 [17/20] |
| F1 (Study R-1135) | 0.95 [19/20] | 0.80 [16/20] | 0.90 [18/20] |
| F2 (Study R-36) | 0.90 [18/20] | 0.95 [19/20] | 0.95 [19/20] |
| **Gestation survival index[b] [surviving to term/total]** | | | |
| F0 (Study R-505) | 1.00 [184/184] | 1.00 [189/189] | 1.00 [218/218] |
| F1 (Study R-1135) | 1.00 [226/226] | 1.00 [184/184] | 1.00 [212/212] |
| F2 (Study R-36) | 1.00 [227/227] | 1.00 [234/234] | 1.00 [225/225] |
| **Fetal weight per pregnant rat; mean fetal weight (g) ± SD** | | | |
| F0 (Study R-505) | 3.90 ± 0.08 | 3.99 ± 0.13 | 3.94 ± 0.06 |
| F1 (Study R-1135) | 3.80 ± 0.07 | 4.06 ± 0.18 | 4.01 ± 0.08 |
| F2 (Study R-36) | 3.75 ± 0.07 | 3.90 ± 0.07 | 3.92 ± 0.07 |
| **Litter size; mean number of live fetuses/pregnant female ± SD** | | | |
| F0 (Study R-505) | 11.5 ± 0.9 | 11.8 ± 0.7 | 12. 8 ± 0.5 |
| F1 (Study R-1135) | 11.9 ± 0.6 | 11.5 ± 0.4 | 11.8 ± 0.6 |
| F2 (Study R-36) | 12.6 ± 0.8 | 12.3 ± 0.4 | 11.8 ± 0.7 |
| **Resorption index[c] [resorptions/total implants]** | | | |
| F0 (Study R-505) | 0.09 [19/203] | 0.06 [12/201] | 0.06 [14/232] |
| F1 (Study R-1135) | 0.05 [13/239] | 0.08 [17/201] | 0.13 [31/243] |
| F2 (Study R-36) | 0.04 [9/236] | 0.08 [21/255] | 0.06 [14/239] |
| **Number of corpora lutea; mean number of live fetuses/pregnant female ± SD** | | | |
| F0 (Study R-505) | 14.8 ± 0.7 | 14.1 ± 0.4 | 14.4 ± 0.8 |
| F1 (Study R-1135) | 14.6 ± 0.8 | 14.4 ± 0.8 | 14.6 ± 0.5 |
| F2 (Study R-36) | 15.9 ± 0.7 | 14.4 ± 0.4 | 14.5 ± 0.4 |

SD: Standard deviation; Source: Markham et al., 1977b

[a] Fertility index is: proportion of pregnant females/ initial number of females in group;

[b] gestation survival index is: proportion of live fetuses / total number of fetuses;

[c] Resorption index is: proportion of conceptuses having resulted in resorptions/total number of conceptuses

*Table 15c. Key findings of teratogenicity study in rats; Visceral examination of fetuses: fetal/litter incidence of unilateral hydronephrosis, bilateral hydronephrosis (and corresponding percentages)*

| Dietary concentration | Dietary concentration (ppm) | | | Historical control data |
|---|---|---|---|---|
| | 0 ppm | 50 ppm | 275 ppm | |
| Dose level (mg/kg bw/day) | 0 | 3.67 | 20.0 | |
| **Unilateral hydronephrosis; individual frequency (as percentage), [litter frequency]** | | | | |
| F1 pups | 0/66 (0%), [0/16] | 2/69 (3%), [2/16] | 3/78 (4%), [3/17] | 332/6223 (5%) |
| F2 pups | 3/83 (4%), [3/19] | 3/65 (5%), [3/16] | 4/75 (5%), [3/18] | 338/6447 (5%) |
| F3 pups | 1/82 (1%), [1/18] | 3/82 (4%), [3/19] | 9/83 (11%), [5/19] | 349/6716 (5%) |

| Dietary concentration | Dietary concentration (ppm) 0 ppm | 50 ppm | 275 ppm | Historical control data |
|---|---|---|---|---|
| **Bilateral hydronephrosis; individual frequency (as percentage), [litter frequency]** | | | | |
| F1 pups | 1/66 (2%), [1/16] | 1/69 (2%), [1/16] | 0/78 (0%), [0/17] | 399/6223 (6%) |
| F2 pups | 2/83 (2%), [2/19] | 1/65 (2%), [1/16] | 1/75 (1%), [1/18] | 408/6447 (6%) |
| F3 pups | 2/82 (2%), [2/18] | 1/82 (1%), [1/19] | 3/83 (1%), [2/19] | 423/6716 (6%) |

Statistical differences not stated; Source: Markham et al., 1977b

***Rabbit***

A GLP-compliant development toxicity study, employed three groups of 25 time-mated female New Zealand White rabbits (Hra:(NZW)SPF), six months old at the time of mating by the supplier, weighing 2902–3630 g on GD 0. The rabbits were administered tricyclazole technical (purity 99.3%, Batch no. 2003030017) dispersed in 0.5% aqueous methylcellulose at 0, 7.5, 25 or 75 mg/kg bw daily by oral gavage from GD 7 to GD 28, at a dosage volume of 2 mL/kg bw. These dose levels were selected on the basis of the results of a preliminary range-finding study (Knapp, 2008), In that study, maternal toxicity was apparent at a dosage level of 100 mg/kg bw per day (food consumption and eventual group termination as evidenced by moribundity, mean body weight losses which showed dose-related reductions).

All rabbits were observed twice daily for clinical signs, moribundity and mortality. Individual detailed clinical observations were recorded from the day of receipt until GD 29. Individual maternal body weights were recorded on GD 0 (by the supplier), 4 and daily from GD 7 to GD 29. Food consumption and individual food consumption was recorded daily from GD 4 to GD 29. On GD 29, gravid uterine weights were recorded and net body weight and net body weight change were calculated for each gravid female at the scheduled laparohysterectomy. A gross necropsy was performed on females that died, delivered or aborted during the course of the study. In the case of any uterus that showed no macroscopic evidence of implantation, it was opened and examined for detection of early implantation loss. Sections of the stomach, liver (with gall bladder), kidney and any gross lesions were preserved for possible future histopathological examination. The number and location of former implantation sites, corpora lutea and viable fetuses/kits were recorded. Recognizable fetuses were examined externally and retained in 10% neutral-buffered formalin. All surviving rabbits were euthanized on GD 29. The number of corpora lutea on each ovary was recorded. The trimmed uterus was weighed, and the number and location of all fetuses, early and late resorptions and the total number of implantation sites were recorded. The placentae were also examined. The detailed external examination of each fetus included examination of the eyes, palate and external orifices. Each fetus was subjected to a visceral examination and its sex was determined.

No treatment related mortalities or clinical signs were observed, although one animal in the 7.5 mg/kg bw per day group was found dead on GD 25, and one female in the 7.5 mg/kg bw per day group aborted two fetuses on GD 22. Food consumption was reduced in the high-dose group starting from GD 13, with a mean reduction compared to controls of 18% when expressed on a per animal basis (15% on a body weight basis) during the period GD 7 to day GD 29. As the daily reductions in food consumption (8.0%–41.0% on a per animal basis, 6.1%–40.0% on a body weight basis) corresponded to reduced body weight gains in the 75 mg/kg bw per day group, the reductions in food consumption were considered to be treatment-related. Food consumption by animals receiving 7.5 and 25 mg/kg bw per day was similar to that for the controls throughout the treatment period.

There was no evidence of teratogenicity in this study. Lower fetal weight (both sexes) was observed at the high dose.

The maternal NOAEL was 25 mg/kg bw per day based on lower mean body weight gains and reduced food consumption at 75 mg/kg bw per day. The embryo/fetal NOAEL was 25 mg/kg. bw per day based on lower mean fetal weight at 75 mg/kg bw per day (Knapp, 2009).

*Table 16a. Key findings of prenatal developmental toxicity study in rabbits; mean body weight, body weight gain, gravid uterus weight and food consumption, organ weights and pregnancy rate*

| | | Dose levels (mg/kg bw per day) | | | |
|---|---|---|---|---|---|
| **Parameter** | | **0** | **7.5** | **25** | **75** |
| Body weight; day 29 (g ± SD) | | 3599 ± 250.1 | 3572 ± 239.6 | 3595 ± 234.7 | 3451 ± 186.2 |
| Body weight gain; days 7–29 (g ± SD) | | 346 ± 153.6 | 299 ± 183 | 309 ± 134.7 | 210 ± 113.8** |
| Food consumption; days 7–29 (g/animal/day ± SD) | | 141 ± 23.4 | 149 ± 25.8 | 145 ± 22.5 | 116 ± 18.3** |
| **Organ weights** | | | | | |
| Gravid uterine weight: | absolute (g ± SD) | 474.8 ± 72.77 | 465.7 ± 56.69 | 459.7 ± 84.98 | 449.7 ± 61.53 |
| Liver weight: | absolute (g ± SD) | 87.38 ± 15.8 | 86.78 ± 12.7 | 91.8 ± 10.2 | 96.17 ± 11.7 |
| Liver weight: | relative (%) | 2.42 ± 0.36 | 2.42 ± 0.27 | 2.55 ± 0.29 | 2.79 ± 0.34** |
| Kidney weight: | absolute (g ± SD) | 15.24 ± 1.2 | 15.75 ± 1.7 | 15.12 ± 1.6 | 15.76 ± 1.4 |
| Kidney weight: | relative (%) | 0.42 ± 0.03 | 0.44 ± 0.04 | 0.42 ± 0.04 | 0.45 ± 0.02** |
| **Pregnancy rate** | | | | | |
| Number pregnant/number in group | | 25/25 | 22/25 | 23/25 | 24/25 |

SD: Standard deviation; Source: Knapp, 2009

** Different from controls using Dunnett's test, with statistical probability $p < 0.01$

*Table 16b. Key findings of prenatal developmental toxicity study in rabbits; reproduction values (shown as mean ± standard deviation)*

| Dose | Reproductive parameter (mean ± standard deviation) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Dose groups (mg/kg bw per day) | Total number of fetuses | Male (%) | Viable fetuses/dam | Total number of dead fetuses | Implantations | Pre-implantation Loss | Post-implantation Loss | Corpora lutea | Resorptions (early) | Resorptions (late) |
| 0 | 217 | 42.6 | 9.0 ± 1.46 | 0 | 9.3 ± 1.43 | 0.7 ± 0.92 | 0.3 ± 0.44 | 10.0 ± 1.63 | 0.3 ± 0.44 | 0.0 ± 0.00 |
| 7.5 | 295 | 48.2 | 9.3 ± 1.59 | 0 | 9.7 ± 1.71 | 0.7 ± 0.96 | 0.4 ± 0.81 | 10.4 ± 2.23 | 0.3 ± 0.64 | 0.1 ± 0.48 |
| 25 | 199 | 49 | 8.7 ± 2.23 | 0 | 8.9 ± 2.14 | 1.2 ± 1.54 | 0.2 ± 0.42 | 10.1 ± 1.73 | 0.2 ± 0.42 | 0.0 ± 0.00 |
| 75 | 222 | 45.7 | 9.3 ± 2.77 | 1 | 9.8 ± 1.99 | 0.6 ± 0.78 | 0.6 ± 2.06 | 10.4 ± 2.12 | 0.0 ± 0.00 | 0.5 ± 2.06 |

SD: Standard deviation; Source: Knapp, 2009

*Table 16c. Key findings of prenatal developmental toxicity study in rabbits; fetal weights*

| Dose groups (mg/kg bw/day) | Fetal weights, male mean ± SD (g) | Fetal weights, female mean ± SD (g) | Fetal weights, combined mean ± SD (g) |
|---|---|---|---|
| 0 | 39.7 ± 0.82 | 38.6 ± 3.41 | 39.1 ± 3.33 |
| 7.5 | 37.7 ± 5.28 | 36.8 ± 5.68 | 37.1 ± 5.42 |
| 25 | 40.3 ± 5.97 | 39.6 ± 6.24 | 39.9 ± 5.87 |
| 75 | 34. 3 ± 4.40* | 32.6 ± 4.22* | 33.4 ± 4.03* |

SD: Standard deviation; Source: Knapp, 2009

** Different from controls using Dunnett's test, with statistical probability $p < 0.01$

***Table 16d. Key findings of prenatal developmental toxicity study in rabbits; fetuses and [litters] with malformations***

| | Dose group (mg/kg bw per day) | | | |
|---|---|---|---|---|
| | **0** | **7.5** | **25** | **75** |
| Number of fetuses examined | 217 | 195 | 199 | 222 |
| Total external malformations | 0 | 0 | 0 | 0 |
| Total visceral malformations | 0 | 0 | 2[2] | 1[1] |
| Stenotic pulmonary trunk | 0 | 0 | 0 | 1 |
| Intraventricular septal defect | 0 | 0 | 1 | 0 |
| Lungs; lobular agenesis | 0 | 0 | 1 | 0 |
| Total skeletal malformations | 1[1] | 3[3] | 1[1] | 0 |
| Vertebral anomaly with or without associated rib anomaly | 1 | 0 | 1 | 0 |
| Sternebrae fused | 0 | 1 | 0 | 0 |
| Rib anomaly | 0 | 1 | 0 | 0 |
| Sternebra(e) malaligned (severe) | 0 | 1 | 0 | 0 |
| Total malformations | 1[1] | 3[3] | 3[3] | 1[1] |

Source: Knapp, 2009

In a teratology study carried out prior to GLP and other guidelines, artificially inseminated female Dutch belted rabbits (age not reported, body weights 1.70–3.04 kg) were randomly assigned to four dose groups of 15 animals. These received tricyclazole (purity 99.4%, Batch no. C53-C48-147) suspended in aqueous acacia gum solution, at dose levels of 0 (controls), 2, 10 or 50 mg/kg bw per day by oral gavage daily from GD 6 to GD 18, in dosage volumes of 5 mL/kg bw. No information on the achieved concentrations, stability or homogeneity of the mixture was provided. Food consumption was recorded daily and body weights were recorded on GDs 0, 6, 12, 18, 21 and 27. Animals were observed daily for clinical signs. Reproduction parameters included number of corpora lutea, number and location of fetuses, number of early and late resorptions, fertility index, gestation survival index, resorption index and implantation index. Fetal parameters included viability, weight, sex and external, visceral and skeletal observations. After 24 hours treatment in a 1% sodium chloride solution, the viscera and brain were examined and carcasses prepared for skeletal examination. Following death, or abortion, rabbits were subjected to a gross necropsy.

No treatment-related maternal effects were observed during the study, with body weight and food consumption comparable between groups and controls. The number of pregnant animals per group was low (lower than recommended by OECD TG 414), however no treatment-related effects were observed on reproductive parameters. No treatment-related fetal abnormalities were observed in this study. During the study some females were seen to suffer from respiratory disease, which resulted in death. This study was not considered to be of regulatory quality. Under the conditions of this test, the NOAEL for maternal toxicity and NOEL for developmental effects were the highest administered dose, 50 mg/kg bw per day (Worth, 1977a).

## 2.6 Special studies

### (a) Neurotoxicity studies

Neurotoxic effects were not observed in short- or long-term feeding studies, however no specific neurotoxicity studies were conducted.

### (b) Immunotoxicity studies

No data were provided on immunotoxicity.

### (c) Mechanistic studies

An in vitro, nuclear receptor activation study was conducted in rat hepatocytes to evaluate the nuclear receptor activation potential of tricyclazole (purity 97.4%) via the assessment of biomarker gene expression responses. Cryopreserved rat primary hepatocytes were used for this study. Rat primary hepatocytes were incubated for a total of 24 hours with six concentrations of tricyclazole ranging from 0.3 µM to 100 µM, or with a single concentration of four positive control compounds: 3-methylchloanthrene (AhR), phenobarbital (CAR), dexamethasone (PXR) and Wyether-14643 (PPARα), which are known activators for each of the nuclear receptors. After an initial exposure period of 18 hours, the test media were removed and replenished with fresh test media and incubated for six additional hours. This replenishment approach was utilized to aid in the evaluation of compounds that might be rapidly metabolized. After the exposure period cells were harvested and gene expression responses measured by quantitative real-time polymerase chain reaction (PCR), normalized to rat β-actin. Cytotoxicity was determined concurrently in an independent set of plates.

The positive control compounds demonstrated an acceptable assay response with a minimum induction of at least 10-fold for the nuclear receptor(s) AhR, CAR, and PPAR-α (respectively, 44.68-fold, 78.31-fold, and 22.87-fold) by their respective positive control compounds 3-methylchloanthrene, phenobarbital and Wyether-14643. In this study, the induction of *Cyp3a1* (PXR) did not meet the 10-fold minimum induction threshold (6.25-fold with the positive control, dexamethasone), however, the specificity of the response for the respective nuclear receptor and the elevated baseline levels of transcript demonstrated the robust nature of the induced response. Therefore, the assay was considered acceptable.

Treatment with tricyclazole did not result in *Cyp1a1*, *Cyp2b1*, *Cyp3a1* or *Cyp4a1* biomarker gene induction responses that were 40% or more of the positive control response at any of the evaluated concentrations. In addition, tricyclazole was not found to be cytotoxic (cell viability was over 70%) at any of the concentrations tested. Based on these results, tricyclazole was considered to be negative for nuclear receptor activation in rat primary hepatocytes (LeBaron & Visconti, 2016).

### (d) Endocrine disruption studies

Using the US EPA ToxCast™ database (data extraction 10 August 2020), tricyclazole tested inactive in:

- four estrogen high-throughput screening (HTS) bioassays – two agonist and two antagonist bioassays in human cell lines;
- five androgen HTS bioassays – two agonist bioassays for protein production in human cell lines and three antagonist bioassays for inactive RNA transcription in human cell lines;
- four thyroid HTS assays – two thyroid receptor (TR) in rat pituitary gland cell lines and two thyroid stimulating hormone receptor (TSHR) assays in a human kidney cell line;
- one steroidogenesis-related HTS assay – aromatase inhibition in human breast cell line).

Tricyclazole was evaluated for its potential to affect the endocrine system by inhibiting the catalytic activity of aromatase, the enzyme responsible for the conversion of androgens to estrogens.

*Study 1*

Five aromatase experiments were performed of which three experiments were considered valid (experiments 1, 3 and 5). In experiments 2 and 4 the full activity controls fell outside the acceptance criteria, so their results were rejected.

Within each experiment, the test item was assayed at eight concentrations (each in triplicate) ranging from 100 pM to 100 µM. Concurrently, a complete dose–response curve for the positive control inhibitor, 4-hydroxy-androstenedione (4-OH-ASDN) was run. Ethanol was used as the vehicle and the concentration of vehicle in the incubations was kept constant at 1% (v/v). The inhibition of the aromatase enzymatic activity was followed by adding to a fixed concentration of 100 nM, tritiated substrate, 4-androstene-3,17-dione (ASDN), to each well.

In experiment 1, 3 and 5 all results obtained for the full activity controls, the background controls and the positive control inhibitor (4-OH-ASDN) were within the acceptance criteria, within

the laboratory historical control data range or accepted after evaluation. Therefore, the results of these aromatase assay experiments were accepted.

The half-maximal inhibitory concentration ($IC_{50}$) lowest points on the dose–response curve (as percentages of control activity) for experiment 1, 3, and 5 were 77.3%, 89.3% and 83.9%, respectively.

No $IC_{50}$ was obtained from any of the three-valid aromatase assay experiments. The average lowest point on the dose–response curve (percentage of control activity) across the replicates was 83.5%. Since this is greater than 75%, the test item was classified as a noninhibitor in the aromatase assay.

In conclusion, three aromatase assay experiments were valid, and tricyclazole was shown not to be an inhibitor in the aromatase assay (Verkaart, 2020).

*Study 2*

In a guideline-compliant, in vitro study, the potential of tricyclazole to modulate the steroidogenic pathway (from cholesterol to production of testosterone and estradiol) was evaluated, using the human cell line H295. While the guideline required three valid experiments, five were performed as the first three did not allow a conclusion to be drawn. All five experiments were determined to be valid since acceptability criteria were met.

In the first four experiments, seven concentrations up to 316 µM tricyclazole were tested for both testosterone and estradiol concentrations, while in the final experiment, five concentrations up to 63.2 µM were tested and only testosterone concentrations were measured. The positive control inducer forskolin (0.1 and 10 µM) and the positive control inhibitor prochloraz (0.1 and 1 µM) were also tested. Dimethylsulfoxide (DMSO) was used as vehicle and the concentration of vehicle in the incubations was kept constant at 0.1% (v/v). A preliminary solubility test was conducted. H295R cells were exposed for 48 hours to the vehicle, test item and positive controls. Immediately after exposure, cell viability was determined using a 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Concentrations of estradiol and testosterone in the exposure medium were determined using commercial enzyme-linked immunosorbent assay systems (ELISAs).

Cytotoxicity was observed in experiment 1 at 316 µM of tricyclazole, and this concentration was therefore excluded from subsequent tests. As a result, the highest tricyclazole concentration tested in experiments 2, 3 and 4 was 100 µM.

In experiment 1, estradiol synthesis was slightly increased (to not more than 1.5-fold control levels) at 0.001 and 100 µM. Testosterone synthesis was decreased at 0.001 and 100 µM. As the results did not show any relationship to dose they were considered equivocal.

In experiment 2 no effects were observed on estradiol synthesis, however there was a statistically significant decrease in testosterone production at 31.6 µM and 100 µM, and the test was therefore considered to be positive for inhibition of testosterone synthesis.

In the experiment 3 no effects were observed on either estradiol or testosterone synthesis, and the results were therefore considered to be negative.

In experiment 4, there was a slight increase in estradiol synthesis at 100 µM, and increased testosterone synthesis at 0.1, 1 and 10 µM. The result were considered to be equivocal for estradiol. While increased testosterone synthesis was observed at three adjacent concentrations, the effects were very minor (not more than 1.3-fold), and there was not a clear dose–response relationship, so the results were considered equivocal.

Effects on testosterone synthesis alone were further investigated in a fifth experiment, where increased testosterone synthesis was observed at 1 and 10 µM. There was no clear dose–response curve, and so the results were considered equivocal.

Overall, the results of this steroidogenic study were considered to be equivocal (Rijik, 2020).

*Study 3*

Tricyclazole (purity 98.2%) was tested in a Hershberger androgen agonistic and antagonistic assay in Wistar rats. A pilot dose range-finding study was conducted, with tricyclazole administered orally by gavage at 0, 30, 60, 120 and 240 mg/kg bw per day to castrated male rats. All rats at 240 mg/kg bw

per day were found moribund on treatment day 7 and were euthanized. Treatment-related effects were observed at 60 and 120 mg/kg bw per day, including dose-related decreases in terminal body weight. On this basis, the main study was conducted at dose levels of 6, 20 and 60 mg/kg bw per day.

Tricyclazole was administered by subcutaneous injection to groups of six castrated male rats, either with or without testosterone propionate, at 0.4 mg/kg bw per day. Control groups included a vehicle control, a positive control group (testosterone propionate), and a group receiving both testosterone propionate and an androgen antagonist (flutamide) that was administered orally at 3.0 mg/kg bw per day. Rats were dosed for ten consecutive days, with dose volume adjusted for most recent body weight. Rats were observed twice daily for mortality, morbidity and clinical signs, and were weighed daily during the treatment period and on the day of necropsy. Food consumption was determined daily. After termination of the study, the following organ weights were measured: ventral prostate, seminal vesicle, levator ani-bulbocavernosus muscle, Cowper's glands, glans penis, liver, kidney, and adrenals.

No mortality, morbidity or adverse clinical signs were observed. Treatment-related reductions compared to controls in food consumption and body weight (by 11%) was observed at 60 mg/kg bw per day from day 7 of treatment. In rats treated with testosterone and tricyclazole at 60 mg/kg bw per day, a slight decrease in body weight (by 8.5%) was observed. While this effect was minimal and not statistically significant, it was considered treatment-related.

Treatment with tricyclazole did not result in an increase in the weight of androgen-dependent tissues when compared to the vehicle control group, indicating no androgenic activity. Combined treatment with testosterone propionate and tricyclazole did not result in changes to the weight of androgen dependent tissues. The positive control group (testosterone propionate and flutamide) showed reduced weights of androgen-dependent tissues. Increased liver weights were observed in all groups treated with tricyclazole, an effect that was considered treatment-related. Slight increases in relative adrenal weight were observed at the high dose. Overall, tricyclazole did not produce either androgenic or anti-androgenic responses in this study (Poshiya, 2020).

*Study 4*

Tricyclazole TGAI, 98.2%, was tested in an uterotrophic estrogen agonistic assay in Wistar rats. In order to select dose levels for a main uterotrophic assay, a pilot dose range-finding study was conducted at dose levels of 0 (vehicle, 1% aqueous CMC), 25, 50 100 and 200 mg/kg bw per day tricyclazole, with intact adult females, under similar conditions to the main uterotrophic assay. A high-dose level of 60 mg/kg bw per day was selected for the main assay based on the resulting moderate treatment-related clinical signs seen in the range-finder, those being, no body weight gain, lower mean body weight, and reduced food consumption observed in animals receiving 100 mg/kg bw per day.

In the main estrogen agonist assay, five groups each composed of six ovariectomized females were treated by oral gavage with the vehicle (1% aqueous CMC), or the test item at dose levels of 6, 20 or 60 mg/kg bw per day, or by subcutaneous injection with the positive control (reference agonist) 17α-ethynyl estradiol at 0.3 µg/kg bw per day. The animals were dosed for three consecutive days and necropsied approximately 24 hours after the final dose administration. Wet and blotted uterine weights, and liver weights were recorded.

No mortality, morbidity or clinical signs were observed. Treatment-related and dose-related effects on body weight, body weight gain and food consumption were observed. At the high dose of 60 mg/kg bw per day animals lost weight compared to the day treatment was initiated, with a corresponding decrease in body weight (by 9%) compared to control (although this was not statistically significant). This was corroborated by a statistically significant decrease in food consumption (by 41% compared to controls). These effects were considered treatment-related and adverse systemic effects of the test item.

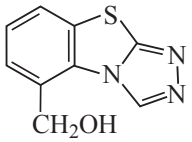
Treatment with tricyclazole did not produce any increase in uterus weight (wet or blotted) indicating no estrogenic effect in the uterotrophic estrogen agonist assay. The negative and the positive control responses were consistent with the expected ranges based on historical control values.

Tricyclazole was found to be negative in an estrogen agonist assay (Poshiya, 2020b).

**(e) Toxicity studies on metabolites**

***Metabolite X355227 (tricyclazole-alcohol)***

***Figure 3. Chemical structure of metabolite X355227 (tricyclazole-alcohol)***




*Study 1*

In an acute oral toxicity study, groups of three fasted female Wistar rats (RCCHan:WIST) (ca 9–11 weeks old at dosing) were given a single oral dose of X355227 (in 0.5% aqueous methyl cellulose) at doses of 300 or 2000 mg/kg bw and observed for 14 days. No mortality was observed in any of the rats treated with at 300 mg/kg bw. Following treatment at 2000 mg/kg bw, all the rats were found dead within 48 hours of dosing. The oral $LD_{50}$ for female rats was therefore between 300 and 2000 mg/kg bw ($LD_{50}$ cut-off value determined as 500 mg/kg bw) (Verma, 2018).

*Study 2*

In an oral (dietary) dose rage-finding study (not GLP), X355227 (purity 98%) was administrated to groups of four female Wistar rats (Crl:WI(Han) at 0, 100, 250 or 625 ppm in the diet (equal to 0, 7.2, 16.6 and 43 mg/kg bw per day) for seven days. Tricyclazole (purity 98.2%) was administrated at 625 ppm (41.7 mg/kg bw per day). There were no treatment-related clinical signs or mortality. A slight and transient reduction in food consumption was observed over the first few days of treatment at 250 and 625 ppm of X355227 and at 625 ppm of tricyclazole, however overall food intake (days 0–7) were not affected. At a dietary level of 625 ppm, slightly elevated liver weights were observed with both X355227 and tricyclazole, which is supported by the results of previous longer term studies conducted with tricyclazole at comparable dose levels (Lina, 2018a).

*Study 3*

A combined repeat-dose oral toxicity study conducted with a reproduction/developmental toxicity screening study was performed using Wistar Han IGS rats (Crl:WI(Han) using tricyclazole, and its plant metabolite X355227. Test substances were administered during a premating period of two weeks (preceded by a two-week pretreatment period), during mating and subsequent postmating up to a total of 39 days of treatment (for male F0 rats), or during subsequent gestation and lactation until LD 22 (for female F0 rats). Pups were sacrificed on LD 21 (the study was essentially based on an OECD 422 study, with an extended lactation period).

The study comprised five groups of 11 male and 11 female animals each. Tricyclazole (purity 98.2%) was administered at 0, 100 and 400 ppm, and metabolite X355227 (98% purity) at 0, 108 and 434 ppm. Dose levels were chosen with reference to doses equimolar to tricyclazole NOAELs (or LOAELs) from existing repeat-dose studies (ca 5 and 20 mg/kg bw per day, respectively).

***Table 17. Achieved mean substance intakes (mg/kg body weight per day) of test substances in a combined repeat-dose oral toxicity with reproduction/developmental toxicity screening study***

| | **Test article/dietary dose groups (ppm)** | | | |
|---|---|---|---|---|
| | **Metabolite X355227** | | **Tricyclazole** | |
| | **108 (low dose)** | **434 (high dose)** | **100 (low dose)** | **400 (high dose)** |
| Premating and postmating males | 6 | 22 | 5 | 20 |
| Premating period females | 7 | 26 | 6 | 23 |
| Gestation period females | 7 | 28 | 7 | 24 |
| Lactation period females | 19 | 74 | 18 | 69 |

Source: Lina, 2018b

The content, homogeneity and stability of the test substances in the diet were confirmed by analysis. Animals were observed daily for clinical signs. A detailed clinical examination was performed

on all adult rats once weekly, although arena testing was not conducted during the last days of pregnancy or first days of lactation. Males and non-mated females were weighed weekly throughout the study, while mated females were weighed on GDs 0, 7, 14 and 20 and on LDs 0, 4, 7, 14 and 21. Food consumption was determined on a per cage basis. Vaginal smears were taken to evaluate state of the estrus cycle during the pretreatment and premating periods, and during mating until confirmation of mating. Blood samples were taken from five rats of each sex per group at terminal sacrifice for examination of haematological and clinical chemistry parameters. Levels of thyroxine (T4) were determined for five males per group and for all females with a litter. Following terminal sacrifice, a gross examination was conducted. Organ weight of all reproductive organs was determined, as well as that of the adrenals, brain, heart, kidneys, liver, spleen, thymus and thyroid. Tissues and all gross lesions were preserved. Reproductive parameters were determined, and pups were observed, with any abnormal behaviour being recorded on LDs 0, 4, 7, 14 and 21. Anogenital distance was measured on LD 4.

There was no mortality and no treatment-related clinical signs or signs of neurotoxicity throughout the study. The overall body weight gain was slightly reduced in females receiving the high dose of tricyclazole during premating and gestation, but there were no significant differences in body weight or consistent differences in food consumption among groups throughout the study.

There were no treatment-related changes in red blood cell or coagulation parameters, or in total and differential white blood cell counts. For the high-dose tricyclazole group, statistically significant increases were noted in sodium concentration in males and in cholesterol concentration in females, however these were within the range of historical control values. No treatment-related changes were observed in T4 levels. The relative weight of the liver compared to controls was increased in both males and females of the 400 ppm tricyclazole group. Relative kidney weight was increased in females at both tricyclazole dose levels. The absolute and the relative weight of the thymus was decreased (statistically significant) in females dosed at 400 ppm of tricyclazole. Macroscopic and microscopic examination of adult animals did not reveal any treatment-related abnormalities. There were no effects due to either test substance on measured fertility parameters: estrus cyclicity, number of pregnant females, precoital time, mating index, fertility index, duration of gestation, or number of implantation sites. There were no effects due to either test substance on litter or pup parameters that were examined: clinical observations, pup viability, pup weights, sex ratio, anogenital distance, nipple retention, macroscopy, thyroid weight.

Based on decreased body weight gain, increased liver weight accompanied by increased cholesterol concentration in the high-dose tricyclazole group, the NOAEL for tricyclazole for parental toxicity was 100 ppm (equal to values ranging from 5 to 18 mg/kg bw per day, at various study phases). The NOEL of tricyclazole for reproduction and development was 400 ppm (equal to values ranging from 20 to 69 mg/kg bw per day), the highest concentration tested. The NOAEL of X355227 (corrected to amounts equimolar to tricyclazole) for parental, reproductive and developmental toxicity was 434 ppm (equal to values ranging from 22 to 74 mg/kg bw per day, at various study phases), the highest concentration tested (Lina, 2018b).

***Table 18a. Key findings of oral dose range-finding study with tricyclazole and X355227, summary of body weight gains; mean ± SD [percentage difference from control] shown***

| Time period | Test article/ dietary dose | | | | |
|---|---|---|---|---|---|
| | Control | Tricyclazole | | X355227 | |
| | 0 ppm | 100 ppm | 400 ppm | 108 ppm [a] | 434 ppm [a] |
| | | | Males | | |
| Days 0–7 | 15.04 ± 3.73 | 16.38 ± 3.53 [+8.9%] | 1 5.72 [–13.1%] | 14.40 ± 6.32 [–4.3%] | 13.45 ± 3.43 [–10.6%] |
| Days 7–14 | 13.09 ± 3.12 | 18.48** ± 2.35 [+41.2%] | 11.05 ± 6.8 [–15.6%] | 15.25 ± 6.28 [+16.5%] | 17.29 ± 3.20 [+32.1%] |
| Days 14–21 | 3.73 ± 3.23 | 1.58 ± 9.59 [–57.6%] | 4.42 ± 3.65 [+18.5%] | 2.39 ± 4.28 [–35.9%] | 3.11 ± 3.45 [–16.6%] |
| Days 21–28 | 10.63 ± 2.94 | 10.18 ± 3.79 [–4.2%] | 8.62 ± 5.48 [–18.9%] | 8.61 ± 4.81 [–19.0%] | 8.02 ± 3.15 [–24.6%] |

| Time period | Test article/ dietary dose | | | | |
|---|---|---|---|---|---|
| | Control | Tricyclazole | | X355227 | |
| | 0 ppm | 100 ppm | 400 ppm | 108 ppm [a] | 434 ppm [a] |
| Days 28–35 | 11.15 ± 4.35 | 7.7 ± 9.64 [–30.9%] | 10.55 ± 3.59 [–5.4%] | 10.99 ± 4.28 [–1.4%] | 11.60 ± 4.84 [+4.0%] |
| Days 0–35 | 53.64 ± 10.12 | 54.33 ± 21.84 [+1.3%] | 47.71 ± 14.09 [–11.1%] | 51.65 ± 19.81 [–3.7%] | 53.47 ± 12.08 [–0.3%] |
| | | | **Females** | | |
| Days 0–7 | 23.66 ± 4.86 | 22.38 ± 5.42 [–5.4%] | 17.38 ± 3.05 [–26.5%] | 19.81 ± 10.48 [–16.3%] | 20.63 ± 2.93 [–12.8%] |
| Days 7–14 | 17.4 ± 3.11 | 18.67 ± 4.15 [+7.3%] | 18.19 ± 3.88 [+4.5%] | 19.37 ± 6.35 [+11.3%] | 19.81 ± 4.07 [+13.9%] |
| Days 14–21 | 61.65 ± 6.46 | 58.95 ± 9.4 [–4.4%] | 53.4 ± 6.52 [–13.4%] | 61.84 ± 8.49 [+0.3%] | 52.60 ± 11.55 [–14.7%] |
| Days 0–20 | 102.71 ± 7.36 | 100 ± 16.11 [–2.6%] | 88.97 ± 8.94 [–13.4%] | 101.02 ± 14.64 [–1.6%] | 93.04 ± 11.78 [–9.4%] |

SD: Standard deviation; Source : Lina, 2018b

[a] 108 and 434 ppm of X355227 (mw 205.24) represent amounts equimolar to 100 and 400 ppm of tricyclazole (mw 189.24)

** Significant difference from controls using Kruskal-Wallis & Dunnett on Ranks at $p < 0.01$

***Table 18b. Key findings of oral dose range-finding study with tricyclazole and X355227, summary of significant clinical chemistry parameters; mean ± SD [percentage difference from control] shown***

| Parameter | Sex | Test article/ dietary dose | | | | | HCD range |
|---|---|---|---|---|---|---|---|
| | | Control | Tricyclazole | | X355227 | | |
| | | 0 ppm | 100 ppm | 400 ppm | 108 ppm [a] | 434 ppm [a] | |
| **Sodium**: day 39 (mmol/L) | M | 144.2 ± 1.9 | 144.6 ± 1.1 [+0.3%] | 147.4** ± 1.1 [+2.2%] | 145.5 ± 0.6 [+0.9%] | 144.4 ± 1.1 [+0.1%] | 139.8–147.6 |
| **Cholesterol**: day 22 (mmol/L) | F | 1.977 ± 0.561 | 1.996 ± 0.517 [+1%] | 2.55* ± 0.348 [+29%] | 1.839 ± 0.392 [–7.0%] | 2.232 ± 0.115 [+12.9%] | 1.69–2.896 |

M: Male; F: Female; SD: Standard deviation; HCD: Historical control data; Source : Lina, 2018b

Significant difference from controls using Ancova/Anova & Dunnett's test at: * $p < 0.05$; ** $p < 0.01$;

[a] 108 and 434 ppm of X355227 (mw 205.24) represent amounts equimolar to 100 and 400 ppm of tricyclazole (mw 189.24)

***Table 18c. Key findings of oral dose range-finding study with tricyclazole and X355227, summary of relative organ weights in males and females***

| Organ weights | Test article/ dietary dose | | | | | HCD range |
|---|---|---|---|---|---|---|
| | Control | Tricyclazole | | X355227 | | |
| | 0 ppm | 100 ppm | 400 ppm | 108 ppm [a] | 434 ppm [a] | |
| **Kidney weights** | | | | | | |
| Relative in males (g) mean ± SD [% difference from control] | 5.541 ± 0.367 | 5.934 ± 0.447 [+7.1%] | 5.965 ± 0.431 [7.7%] | 5.954 ± 0.186 [+7.5%] | 5.95 ± 0.451 [+7.4%] | 5.622–6.285 |
| Relative in females (g) mean ± SD [% difference from control] | 6.779 ± 0.427 | 7.673 ± 0.501** [+13.2%] | 7.754 ± 0.219** [14.4%] | 6.933 ± 0.262 [+2.3%] | 7.036 ± 0.379 [+3.8%] | 5.860–6.519 |

| | Test article/ dietary dose | | | | | HCD range |
|---|---|---|---|---|---|---|
| | Control | Tricyclazole | | X355227 | | |
| Organ weights | 0 ppm | 100 ppm | 400 ppm | 108 ppm [a] | 434 ppm [a] | |
| **Liver weights** | | | | | | |
| Relative in males (g)<br>mean ± SD<br>[% difference from control] | 22.4<br>± 1.05 | 23.93<br>± 1.94<br>[+6.8%] | 26.22<br>± 1.73<br>[+17.1%] | 25.44<br>± 4.11<br>[+13.6%] | 24.22<br>± 1.33<br>[+8.1%] | 23.25–30.76 |
| Relative in females (g)<br>mean ± SD<br>[% difference from control] | 40.07<br>± 2.34 | 40.42<br>± 3.76<br>[+0.9%] | 45.66<br>± 2.09**<br>[+14%] | 39.93<br>± 1.34<br>[↓0.3%] | 43.31<br>± 1.76<br>[+8.1%] | 32.95–47.02 |

M: Male; F: Female; SD: Standard deviation; HCD: Historical control data; Source : Lina, 2018b
** Different from controls with statistical probability $p < 0.01$;

[a] 108 and 434 ppm of X355227 (mw 205.24) represent amounts equimolar to 100 and 400 ppm of tricyclazole (mw 189.24)

***Table 19. Genotoxicity studies on metabolite X355227***

| Type of study | Organism/cells | Dose range tested | Purity | Result | Reference |
|---|---|---|---|---|---|
| | | **In vitro genotoxicity tests** | | | |
| In vitro bacterial reverse mutation | *Salmonella typhimurium*, G46, TA1537, TA1535, TA98, TA100 and TA102 | Test 1<br>156.25, 312.5, 625, 1250, 2500 and 5000 µg/plate (± S9)<br>Test 2<br>51.2, 128, 320, 800, 2000 and 5000 µg/plate (± S9) | 99% | Negative | Tekale, 2017 |
| In vitro gene mutation assay | Chinese hamster ovary CHO-K1 cell line | 125, 250, 500, 1000 and 2000 µg/mL, (± S9) | 99% | Negative | Desphand, 2017 |
| In vitro mammalian cytogenetics – micronucleus test | Human peripheral lymphocytes | 100, 200, 400, 800 and 1200 µg/mL , (± S9) | 99% | Negative | Nagane, 2017 |

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*

Under the conditions of these tests, X355227 was not genotoxic.

***Metabolite X12691895***

***Figure 4. Chemical structure of metabolite X12691895***

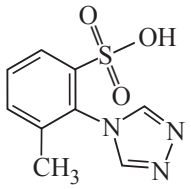



***Table 20. Genotoxicity studies on X12691895***

| Type of study | Organism/cells | Dose range tested | Purity | Result | Reference |
|---|---|---|---|---|---|
| **In vitro genotoxicity tests** | | | | | |
| In vitro bacterial reverse mutation | *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2 *uvr*A, (± S9) | 33.3, 66.7, 100, 333, 667, 1000, 3333 and 5000 µg/plate | 99% | Negative | Myhre, 2020a |
| In vitro mammalian cytogenetics – micronucleus test | Human peripheral lymphocytes (± S9) | 100, 250, 500, 1000, 1500 and 2000 µg/mL | 99% | Negative | Kellum, 2020a |

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*

Under the conditions of these tests, X12691895 was not genotoxic.

***Metabolite X484574***

***Figure 5. Chemical structure of metabolite X484574***

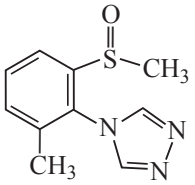



***Table 21. Genotoxicity studies on X484574***

| Type of study | Organism/cells | Dose range tested | Purity | Result | Reference |
|---|---|---|---|---|---|
| **In vitro genotoxicity tests** | | | | | |
| In vitro bacterial reverse mutation | *Salmonella typhimurium* TA98, TA100, TA1535 and TA 1537 and *Escherichia coli* WP2 *uvr*A , (± S9) | 333, 667, 1000, 3333, and 5000 µg/plate | 99% | Negative | Myhre, 2020b |
| In vitro mammalian cytogenetics – micronucleus test | Human peripheral lymphocytes (± S9) | 100, 250, 500, 1000, and 2000 µg/mL | 99% | Negative | Kellum, 2020b |

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*

Under the conditions of these tests, X484574 was not genotoxic.

***Metabolite X11967410***

***Figure 6. Chemical structure of metabolite X11967410***

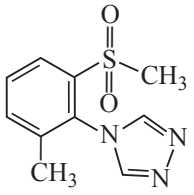


***Table 22. Genotoxicity studies on X11967410***

| Type of study | Organism/cells | Dose range tested | Purity | Result | Reference |
|---|---|---|---|---|---|
| | | ***In vitro* genotoxicity tests** | | | |
| In vitro bacterial reverse mutation | *Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA 1537 (± S9) | Trial 1<br>39, 78, 156, 312, 625 and 1250 µg/plate, (± S9)<br>Trial 2<br>12, 32, 80, 200 and 500 µg/plate, (± S9) | 99% | Negative | Pillai, 2009 |
| In vitro gene mutation assay | Chinese hamster ovary CHO-K1-BH4 cell line | Test 1<br>75, 150, 300, 600, 1200, and 2400 µg/mL, (± S9)<br>Test 2<br>100, 200, 400, 800, 1600 and 2400 µg/mL, (± S9) | 99% | Not mutagenic | Nagane, 2012a |
| In vitro chromosome aberration test | Human peripheral lymphocytes | Phase 1 150, 300, 600, 1200 and 2400 µg/mL, (± S9)<br>Phase 2 150, 300, 600, 1200 and 2400 µg/mL, (± S9) | 99% | Negative | Nagane, 2012b |

S9: Rat liver supernatant fraction obtained by centrifuging at 9000 *g*

Under the conditions of these tests, X11967410 was not genotoxic.

## 3. Observations in humans

**Medical surveillance on manufacturing plant personnel**

Tricyclazole is a systemic fungicide for the control of rice blast disease (*Pyricularia oryzae)*. It has been manufactured by Dow AgroSciences Ihara (Japan) until June 2004 and under contract by Ihara Chemical (Japan) since early 2004. Review of available medical surveillance data from the DAS Ihara site is as follows: annual medical examinations of 22 manufacturing workers including blood tests disclosed no abnormalities. Annual medical exams including blood tests of 13 Beam 75 WP formulation and packaging workers disclosed no abnormalities. Tricyclazole is being manufactured at Nagarjuna Agrichem Limited (India) since 1998 with 34 workers at the tricyclazole manufacturing plant and is being manufactured at Deccan Fine Chemicals Limited (India) since April 2011 with 20 workers in the tricyclazole manufacturing plant. The workers at both Nagarjuna and Deccan sites have 100% participation in the voluntary health surveillance which revealed no adverse effects.

## 4. Microbiological aspects

There was no information available in the public domain and no experimental data were submitted that addressed the possible impact of tricyclazole residues on the human intestinal microbiome.

# Comments

## Biochemical aspects

Following administration of $^{14}$C-labelled tricyclazole as a single oral gavage dose to rats at 2 or 40 mg/kg bw, tricyclazole was readily and rapidly absorbed, and this was followed by rapid and extensive biphasic elimination (Andre, 2004). Following dosing at 2, 7, 28 or 50 mg/kg bw, the maximum plasma concentration ($C_{max}$) was achieved between five minutes (at 7 mg/kg bw) and one hour (at 50 mg/kg bw) post dose, with the plasma concentration curves and area under the concentration–time curve (AUC) indicating that absorption starts to saturate from 28 mg/kg bw upwards (Dubnicka, 2017a). Following oral and intravenous dosing at 2 mg/kg bw, the AUCs for 0–96 hours for the two methods of delivery were 11.7 µg.hour/mL and 12.5 µg.hour/mL, respectively (Johnson, 2004). The total absorption of the orally administered dose was at least 90% at 2 mg/kg bw. Only between 0.85% and 2.0% of the orally administered [$^{14}$C]tricyclazole, whether administered at 2 or 40 mg/kg bw, remained in the tissues after 168 hours, with some affinity for red blood cells (RBCs) being apparent. Absorbed tricyclazole was extensively metabolized with 29 metabolites found in faeces and urine. Tricyclazole alcohol was the most significant metabolite identified in animal studies, along with derivatives of tricyclazole acid, however each of these were present individually at less than 5% of the administered radioactivity. In combination, tricyclazole alcohol and its three derivatives were present as metabolites totalling 13% of administered dose (AD). The terminal elimination phase was 34–51 hours. Between 31% and 61% of orally administered radiolabel was excreted in the urine, with slightly higher recoveries in females; faecal elimination of radiolabel was 39%–65%. Following intravenous dosing, 35% to 47% of the dose was excreted in urine, with up to 58% excreted in faeces, indicating extensive biliary elimination.

No potential for accumulation was demonstrated in the available studies.

## Toxicological data

The acute oral median lethal dose ($LD_{50}$) of tricyclazole was 237 mg/kg bw (Durando, 2005a) and the dermal $LD_{50}$ was greater than 5000 mg/kg bw (Durando, 2005b). The inhalation median lethal concentration ($LC_{50}$) of tricyclazole was greater than 2.58 mg/L (Blagden, 1998). Tricyclazole was not irritating to the skin or eyes of rabbits (Durando, 2005), and was not skin sensitizing in the Guinea pig maximization or mouse local lymph node assay (LLNA) (Prinsen, 2003b; Woolhiser & Wiescinski, 2005).

In repeat-dose toxicity studies with tricyclazole, effects on body weight, body weight gain and on food consumption were commonly seen in rats, mice and dogs. The liver was the target organ in rats, mice and dogs, with changes in clinical chemistry and increases in liver weight.

In a preGLP, 90-day dietary toxicity study in mice, tricyclazole was administered at concentrations of 0, 400, 1000, 2500 or 3600 ppm (equivalent to 0, 60, 150, 375 and 540 mg/kg bw per day). The NOAEL was 400 ppm (equivalent to 60 mg/kg bw per day), based on mortality at 1000 ppm (equivalent to 150 mg/kg bw per day) (Worth et al., 1978a).

In a six-month preGLP toxicity study, tricyclazole was administered via the diet to mice at concentrations of 0, 310, 800, 1900 or 3000 ppm (equivalent to 0, 47. 120, 285 and 450 mg/kg bw per day). A NOAEL of 310 ppm (equivalent to 47 mg/kg bw per day) was identified based on increased absolute and relative liver weights and proliferation of small bile ducts at 800 ppm (equivalent to 120 mg/kg bw per day) and above (Howard & Owen, 1979a).

In a 90-day, preGLP dietary toxicity study in rats, tricyclazole was administered at dietary concentrations of 0, 282, 635 or 1640 ppm (equal to 0, 21, 47 and 180 mg/kg bw per day for males, 0, 23, 54 and 190 mg/kg bw per day for females). The NOAEL was 282 ppm (equal to 21 mg/kg bw per day) based on decreased body weight at 635 ppm (equal to 47 mg/kg bw per day) (Worth et al., 1978b).

In a one-year oral toxicity study in dogs, tricyclazole was administered in gelatine capsules at dose levels of 0, 5, 15 or 45 mg/kg bw per day. The NOAEL was 15 mg/kg bw per day, based on decreased body weight and increased liver and kidney weights, at 45 mg/kg bw per day (Holzhausen, 1986).

In a 12-month preGLP oral study in mice, tricyclazole was administered at dietary concentrations of 50, 140, 400 or 620 ppm (equivalent to 0, 7.5, 21, 60 and 93 mg/kg bw per day). The NOAEL was 620 ppm (equivalent to approximately 93 mg/kg bw per day), the highest dose tested (Howard & Owen, 1979b).

In a two-year, preGLP dietary study, mice were treated at dietary concentrations of 0, 50, 140 or 400 ppm (equivalent to approximately 0, 3.5, 10 and 29 mg/kg bw per day for males, 0, 3.6, 10.4, and 30 mg/kg bw per day for females). The NOAEL was 400 ppm (equivalent to approximately 29 mg/kg bw per day), the highest dose tested The NOAEL was 620 ppm (equivalent to approximately 93 mg/kg bw per day), the highest dose tested (Howard et al., 1978).

In a 95-week study, mice were administered tricyclazole in the diet at 0, 25, 75, 250 or 1000 ppm (equal to 0, 2.59, 7.98, 24.9 and 101 mg/kg bw per day for males, 0, 2.2, 6.67, 21.8 and 91 mg/kg bw per day for females). The NOAEL was 75 ppm (equal to 6.67 mg/kg bw per day) based on increased liver weight and periportal hepatocellular fatty change and hepatocyte degeneration, observed at 250 ppm (equal to 21.8 mg/kg bw per day). The NOAEL for carcinogenic effects was 1000 ppm (equal to 91 mg/kg bw per day), the highest dose tested (Harada, 1985).

In a two-year dietary study, carried out before internationally agreed GLP or guidelines, rats were administered tricyclazole at 0, 100, 275 or 620 ppm (equal to 0, 4.1, 11 and 26 mg/kg per day for males, 0, 5.3, 16, and 40 mg/kg per day for females). The NOAEL was 275 ppm (equal to 11 mg/kg bw per day) based on body weight decreases at 620 ppm (equal to 26 mg/kg bw per day). There were no treatment-related increases in neoplasms in any treated group (Howard et al., 1977; Stebbins, Golden & McFadden, 2016).

In a two-year dietary study, rats were administered tricyclazole at 0, 40, 126 or 400 ppm (equal to 0, 1.8, 5.9 and 18 mg/kg bw per day for males, 0, 2.3, 7.1 and 22 mg/kg bw per day for females). The NOAEL was 126 ppm (equal to 5.9 mg/kg bw per day), based on suppression of body weight gain and reduced food consumption in females, and suppression of body weight gain in males, at 400 ppm (equal to 18 mg/kg bw per day). There was no increase in treatment-related neoplasms in any treated group (Heward, 2020).

The overall NOAEL for chronic toxicity in rats was 275 ppm (equal to 11 mg/kg bw per day) based on body weight decreases at 400 ppm (equal to 18 mg/kg bw day). The overall NOAEL for carcinogenicity was 620 ppm (equal to 26 mg/kg bw), the highest dose tested.

The Meeting concluded that tricyclazole is not carcinogenic in mice or rats.

Tricyclazole was tested for genotoxicity in an adequate range of in vitro and in vivo assays. It was positive in two in vitro mouse lymphoma assays (Steenwinkel, 2003, 2004), however no mutagenicity was observed in the in vivo mutagenicity test (lacZ bacteriophage mouse transgenic rodent assay; Matsumori, 2017), and tricyclazole was negative in an in vivo mouse micronucleus assay (Kehr, Parton & Garriott, 1988).

The Meeting concluded that tricyclazole is unlikely to be genotoxic in vivo.

In view of the lack of genotoxicity in vivo and the absence of carcinogenicity in mice and rats, the Meeting concluded that tricyclazole is unlikely to pose a carcinogenic risk to humans at levels occurring in the diet.

In a two-generation reproductive toxicity study in rats administered tricyclazole at 0, 30, 100 or 400 ppm in the diet (equivalent to 0, 2.1, 7.0 and 27.7 mg/kg bw per day for males, 0, 2.3, 7.0 and 30.6 mg/kg bw per day for females) the NOAEL for parental toxicity was 100 ppm (equivalent to 7.0 mg/kg bw per day) based on decreased mean body weight in males and females in the F0 and F1 generations, along with decreased mean body weight gain, decreased food consumption and increased liver weight (absolute and relative) in F0 and F1 parental animals at 400 ppm (equivalent to 27.7 mg/kg bw per day). The NOAEL for reproductive toxicity was 400 ppm (equivalent to 28 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 100 ppm (equivalent to 7.0 mg/kg bw per day), as decreases in body weight gain and delayed sexual maturity in the pups were observed at 400 ppm (equivalent to 28 mg/kg bw per day) (Wolterbeek, 2004a).

In a developmental toxicity study, rats were dosed daily with tricyclazole by gavage at 0, 5,

20 or 50 mg/kg bw per day from gestation day (GD) 0 to GD 20. The NOAEL for maternal toxicity was 5 mg/kg bw per day based on suppressed body weight gain and food consumption at 20 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 20 mg/kg bw per day due to incomplete ossifications at 50 mg/kg bw per day (Wolterbeek, 2004b).

In a developmental toxicity study, rabbits were dosed daily by oral gavage with tricyclazole at 0, 7.5, 25 or 75 mg/kg bw per day from GD 7 to GD 28. The maternal NOAEL was 25 mg/kg bw per day based on lower mean body weight gains and reduced food consumption at 75 mg/kg bw per day. The embryo/fetal NOAEL was 25 mg/kg bw per day based on lower mean fetal weight at 75 mg/kg bw per day (Knapp, 2009).

The Meeting concluded that tricyclazole is not teratogenic.

No specific neurotoxicity studies were conducted, however no signs of neurotoxicity were observed in other acute or repeat-dose studies.

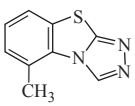
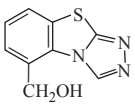
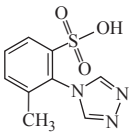
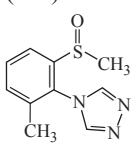
The Meeting concluded that tricyclazole is unlikely to be neurotoxic.

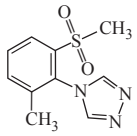
No specific data were submitted regarding immunotoxicity, but no evidence of immunotoxicity was reported in routine toxicological studies with tricyclazole.

The meeting concluded that tricyclazole is unlikely to be immunotoxic.

## Toxicological data on metabolites and/or degradates

***Table 22. Summary of toxicological characterization of plant/livestock metabolites***

| **Compound, codes and structure** | **Major rat metabolite** (> 10% AD) | **Genotoxicity assessment** (data, QSAR, read-across) | **General toxicity** | **Toxicological reference values** |
|---|---|---|---|---|
| **Tricyclazole**  | Parent | Not genotoxic in vivo (data) | Full data set | ADI: 0–0.05 mg/kg bw<br>ARfD: 0.05 mg/kg bw |
| **X355227** Tricyclazole-alcohol,  | While tricyclazole alcohol and derivative made up more than 13% of AD, additional toxicity tests were also conducted | Not genotoxic (in vitro tests) | Oral $LD_{50}$ between 200 and 300 mg/kg bw<br>Combined repeat-dose oral toxicity with screening for reproductive/ developmental toxicity indicated NOAEL of 22 mg/kg bw per day, compared with parent at 5 mg/kg bw per day in the same study | Covered by parent based on lower toxicity of metabolite |
| **X12691895** (M3)  | No | Not genotoxic (in vitro tests) | | Limit to Cramer class III TTC: 1.5 µg/kg bw per day |
| **X484574** (M1)  | No | Not genotoxic (in vitro tests) | | Limit to Cramer class III TTC: 1.5 µg/kg bw per day |

| Compound, codes and structure | Major rat metabolite (> 10% AD) | Genotoxicity assessment (data, QSAR, read-across) | General toxicity | Toxicological reference values |
|---|---|---|---|---|
| **X11967410** (Metabolite D)  | No | Not genotoxic (in vitro tests) | | Limit to Cramer class III TTC: 1.5 µg/kg bw per day |

QSAR Quantitative structure–activity relationship; TTC Threshold of toxicological concern; AD Administered dose; ADI Acceptable daily intake; ARfD Acute reference dose

Finally, some of the common triazole metabolites, including triazole-alanine, triazole-acetic acid and triazole-lactic acid were observed at variable levels in crops but not in rat absorption, distribution, metabolism and excretion studies with tricyclazole. No further data on these metabolites was presented in the dossier. However, as reference doses have been previously established for these triazole metabolites by JMPR in 2008, additional toxicological consideration was not thought necessary at this time.

The Meeting concluded that metabolite X355227 (tricyclazole-alcohol) is toxicologically relevant and not of greater potency than the parent The Meeting concluded that metabolites X484574, X12691895 and X11967410 were not genotoxic, but could not conclude on the toxicological relevance of these metabolites. On this basis, they are considered to fall within Cramer class III for toxicity.

## Microbiological aspects

There was no information available in the public domain and no experimental data were submitted that addressed the possible impact of tricyclazole residues on the human intestinal microbiome.

## Human data

In reports on manufacturing plant personnel, no adverse health effects have been noted.

The Meeting concluded that the existing database on tricyclazole was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

# Toxicological evaluation

The Meeting established an ADI of 0–0.05 mg/kg bw based on the NOAEL of 5 mg/kg bw per day in the rat development study and using a safety factor of 100. This was supported by the NOAEL of 6.7 mg/kg bw per day observed in a 95-week study in mice. This ADI also applies to tricyclazole alcohol.

The Meeting established an ARfD of 0.05 mg/kg bw based on the NOAEL of 5 mg/kg bw per day in the rat development study that showed suppressed maternal body weight gain, seen in the first three days of the study. A safety factor of 100 was used. This ARfD also applies to tricyclazole alcohol.

***Levels relevant to risk assessment of tricyclazole***

| Species | Study | Effect | NOAEL | LOAEL |
|---|---|---|---|---|
| Mouse | 95-week study of toxicity and carcinogenicity [a] | Toxicity | 75 ppm, equal to 6.67 mg/kg bw per day | 250 ppm, equal to 21.8 mg/kg bw per day |
| | | Carcinogenicity | 1000 ppm, equal to 91 mg/kg bw per day [c] | - |

| Species | Study | Effect | NOAEL | LOAEL |
|---|---|---|---|---|
| Rat | Two-year studies of toxicity and carcinogenicity [a, d] | Toxicity | 275 ppm, equal to 11 mg/kg bw per day | 400 ppm, equal to 18 mg/kg bw per day |
| | | Carcinogenicity | 620 ppm, equal to 26 mg/kg bw per day [c] | - |
| | Two-generation study of reproductive toxicity [a] | Reproductive toxicity | 400 ppm, equivalent to 27.7 mg/kg bw per day [c] | - |
| | | Parental toxicity | 100 ppm, equivalent to 7.0 mg/kg bw per day | 400 ppm, equivalent to 27.7 mg/kg bw per day |
| | | Offspring toxicity | 100 ppm, equivalent to 7.0 mg/kg bw per day | 400 ppm, equivalent to 27.7 mg/kg bw per day |
| | Developmental toxicity study [b] | Maternal toxicity | 5 mg/kg bw per day [e] | 20 mg/kg bw per day |
| | | Embryo/fetal toxicity | 20 mg/kg bw per day | 50 mg/kg bw per day |
| Rabbit | Developmental toxicity study [b] | Maternal toxicity | 25 mg/kg bw per day | 75 mg/kg bw per day |
| | | Embryo/fetal toxicity | 25 mg/kg bw per day | 75 mg/kg bw per day |
| Dog | One-year studies of toxicity [f] | Toxicity | 15 mg/kg bw per day | 45 mg/kg bw per day |
| **Tricyclazole alcohol** | | | | |
| Rat | Combined repeat oral dosing with reproduction/ development toxicity screening [a] | Parental, reproduction and developmental toxicity | 22 mg/kg bw per day [c] | - |

[a] Dietary administration; [b] Gavage administration; [c] Highest dose tested; [d] Two or more studies combined; [e] Lowest dose tested; [f] Capsule administration

*Acceptable daily intake (ADI) applies to tricyclazole and tricyclazole alcohol, expressed as tricyclazole*

0–0.05 mg/kg bw

*Acute reference dose (ArfD) applies to tricyclazole and tricyclazole alcohol, expressed as tricyclazole*

0.05 mg/kg bw

## Information that would be useful for the continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure.

***Critical end-points for setting guidance values for exposure to tricyclazole***

| | |
|---|---|
| **Absorption, distribution, excretion and metabolism in mammals** | |
| Rate and extent of oral absorption | Fast absorption ($T_{max}$ ca 10–30 minutes)<br>Almost completely absorbed (ca 89% in males, 96% females) |
| Dermal absorption | No data |
| Distribution | Distributed according to relative organ blood flow;<br>highest amounts in liver and kidney, with lower amounts in plasma, stomach and bone marrow |
| Potential for accumulation | No evidence of accumulation |
| Rate and extent of excretion | Urinary and faecal elimination, via the bile were the primary routes of elimination (ca 97%);<br>urinary excretion (ca 27–48%), biliary excretion (58%) |
| Metabolism in animals | Primary metabolite is tricyclazole alcohol |
| Toxicologically significant compounds in animals and plants | Tricyclazole, tricyclazole alcohol, X11967410 (metabolite D) |
| **Acute toxicity** | |
| Rat, $LD_{50}$, oral | 237 mg/kg bw |
| Rat, $LD_{50}$, dermal | > 2000 mg/kg bw |
| Rat, $LC_{50}$, inhalation | > 2.58 mg/L |
| Rabbit, dermal irritation | Not irritating |
| Rabbit, ocular irritation | Not irritating |
| Guinea pig, dermal sensitization | Not sensitizing (Magnusson & Kligman/Buehler) |
| **Short-term studies of toxicity** | |
| Target/critical effect | Decreased body weight (mouse, rat, dog)<br>Liver; increased organ weight and proliferation of small bile ducts (mouse) |
| Lowest relevant oral NOAEL | 15 mg/kg bw per day (dog) |
| Lowest relevant dermal NOAEL | 300 mg/kg bw per day (rat) . |
| **Long-term studies of toxicity and carcinogenicity** | |
| Target/critical effect | Mouse: liver (increased organ weights, periportal hepatocellular fatty change, peripheral acidophilic degeneration of hepatocytes)<br>Rat: reduced body weights, and feed consumption<br>Liver (slight increase in bile duct hyperplasia) |
| Lowest relevant NOAEL | 6.67 mg/kg bw per day (mouse) |
| Carcinogenicity | Not carcinogenic |
| **Genotoxicity** | Unlikely to be genotoxic |
| **Reproductive toxicity** | |
| Target/critical effect | Parental effects: decreased body weight, body weight gain, and food consumption (both sexes, rat), increased liver weight (both sexes, rat)<br>Reproductive effects: none<br>Developmental effects: reduced pup weights, delayed maturity |
| Lowest relevant parental NOAEL | 100 ppm, equal to 7.0 mg/kg bw per day (rat) |
| Lowest relevant offspring NOAEL | 100 ppm, equal to 7.0 mg/kg bw per day (rat) |
| Lowest relevant reproductive NOAEL | 400 ppm, equal to 28 mg/kg bw per day (rat) |

| | |
|---|---|
| **Developmental toxicity** | |
| Target/critical effect | Rat:<br>Maternal effects: decreased body weight, body weight gain, feed consumption and adjusted body weight<br>Developmental effects: decreased body weight, delayed ossification<br>Rabbit:<br>Maternal effects: effects on body weight gain and feed consumption<br>Developmental effects: lower mean fetal weight<br>No teratogenic effects |
| Lowest relevant maternal NOAEL | 5 mg/kg bw per day (rat) |
| Lowest relevant embryo/fetal NOAEL | 20 mg/kg bw per day (rat) |
| **Neurotoxicity** | No specific neurotoxicity studies were conducted, however no signs of neurotoxicity were observed in other routine studies |
| **Other toxicological studies** | |
| Immunotoxicity | No data |
| **Studies on toxicologically relevant metabolites** | |
| Tricyclazole alcohol | Acute oral $LD_{50}$: between 200 and 300 mg/kg bw (rat)<br>Combined dietary and reproduction study basis for a NOAEL: 22 mg/kg bw per day (rat)<br>Not genotoxic (Ames, micronucleus in vitro) |
| X484574 (M1), X12691895 (M3) | Not genotoxic (Ames, micronucleus in vitro) |
| X11967410 (Metabolite D) | Not genotoxic (Ames, micronucleus in vitro) |
| **Microbiological data** | No data submitted |
| **Human data** | No clinical cases or poisoning incidents have been recorded |

## Summary

| | Value | Study | Safety factor |
|---|---|---|---|
| ADI | 0–0.05 mg/kg bw [a] | Rat developmental study | 100 |
| ARfD | 0–0.05 mg/kg bw [a] | Rat developmental study | 100 |

[a] Applies to tricyclazole and tricyclazole alcohol, expressed as tricyclazole

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# Annex 1

## 2023 Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group on Pesticide Residues

**19–28 September 2023**

**List of WHO participants**

***WHO Experts***

**Prof Alan R. Boobis**, National Heart & Lung Institute, Imperial College London, London United Kingdom of Great Britain and Northern Ireland

**Dr Jessica Broeders**, Board for the Authorisation of Plant Protection Products and Biocides (Ctgb), Kingdom of the Netherlands

**Dr Susy Brescia**, Health & Safety Executive, Chemicals Regulation Division (CRD), Bootle, United Kingdom of Great Britain and Northern Ireland

**Ms Marloes Busschers**, Courcenay, Mardore (Thizy les Bourgs), France, (*WHO Rapporteur*)

**Dr Rhian Cope**, Australian Pesticides and Veterinary Medicines Authority, Armidale, NSW, Australia

**Dr Amélie Crépet**, French Agency for Health and Safety, Maisons-Alfort Cedex, France

**Mr Peter Cressey**, Institute of Environmental Science and Research Limited (ESR), Christchurch, New Zealand

**Dr Stuart Creton**, Food Standards Australia New Zealand, Wellington, New Zealand

**Dr Ian Dewhurst**, Leavening, North Yorkshire, United Kingdom of Great Britain and Northern Ireland

**Dr Hubert Dirven**, Norwegian Institute of Public Health, Oslo, Norway

**Dr Silvana Gorniak**, University of São Paulo, São Paulo, Brazil

**Ms Tracy Hambridge**, Food Standards Australia New Zealand, Kingston, ACT, Australia

**Mrs Judith de Heer**, National Institute for Public Health and the Environment (RIVM), Bilthoven, Kingdom of the Netherlands (did not attend Washington meeting)

**Dr Salmaan H. Inayat-Hussain**, Product Stewardship and Toxicology Section, Petroliam Nasional Berhad, Kuala Lumpur, Malaysia

**Dr Debabrata Kanungo**, Faridabad-121012, India

**Dr Sheila Logan**, Australian Pesticides and Veterinary Medicines Authority, Armidale, NSW, Australia (attended via digital link)

**Ms Kimberley Low**, Health Evaluation Directorate, Pest Management Regulatory Agency, Ottawa, Ontario, Canada

**Dr Glenn Lurman**, German Federal Institute for Risk Assessment (BfR), Berlin, Germany

**Dr Elizabeth Mendez**, United States Environmental Protection Agency, Washington DC, United States of America

**Prof Angelo Moretto**, University of Padova, Occupational Health Unit, Padova University Hospital, Padova, Italy (*Chair*)

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**Dr Midori Yoshida**, Setagaya-ku, Tokyo, Japan

**Dr Katsuhiko Yoshizawa**, Mukogawa Women's University, Hyogo, Japan

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