CYFLUMETOFEN

First draft prepared by
Emanuel Hänggi[1] and Maria Tasheva[2]

[1] Federal Food Safety and Veterinary Office, Zurich, Switzerland
[2] Associate Professor Toxicologist, Consultant, Bulgarian Food Safety Agency, Sofia, Bulgaria

Explanation....43
Evaluation for acceptable daily intake....44
1. Biochemical aspects....44
1.1 Absorption, distribution and excretion....44
(a) Oral route....44
(b) Dermal route....50
1.2 Biotransformation....50
2. Toxicological studies....52
2.1 Acute toxicity....52
(a) Lethal doses....52
(b) Dermal irritation....54
(c) Ocular irritation....54
(d) Dermal sensitization....54
2.2 Short-term studies of toxicity....55
(a) Oral administration....55
(b) Dermal application....61
2.3 Long-term studies of toxicity and carcinogenicity....63
2.4 Genotoxicity....69
(a) In vitro studies....69
(b) In vivo studies....71
2.5 Reproductive and developmental toxicity....71
(a) Multigeneration studies....71
(b) Developmental toxicity....75
2.6 Special studies....77
(a) Neurotoxicity....77
(b) Immunotoxicity....78
(c) Toxicity of metabolites....79
(d) Mode of action for Leydig cell adenoma....84
(e) Other special studies....87
3. Observations in humans....88
Comments....89
Toxicological evaluation....94
References....97

Explanation

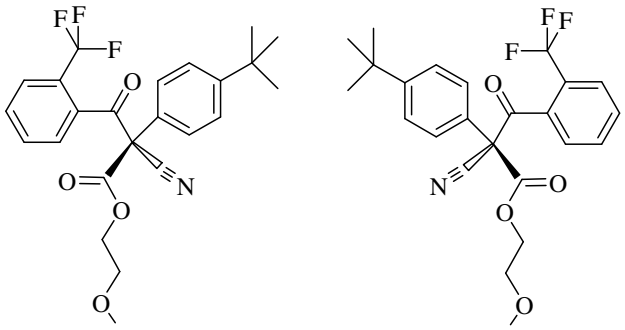
Cyflumetofen is the International Organization for Standardization (ISO)–approved common name for 2-methoxyethyl (*RS*)-2-(4-*tert*-butylphenyl)-2-cyano-3-oxo-3-(α,α,α-trifluoro-*o*-tolyl)propionate (International Union of Pure and Applied Chemistry), with Chemical Abstracts Service (CAS) number 400882-07-7. It is an acaricide and interferes with energy production (inhibition of complex II in mitochondria) on contact with spider mites. Cyflumetofen is a racemic mixture.

Cyflumetofen has not been evaluated previously 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.

All critical studies contained statements of compliance with good laboratory practice (GLP).

The chemical structure of cyflumetofen is shown in Fig. 1.

Fig. 1. Chemical structure of cyflumetofen



S-cyflumetofen *R*-cyflumetofen

Evaluation for acceptable daily intake

1. Biochemical aspects

Cyflumetofen is a racemic mixture, but the specific metabolism or degradation of the individual enantiomers in experimental animals was not investigated. The radiolabelled compounds used in the studies described below were [U-^{14}C-labelled 4-*tert*-butylphenyl ring]cyflumetofen (designated as A-ring-labelled cyflumetofen) and [U-^{14}C-labelled α,α,α-trifluoro-*o*-tolyl ring]cyflumetofen (designated as B-ring-labelled cyflumetofen).

1.1 Absorption, distribution and excretion

(a) Oral route

Mice

To investigate the change in kinetic parameters with increasing doses of cyflumetofen in mice in order to establish the dose range for further toxicological studies (Fabian & Landsiedel, 2011a), a single dose of 98.8% radiochemically pure B-ring-labelled cyflumetofen (specific radioactivity range: 0.04–0.8 MBq/mg) at 50, 250, 500 or 1000 mg/kg body weight (bw) was administered by oral gavage to 30 Crl:CD1 (ICR) male and female mice per dose. Blood was collected from animals in each dose group at 10 sampling times covering 0.5–72 hours post-dosing (three males and three females per time point), means of plasma concentrations were calculated and time course concentrations were analysed.

The pharmacokinetics of cyflumetofen in the plasma of mice differed between males and females (see Table 1): peak concentrations in plasma (C_{max} values) were on average 2.4 times higher in females (range: 34.3–104 µg equivalents [eq]/g) than in males (range: 10.6–65.4 µg eq/g). In both sexes, cyflumetofen was absorbed rapidly, leading to times to reach C_{max} (T_{max} values) of 0.5 hour post-dosing in males and 0.5–1 hour post-dosing in females. Moreover, plasma concentrations in females showed a second C_{max} (range: 6–47 µg eq/g) at 2 hours (dose level: 250 mg/kg bw) or 8 hours post-dosing (all other dose groups), which could indicate enterohepatic recirculation. After T_{max} was reached, concentrations in plasma declined rapidly (initial half-lives ranging from 0.4 to 2.3 hours post-dosing in females and from 0.7 to 2.8 hours post-dosing in males). Terminal half-lives were 1.2–1.8 times higher in males (range: 39.8–60 hours post-dosing) than in females (22–44 hours post-dosing). Finally, 1.4- to 2.0-fold higher internal concentrations, represented by areas under the

concentration–time curve from time 0 to infinity ($AUC_{0-\infty}$), were observed in females (177–1106 µg eq × h/g) than in males (89–717 µg eq × h/g), probably due to enterohepatic recirculation and higher oral absorption in females. In both sexes, $AUC_{0-\infty}$ values increased sublinearly, but continuously, with increasing dose (Fabian & Landsiedel, 2011a).

Table 1. Pharmacokinetic parameters of cyflumetofen in mice after single oral administration

	50 mg/kg bw		250 mg/kg bw		500 mg/kg bw		1 000 mg/kg bw	
Parameter	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
T_{max}[a] (h)	0.5	1; 8	0.5	0.5; 2	0.5	1; 8	0.5	0.5; 8
C_{max}[a] (µg eq/g)	10.61	51.84; 5.96	27.12	34.27; 46.54	43.28	65.32; 27.12	65.4	103.83; 36.87
$t_{½\ init.}$ (h)	2.02	0.42	0.7	2.3	2.82	2.25	1.38	1.02
$t_{½\ term.}$ (h)	39.79	22.04	60.07	41.33	53.16	43.87	54.89	35.22
$AUC_{0-\infty}$ (µg eq × h/g)	89.15	176.74	252.99	365.9	490.41	771.06	716.83	1 106.46

$AUC_{0-\infty}$: area under the concentration–time curve from time 0 to infinity; bw: body weight; C_{max}: peak concentration; eq: equivalent; $t_{½\ init.}$: initial half-life; $t_{½\ term.}$: terminal half-life; T_{max}: time to reach C_{max}

[a] An additional C_{max} and corresponding T_{max} were observed in females.

Source: Fabian & Landsiedel (2011a)

Rats

In a preliminary study to establish test conditions and methods for metabolism studies in rats (Nakamura, 2004a), the excretion pattern, pharmacokinetics and tissue distribution of cyflumetofen were investigated. Single oral doses of 99.2% radiochemically pure A-ring-labelled cyflumetofen or 98.1% radiochemically pure B-ring-labelled cyflumetofen were administered by gavage at 3 or 250 mg/kg bw (3.06 and 0.038 MBq/mg, respectively) to F344/DuCrj-Fischer rats (one rat of each sex per dose and label). For pharmacokinetic analysis, plasma and blood sampling and ^{14}C elimination in urine, faeces and expired air were investigated, covering several time points within 72 hours (24 hours for respiratory excretion) after administration. Tissue ^{14}C concentrations were measured at 72 hours after administration (termination time). Based on the pharmacokinetic analysis from single oral applications, the average (steady-state) concentration of radioactivity, assuming repeated dosing at 24-hour intervals, was estimated for each dose group and used to calculate the ^{14}C repetitive accumulation ratio ($Rac_{24\ h}$), defined as the ratio of the estimated minimum concentration at steady state after repeated dosing to the measured concentration at 24 hours after a single administration. $Rac_{24\ h}$ is a parameter predicting the bioaccumulative potential. Metabolic profiling was performed by high-performance liquid chromatography (HPLC).

No significant radioactivity was found in expired air from any test group. No pronounced differences between radiolabels were observed. At 3 mg/kg bw, the major route of elimination was the urine, ranging from 56.6% to 59.9% of the applied radioactivity in males and from 67.6% to 70.6% of the applied radioactivity in females; excretion in faeces accounted for 35.2–36.5% of the applied radioactivity in males and 23.8–25.3% of the applied radioactivity in females. At 250 mg/kg bw, the major elimination pathway was the faeces, accounting for 72.6–80.8% of the applied radioactivity in males and 68.6–76.4% of the applied radioactivity in females; urinary excretion was lower than at 3 mg/kg bw, amounting to 13.2–19.7% of the applied radioactivity in males and 15.1–25.1% of the applied radioactivity in females. Irrespective of dose level and label, excretion of radioactivity via urine was higher in female rats (about 6–12% of the applied radioactivity) than in male rats, but was lower in both sexes at the high dose than at the low dose. Approximately 92–99% of the applied radioactivity was recovered within 72 hours, and 0.3–2.1% of the applied radioactivity remained in the body. The concentration in plasma declined with biphasic first-order kinetics in all test groups,

with elimination half-lives (β-phase) of 11–20 hours. The T_{max} was in the range of 0.5–1 hour at the low dose and 1–4 hours at the high dose. The C_{max} was 1.3–2.9 mg eq/L at the low dose and 12.5–29.8 mg eq/L at the high dose. The ratio of the $AUC_{0-\infty}$ values between the high- and low-dose groups was 1.6–5 times lower than the dose ratio (approximately 80-fold) for both labels, suggesting saturation of oral absorption at 250 mg/kg bw. $Rac_{24\,h}$ was calculated to be 1.3–1.8, indicating that cumulative absorption was low. The highest total radioactive residue (TRR) concentration was consistently found, aside from the gastrointestinal tract and its contents, in liver (up to 52 times higher than the plasma concentration) and kidney (up to 10 times higher than the plasma concentration), regardless of the label position, dose level or sex. The TRR concentrations from the majority of the other organs or tissues were equal to or less than the plasma concentration in all test groups (Nakamura, 2004a).

To investigate the biliary excretion pattern of cyflumetofen and the radioactive residue levels in rats over 48 hours (Nakamura, 2004b), single doses of [^{14}C]cyflumetofen labelled in the A- or B-ring (99.2% and 98.1% radiochemical purity, respectively) were administered by oral gavage at 3 mg/kg bw (1.88 MBq/mg) or 250 mg/kg bw (0.023 MBq/mg) to bile duct–cannulated F344/DuCrj-Fischer rats (3–4 rats of each sex per dose and label). Urine, bile, faeces and cage wash were collected over 48 hours (termination time), and terminal blood samples were collected, together with the gastrointestinal tract and residual carcass; each sample taken was then analysed for the distribution of orally administered radioactivity. Bile samples were also generated for the metabolite distribution study (see section 1.2), summarized under the study of Ohyama (2004a).

In all test groups, more than 90% of the applied radioactivity was recovered from bile, urine, faeces, cage wash and the carcass. As no pronounced differences between radiolabels were generally observed, ranges and means (if the difference in mean values between radiolabels was < 3% of the applied radioactivity) are given for both radiolabels together. At 3 mg/kg bw, the major routes of elimination of radioactivity recovered over 48 hours post-dosing in both males and females were the urine (means of 30% of the applied radioactivity in males and 41–51% of the applied radioactivity in females) and the bile (means of 37% of the applied radioactivity in males and 24.5% of the applied radioactivity in females). Faecal excretion accounted for about 6–17% of the applied radioactivity in males and 6.5–10% of the applied radioactivity in females. At 250 mg/kg bw, the urinary excretion rate was less than at the low dose (11–15% of the applied radioactivity in males and 16–23.5% of the applied radioactivity in females), whereas the biliary excretion rate was similar to that at the low dose (means of 30.5% of the applied radioactivity in males and 18–21% of the applied radioactivity in females). The major route of elimination at 250 mg/kg bw was faecal excretion (means of 35% of the applied radioactivity in males and 35–41.4% of the applied radioactivity in females), which was higher than at the low dose. Generally, the biliary excretion rate was higher in males than in females, irrespective of the dose level and label position (the difference was about 8–14% of the applied radioactivity), and the urinary excretion rate was higher in females than in males, regardless of the dose level and label position. The applied radioactivity remaining in the body (excluding the gastrointestinal tract and its contents) was about 2.2% in males and 2–6% in females at 3 mg/kg bw and about 1.4% in males and 1.3% in females at 250 mg/kg bw. The estimated absorption rate (up to 48 hours) of cyflumetofen (sum of the radioactivity detected in bile, urine and the body, excluding the gastrointestinal tract and its contents) was a mean of 69% of the applied radioactivity in males and 70.8–78.4% of the applied radioactivity in females at 3 mg/kg bw and a mean of 45% of the applied radioactivity in males and 35.3–45.7% of the applied radioactivity in females at 250 mg/kg bw (Nakamura, 2004b).

To investigate the pharmacokinetics of cyflumetofen (Nakamura, 2004d), single doses of [^{14}C]cyflumetofen labelled in the A- or B-ring (99.2% and 98.1% radiochemical purity, respectively) were administered by oral gavage at 3 mg/kg bw (3.06 MBq/mg) or 250 mg/kg bw (0.039 MBq/mg) to F344/DuCrj-Fischer rats (four rats of each sex per dose and label). For pharmacokinetic analysis, means of plasma concentrations were calculated for every test group for each of nine sampling times spanning 72 hours. Owing to the formation of thrombi starting at 48

hours after dosing, some cannulas were damaged, and some blood samples could not be taken. Therefore, blood samples of only three instead of four animals could be taken in some test groups. $Rac_{24\,h}$ was calculated for each dose to predict the bioaccumulative potential. Additionally, urine and faeces for metabolite profiling were collected and analysed in a separate study (see section 1.2; Ohyama, 2004b).

At 3 mg/kg bw, maximum plasma TRR concentrations were achieved more rapidly (1 hour) than at 250 mg/kg bw (2–4 hours) in male and female rats (Table 2). The TRR concentrations in plasma decreased following biphasic first-order kinetics at about 8 hours after dosing. The slow-phase elimination half-life was shorter (14–17 hours) for the radioactivity from the A-label compared with the radioactivity from the B-label (17–21 hours). The $AUC_{0-\infty}$ did not proportionally increase with dose (the increase was 2- to 5.5-fold less than expected), which is an indication that saturational processes in systemic absorption could occur. There were no pronounced differences in pharmacokinetic parameters between sexes at the low dose; however, at the high dose, the C_{max} (see Table 2) and the $AUC_{0-\infty}$ values were higher in females and the duration of peak concentrations was generally increased in females (1–8 hours after dosing) compared with all other test groups (0.5–2 hours). The $Rac_{24\,h}$ was low in all test groups, indicating that cyflumetofen has no bioaccumulative potential (Table 2) (Nakamura, 2004d).

Table 2. Plasma pharmacokinetic parameters in rats following single oral administration of cyflumetofen at 3 or 250 mg/kg bw

	3 mg/kg bw		250 mg/kg bw	
	Males	**Females**	**Males**	**Females**
A-ring label				
T_{max} (h)	1	1	2	4
C_{max} (µg eq/g)	1.394	0.946	10.01	15.33
Final elimination phase half-life (h)	13.9	14.1	16.7	12.4
$AUC_{0-\infty}$ (µg eq × h/g)	10.388	6.562	159	251.1
$Rac_{24\,h}$	1.4	1.4	1.6	1.4
B-ring label				
T_{max} (h)	1	1	2	2
C_{max} (µg eq/g)	1.059	1.014	10.79	15.37
Final elimination phase half-life (h)	18.2	21.8	21.8	16.9
$AUC_{0-\infty}$ (µg eq × h/g)	10.176	9.196	165.9	327.8
$Rac_{24\,h}$	1.7	1.9	1.9	1.6

$AUC_{0-\infty}$: area under the concentration–time curve from time 0 to infinity; bw: body weight; C_{max}: peak concentration; eq: equivalent; $Rac_{24\,h}$: ^{14}C repetitive accumulation ratio; T_{max}: time to reach C_{max}

Source: Nakamura (2004d)

To investigate the tissue distribution of cyflumetofen (Nakamura, 2004e), single oral doses of 99.2% radiochemically pure A-ring-labelled cyflumetofen or 98.1% radiochemically pure B-ring-labelled cyflumetofen were administered by gavage at 3 or 250 mg/kg bw (1.88 and 0.023 MBq/mg, respectively) to F344/DuCrj-Fischer rats (six rats of each sex per dose and label). To analyse the TRR concentrations in organs and tissues, three rats of each sex per treatment group were terminated near the T_{max}, at either 1 hour for the low-dose group or 2 hours for the high-dose group, and another three rats of each sex per treatment group were terminated at 24 hours after application. To derive tissue

elimination curves and calculate half-lives of ^{14}C concentrations in selected tissues, the mean ^{14}C concentrations in tissues near the T_{max} and at 24 and 72 hours after administration were considered. The results after 72 hours were obtained from a separate study (elimination balance study of Nakamura, 2004c).

Regardless of the label position, dose level or sex, the majority of the applied radioactivity (46–83%, 5.9–31% and ≤ 1.4% after 1–2, 24 and 72 hours, respectively) was observed in the gastrointestinal tract including contents. For all time points, and irrespective of label, sex and dose, the highest TRR concentrations were found in the liver, followed by the kidney. At 3 mg/kg bw, the highest concentrations of TRR in these organs were 7.6–9 mg eq/kg (attributed to 10.4–12% of the applied radioactivity) and 4.7–8 mg eq/kg (attributed to 1.2–1.9% of the applied radioactivity) after 1 hour (T_{max}) in liver and kidney, respectively, regardless of sex or label position. At 250 mg/kg bw, the highest TRR concentrations of 66–117 mg eq/kg (reflecting 1.1–1.8% of the applied radioactivity) and 40–61 mg eq/kg (reflecting 0.12–0.19% of the applied radioactivity) were measured after 2 hours (T_{max}) in liver and kidney, irrespective of sex or label position. At 1–2 hours and 24 hours after application, mean TRR concentrations in kidney were 4 times higher than those in plasma, and those in liver were 12 times higher. TRR concentrations in other tissues were equal to or less than those in plasma at these two time points. However, some other tissues showed higher TRR concentrations compared with plasma concentrations at 72 hours. Additionally, some TRR concentrations in tissues were higher in B-ring-labelled cyflumetofen experiments than in A-ring-labelled cyflumetofen experiments at 72 hours (see also below: Nakamura, 2004c). Regardless of sex, label position or dose group, TRR concentrations in tissues declined with time. At 72 hours post-dosing, the TRR remaining in the carcass and tissues accounted for less than about 2.5% of the applied radioactivity at the low dose and about 1% of the applied radioactivity at the high dose, regardless of label position or sex. At 3 mg/kg bw, the half-life for the elimination of radioactivity from plasma was 9–13 hours, somewhat lower than the half-life for the elimination of radioactivity from bone marrow (14–22 hours) and remarkably lower than the half-life for the elimination of radioactivity from adipose tissue (19–24.5 hours). At 250 mg/kg bw, the half-life for the elimination of radioactivity from plasma was 10–15 hours, slightly lower than the half-lives for the elimination of radioactivity from blood, bone marrow and liver (ranges were 14–18.5, 20–30 and 16–19 hours, respectively), but remarkably lower than the half-life for the elimination of radioactivity from adipose tissue (31–42.5 hours). The elimination pattern of radioactivity from most selected tissues (except liver) appeared to be biphasic at the lower dose, particularly in female rats (Nakamura, 2004e).

To investigate the excretion of radioactivity in urine and faeces and the systemic distribution of radioactivity after 72 hours (Nakamura, 2004c), single oral doses of 99.2% radiochemically pure A-ring-labelled cyflumetofen or 98.1% radiochemically pure B-ring-labelled cyflumetofen were administered by gavage at 3 or 250 mg/kg bw (3.06 and 0.038 MBq/mg, respectively) to F344/DuCrj-Fischer rats (four rats of each sex per dose and label). Samples were also generated to analyse the metabolite distribution in the study of Ohyama (2004b) (see section 1.2), and the TRR results of this study were used to establish tissue elimination half-lives in the study of Nakamura (2004d).

At the end of the study (72 hours post-dosing), overall recovery of radioactivity was at least 95% of the applied radioactivity. The major pathway of elimination of radioactivity was the urine at 3 mg/kg bw and the faeces at 250 mg/kg bw. Irrespective of dose level or label, excretion of radioactivity via urine was higher in female rats than in male rats, but was lower in both sexes at the high dose than at the low dose. Excretion of radioactivity in urine at 3 mg/kg bw was 58.1–59.4% of the applied radioactivity in males and 66.1–66.5% of the applied radioactivity in females, whereas excretion of radioactivity in faeces at 3 mg/kg bw was 32.5–32.9% of the applied radioactivity in males and 25–27.3% of the applied radioactivity in females. At 250 mg/kg bw, excretion of radioactivity in urine was 14.3–16.2% of the applied radioactivity in males and 21.8–25.8% of the applied radioactivity in females, whereas excretion of radioactivity in faeces was 76.8–79.6% of the applied radioactivity in males and 68.3–74.4% of the applied radioactivity in females. No pronounced differences in excretion were observed between radiolabels. The tissues and carcass contained 0.9–2.5% of the applied radioactivity at 3 mg/kg bw and 0.4–0.8% of the applied radioactivity at 250

mg/kg bw for both labels at 72 hours post-dosing. The highest radioactivity concentrations in tissues were measured (aside from the gastrointestinal tract including contents and residual carcass) in the liver (> 6-fold higher than plasma concentrations) and kidney (> 4-fold higher than plasma concentrations), regardless of treatment group or sex, and in the bone marrow (> 5-fold higher than plasma concentrations) in females treated with A-ring-labelled cyflumetofen, regardless of the dose. Other tissues and organs with a ratio of tissue to plasma concentrations above 2 were adipose tissue (predominantly high-dose groups with A-ring label only), adrenal gland (females in low-dose group with A-ring label only) and erythrocytes (females in high-dose group with A-ring label only). Generally, radioactivity concentrations in tissues showed no remarkable sex differences, but (with the exception of liver and bone marrow) radioactivity concentrations in tissues were generally higher in B-ring-labelled than in A-ring-labelled cyflumetofen experiments. Concentrations of radioactivity in tissues measured in animals in the high-dose groups were approximately 53 times higher than those measured in animals in the low-dose groups (Nakamura, 2004c).

To investigate the excretion and tissue distribution of cyflumetofen (Hardwick, 2008), 14 daily repeated oral doses of 98.1% radiochemically pure B-ring-labelled cyflumetofen were administered by gavage at 3 mg/kg bw (1.88 MBq/mg) to female F344/DuCrj-Fischer rats. Groups consisting of four female rats were terminated 24 hours after the fourth and seventh repeated doses and 2, 24 and 72 hours after the 14th dose. Moreover, urine and faeces were collected from an additional group of four females at three (faeces) and five (urine) different time points during the 72 hours after the 14th dose. This group was terminated at 72 hours after the last applied dose as well. From all terminated animals, selected tissues were sampled to analyse TRR concentrations. The urinary and faecal samples were collected to characterize excretion patterns and were pooled across sample collection periods to analyse and quantify metabolites of cyflumetofen (summarized in section 1.2).

Cyflumetofen was widely distributed throughout tissues. After only four daily doses, TRR appeared to reach steady-state concentrations in organs. At all sampling times, TRR concentrations were (with the exception of the gastrointestinal tract and contents) highest in liver, followed by kidney and erythrocytes, and exceeded (except at 2 hours after the last dose in erythrocytes) TRR concentrations in plasma. In all other organs, TRR concentrations were lower than or equal to those in plasma. No remarkable accumulation occurred in any of the tissues or organs investigated. However, the increasing erythrocyte to plasma TRR concentration ratio with respect to the period 24–72 hours after the last dose suggests an association of cyflumetofen or its metabolites with the cellular fraction in blood. The highest TRR concentrations were measured 2 hours after the 14th dose in liver (12.9 mg eq/kg), kidney (7.1 mg eq/kg), erythrocytes (1.15 mg eq/kg) and plasma (1.5 mg eq/kg). Although the TRR concentrations in organs and tissue decreased relatively rapidly until 72 hours post-dosing, TRR concentrations in tissues at 72 hours after the last dose were on average still about 11% of TRR concentrations after 2 hours, with the slowest decline occurring in erythrocytes (25% of TRR concentrations after 2 hours) and skin (30% of TRR concentrations after 2 hours), and TRR concentrations in liver, kidney and erythrocytes were still about 5- to 9-fold higher than TRR concentrations in plasma at that time point. Cumulative elimination of radioactivity at 72 hours after the 14th dose of cyflumetofen administered at 3 mg/kg bw occurred in almost equal proportions in urine (64% of the last dose, equivalent to 4.5% of the total dose) and faeces (59% of the last dose, equivalent to 4.2% of the total dose). Retention of radioactivity in the tissues and carcass was 4.5% (based on the radioactivity in the final dose) or 0.3% (based on the total applied radioactivity). Overall recovery of radioactivity was 132% (based on the radioactivity in the final dose) or 9.1% (based on the total applied radioactivity) at 72 hours post-dosing (Hardwick, 2008).

(b) Dermal route

In vitro

It was not possible to prepare a homogeneous formulation of the representative solution, Scelta 20SC, containing B-ring-labelled cyflumetofen; therefore, B-ring-labelled cyflumetofen was dissolved in acetonitrile. The concentrate, 200 g/L cyflumetofen, and a representative dilution for Scelta 20SC, 0.2 g/L, were applied to four human dermatomed skin discs from two donors and four human skin discs from four donors for 8 hours, respectively, and receptor fluid was analysed for 24 hours. The results show that the actual dermal absorption into the receptor fluid was low: only about 0.6% was absorbed 24 hours after application of a low or high dose. The vast majority remained in the skin as a dermal depot, representing about 20% and 27% for the low and high doses, respectively. As a worst case, the depot in skin can be considered as potentially absorbable material, which sums up to dermal absorptions of cyflumetofen of 21% for the low dose and 28% for the high dose (Corral, 2009).

In vivo

Radiolabelled cyflumetofen formulated in BAS 9210 01 at 200 g/L and 2 and 0.2 g/L cyflumetofen dilutions were applied to the skin of 12 male Wistar rats (per dose) for 8 hours, and urine and faeces were collected and analysed 8, 24 or 120 hours (four animals per time point) after application. At the respective time points, animals were terminated, and residues in carcass, skin and application site were measured. Dermal absorption ranged from 0.26% of the applied dose at 8 hours to 0.96% at 120 hours for the 200 g/L cyflumetofen concentrate. For the 2 and 0.2 g/L cyflumetofen dilutions, 0.63% and 4.93% of the applied dose were found at 8 hours, respectively, reaching a maximum of 2.2% and 8% at 120 hours, respectively. The residue in the application site decreased after the exposure period, and radioactivity was still being excreted at 120 hours, suggesting that a certain part of the depot at the application site becomes systemically available after exposure and probably after the observation period (Fabian & Landsiedel, 2011b).

1.2 Biotransformation

One preliminary (Nakamura, 2004a) and three main metabolism studies (Ohyama, 2004a,b; Hardwick, 2008) were performed. The metabolism of cyflumetofen was investigated by analysing bile, faeces and urine samples retained from the previous studies: single oral administration of 3 or 250 mg/kg bw of A- or B-ring-labelled cyflumetofen to male and female rats (Nakamura, 2004a,b,c) and 14 consecutive oral doses of 3 mg/kg bw per day of B-ring-labelled cyflumetofen to female rats only (Hardwick, 2008).

In a preliminary metabolism study (Nakamura, 2004a), unchanged parent was not detected in urine, and the metabolite profile (peaks not identified) differed between the A- and B-ring labels. In total, four specific peaks from each of the labels were noted. Unchanged parent was found as the main components in faecal extract in all test groups. In addition, three peaks for A-ring label and one for B-ring label were detected. From the metabolite profile, it was assumed that cleavage between the A- and B-rings and subsequent degradation to specific metabolites for each label occur. In addition, most of the metabolites seemed to be excreted via urine.

In the first main metabolism study (Ohyama, 2004b), in which samples of urine and faeces were analysed, cyflumetofen was extensively metabolized in rats. Three groups of metabolites were observed: *tert*-butylphenyl (A-ring) specific, trifluorotolyl (B-ring) specific, and A- and B-ring common metabolites. Most of the main metabolites derived from cleavage between the A-ring and the B-ring moieties, and the levels of the uncleaved metabolites, including both the A- and B-ring moieties, which are predominantly represented by metabolites AB-1, AB-2 and AB-3, were relatively low.

At 3 mg/kg bw in non-cannulated rats, most of the major metabolites were excreted in urine. The major metabolites containing the A-ring only in urine and faeces were A-20 (HPLC fraction: M8; urine: 1–4% of the applied radioactivity; faeces: about 3% of the applied radioactivity), A-21 (M10;

urine: 7–21% of the applied radioactivity; only traces in faeces) and A-18 (M28; urine: 14–34% of the applied radioactivity; only traces in faeces). The major metabolites containing the B-ring only were B-1 (M14; urine: 8–10% of the applied radioactivity; faeces: about 17% of the applied radioactivity), [B-1]-MA (M16; urine: 6–13.5% of the applied radioactivity; only traces in faeces) and [B-1]-TLA (M17; urine: 17–20% of the applied radioactivity; only traces in faeces). Metabolite AB-3 (M27; containing the A- and B-ring moieties) was urine specific and occurred at greater than 100-fold higher levels in females (8–9% of the applied radioactivity) than in males. However, AB-2 (M26), which is derived from AB-3 by further oxidation, was found at a similar range of concentrations between sexes. With the exception of AB-3, the profiles of the metabolites were similar in both sexes, but some differences in quantity were apparent. The amounts of A-21 (M10), A-18 (M28), A-20 (M8) and [B-1]-MA (M16) were 1.1%, 14.7%, 3.9% and 6.2% of the applied radioactivity for males, but 6.7%, 33.9%, 1.0% and 13.5% of the applied radioactivity for females, respectively. Unchanged parent was not detected or occurred in minor amounts in urine and was about 2–4% of the applied radioactivity in faeces for both labels and sexes.

At 250 mg/kg bw, the profiles of the identified metabolites and the quantitative differences between sexes were similar to those at 3 mg/kg bw. However, metabolites at 250 mg/kg bw occurred at lower percentages (about ≤ 9-fold lower) of the applied radioactivity in urine and faeces than at 3 mg/kg bw. In contrast, the fraction of the unchanged parent in faeces was 14- to 27-fold higher in the high-dose groups than in the low-dose groups (≤ 66% versus ≤ 4.3% of the applied radioactivity, respectively), which might be due to saturation of oral absorption processes. As for the low dose, unchanged parent was not detected or occurred in minor amounts in urine at the high dose. Unfiltered/unextractable fractions (from different label positions, sexes and doses) in urine and faeces were all less than or equal to 4%.

In the second main metabolism study, in which bile samples were analysed following a dose of 3 or 250 mg/kg bw in rats, unchanged parent was not detected or occurred in minor amounts in bile, regardless of dose, label or sex. The metabolite profile for bile was significantly different from those for urine and faeces in the non-cannulated animals. In bile, the highest portion of applied radioactivity consisted of metabolites containing both the A- and B-ring moieties. With the exception of AB-2 (Mb30), metabolites above 1% of the applied radioactivity in bile were all subjected to phase 2 biotransformation (conjugation to glucuronic acid [GA] and glutathione [SG]) (Ohyama, 2004a).

GA-conjugated AB-1 and AB-3 were eluted in more than one fraction (AB-1 in Mb32 and Mb33; AB-3 in Mb18 and Mb21), possibly due to keto-enol tautomerism and subsequent different positioning of GA on each of the AB-1 and AB-3 molecules.

The major metabolites identified at levels above 5% of the applied radioactivity in bile were the GA-conjugated AB-1 (Mb32 and Mb33; low dose: 5.7–7.5% of the applied radioactivity; high dose: 7.6–13.3% of the applied radioactivity) and the GA-conjugated AB-3 (Mb18; low dose: 5–6.8% of the applied radioactivity; high dose: 3.5–5.5% of the applied radioactivity). A certain portion of the GA-conjugated AB-3 was also detected in the Mb21 fractions (see above). However, the amount was determined only for males in the low-dose group (A-ring label) and accounted for 1.4% of the applied radioactivity, indicating that total levels of GA-conjugated AB-3 in bile might be underestimated. Furthermore, metabolites occurring at levels above 1% of the applied radioactivity were AB-2 (≤ 3.2% of the applied radioactivity) and cleavage products (containing either A- or B-ring moieties), such as the SG-conjugated B-1 ([B-1]-SG: ≤ 2.6% of the applied radioactivity) and the GA-conjugated A-6 ([A-6]-GA: ≤ 3.7% of the applied radioactivity) and A-22 ([A-22]-GA: ≤ 3.8% of the applied radioactivity). All other metabolites were found at levels below 1% of the applied radioactivity. The percentages of the applied radioactivity in bile of SG-conjugated B-1 in males and of AB-2 in all treatment groups were 4- and 5.7-fold, respectively, lower at the high dose than at the low dose, and GA-conjugated AB-1 showed a higher portion of the applied radioactivity in bile at the high dose than at the low dose (see above). Otherwise, all percentages of the applied radioactivity for the other main metabolites were in a similar range in the high- and low-dose groups.

In the third main metabolism study (Hardwick, 2008), B-ring-labelled cyflumetofen at 3 mg/kg bw was administered daily for 14 days to female rats, and samples of urine and faeces were collected and pooled during the 72 hours after the last dose. The only reference standards used for co-chromatography during HPLC were parent and 2-trifluoro-methyl benzoic acid (B1, M14), and no mass spectrometry was performed on the fractions. According to 25 chromatographic regions derived from urine and up to at least six regions in the faeces, cyflumetofen appears to be extensively metabolized.

Unchanged parent was not detected in the urine of female rats, whereas B-1 was observed at 13% of the final dose. The urine contained nine other major unidentified fractions (0.7–11.7% of the applied radioactivity of the final dose). Unchanged parent was observed at 24.7% of the applied radioactivity of the final dose in faeces, and B1 (M14) was found at 22.2% of the applied radioactivity of the final dose in faeces. Other (unidentified) fractions were each less than 1% of the applied radioactivity of the final dose. As the fraction of unchanged parent was higher in the repeated-dose study than in an equivalent single-dose oral study in faeces (25% versus 2.5% of the applied radioactivity, respectively), saturation of oral absorption might occur following repeated dosing.

In conclusion, the major route of metabolism of cyflumetofen is suggested to be cleavage into A-ring and B-ring moieties (Fig. 2). The A-ring moiety was hydrolysed to A-18 (M28 in urine) via A-l (putative intermediate), followed by hydration of the A-ring to A-21 (M10). As another minor route for A-ring metabolism, A-21 was decarboxylated to form the intermediate A-6, which was glucuronidated or transformed to A-20 (M8). The main metabolic pathway of the B-ring moiety consists of two routes: one is the hydrolytic pathway producing B-1 (M14), and the other is the formation of mercapturic acid conjugate ([B-1]-MA, M16) and thiolactic acid conjugate ([B-1]-TLA, M17) via the conjugation of the B-ring moiety with glutathione ([B-1]-SG; bile-specific metabolite). A minor route of metabolism of cyflumetofen is supposed to be the transformation of metabolites maintaining the A- and B-rings. The transformation pathway of these metabolites starts with cleavage of the carboxylic ester moiety, resulting in the formation of AB-1, followed by oxidation of the *tert*-butyl group to alcohol (AB-3; M27) and carboxylic acid (AB-2; M26). Glucuronidated AB-1 and AB-3 were the main metabolites in bile; however, the precise position of glucuronidation could not be determined (see above), and glucuronidated AB-1 and AB-3 metabolites were not identified in urine and faeces of non-cannulated rats, suggesting that rapid degradation of these metabolites occurs.

2. Toxicological studies

2.1 Acute toxicity

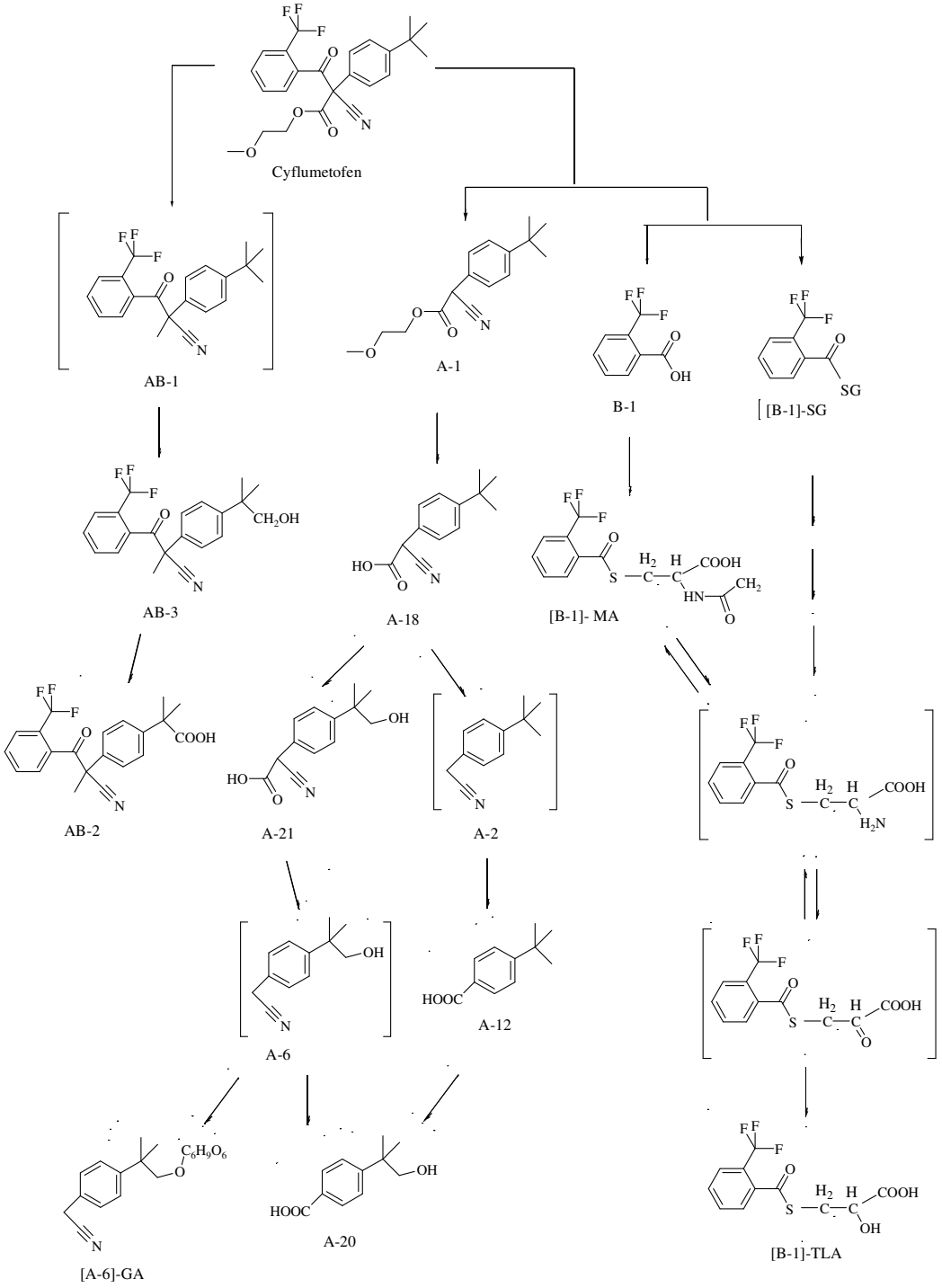
(a) Lethal doses

The acute oral toxicity of cyflumetofen (batch no. 01H1; purity 98.0%) was examined after a single dose administered by gavage to five fasted adult female Han Wistar rats (Table 3). Animals were observed for 14 days. No mortality occurred at 2000 mg/kg bw. Clinical signs were restricted to loose faeces from approximately 5 hours post-dosing in one female. No other apparent abnormalities were observed in any animal at necropsy at the termination of the study. Therefore, the oral median lethal dose (LD_{50}) of cyflumetofen in female rats was found to be greater than 2000 mg/kg bw (Moore, 2003a).

The acute dermal toxicity of cyflumetofen (batch no. 01H1; purity 98.0%) was assessed at 5000 mg/kg bw in five male and five female Han Wistar rats. There were no mortalities, no systemic clinical signs and no local dermal signs of toxicity. The median dermal LD_{50} of cyflumetofen in male and female rats was therefore greater than 5000 mg/kg bw (Moore, 2003b).

The acute inhalation toxicity of cyflumetofen (batch no. 01H1; purity 98.0%) after a 4-hour nose-only exposure was assessed at an actual concentration of 2.65 mg/L (maximum attainable concentration) in five male and five female Han Wistar rats, which were observed for 14 days post-exposure. During treatment, exaggerated breathing was evident in rats 15 minutes after the start of exposure. During the observation period, exaggerated breathing and brown staining around the snout/jaws were observed in rats immediately after exposure, and the exaggerated breathing persisted

Fig. 2. Metabolism of cyflumetofen in the rat



GA: glucuronic acid conjugate; SG: glutathione conjugate; MA: mercapturic acid conjugate; TLA: thiolactic acid conjugate; [ ]: possible intermediate

Table 3. Acute toxicity of cyflumetofen

Species	Strain	Sex	Route	LD_{50} (mg/kg bw)	LC_{50} (mg/L)	Purity (%)	Reference
Rat	Han Wistar	Female	Oral	> 2 000	–	98	Moore (2003a)
Rat	Han Wistar	Male, female	Dermal	> 5 000	–	98	Moore (2003b)
Rat	Han Wistar	Male, female	Respiratory	–	> 2.65	98	Bowden (2003)

bw: body weight; LC_{50}: median lethal concentration; LD_{50}: median lethal dose

to day 1 of the observation period. All rats were of normal appearance and behaviour from day 2 of the observation period to termination of the study. A slightly reduced mean body weight gain was apparent for males and females during the first week. During the second week, body weight gain was considered normal. The median respiratory lethal concentration (LC_{50}) of cyflumetofen in male and female rats was found to be greater than 2.65 mg/L. The test substance was of low toxicity via inhalation (Bowden, 2003).

(b) Dermal irritation

The primary dermal irritation potential of 0.5 g cyflumetofen (batch no. 01H1; purity 98.0%) was assessed in three male albino New Zealand White rabbits. Animals were treated for 4 hours and observed for 4 days after exposure. No dermal reactions were observed after exposure, and no sign of toxicity or ill-health was detected during the observation period (Rees, 2003a).

(c) Ocular irritation

The primary eye irritation potential of 0.1 g cyflumetofen (batch no. 01H1; purity 98.0%) per animal was tested in four (one in a preliminary study and three in the main study) female albino New Zealand White rabbits by instillation into the right eye of the animals. In the preliminary study, after a slight initial pain response after instillation, injection of the conjunctival blood vessel was observed during the first week after instillation. In the main study, instillation caused no or slight pain responses. Injection of the conjunctival blood vessel in two animals or, in a single case, a crimson red conjunctival appearance was evident during the first week after instillation, persisting in one case until day 15. Additionally, very slight discharge in two animals was seen 1 hour after instillation. Treated eyes of all animals were normal on day 22 (Rees, 2003b). Cyflumetofen was considered to be a slight eye irritant.

(d) Dermal sensitization

The skin sensitizing potential (Hooiveld, 2003) of cyflumetofen (batch no. 01H1; purity 98.0%) was assessed using the maximization assay with 15 female guinea-pigs (five animals were used as concurrent controls and 10 animals for the treatment group). The highest irritating dose for intradermal injection was 1% cyflumetofen (slight erythema); however, for topical application, 50% cyflumetofen did not reveal any irritation reactions in a preliminary irritation test. As no irritation was evident during topical induction, the application site was pretreated with 10% sodium dodecyl sulfate at 24 hours prior to topical induction in the main study. The same concentration (50% cyflumetofen) was used for the challenge application (as the highest non-irritating dose). At challenge, well defined to moderate erythema in 100% of females (10/10) was noted after 24 and 48 hours. In one and two females, scabbing was seen after 24 and 48 hours, respectively. Additionally, scaliness was observed in all females at 48 hours. Overall, cyflumetofen was considered to be a skin sensitizer. No mortality, symptoms of systemic toxicity or changes in body weight were observed.

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

Cyflumetofen (batch no. 01D1; purity 97.67%) was administered for 28 days in the diet to groups of six male and six female 5-week-old ICR (Crj:CD-1) mice. The concentrations in feed were 0, 100, 500, 1000 and 5000 parts per million (ppm) (equal to 0, 13.1, 67.2, 135 and 663 mg/kg bw per day for males and 0, 14.5, 74.9, 150 and 763 mg/kg bw per day for females, respectively) (Yoshida, 2004d). Clinical observations of all animals were recorded daily. Body weights were measured at initiation and weekly thereafter, and feed consumption was measured on 4 consecutive days every week. Haematological analysis and clinical chemistry were performed on all animals after 4 weeks of treatment. At study termination, organ weights and macroscopy were investigated in all dose groups. Histological analysis of adrenals was performed in all animals at study termination.

No mortality and no treatment-related effects on clinical signs, body weight or feed consumption were noted. Haematological analysis revealed a statistically significantly reduced platelet count in females at 5000 ppm. However, a clear dose–response relationship was not apparent. Clinical chemistry showed a non-statistically significant increase in blood urea nitrogen (+14%) at 5000 ppm in females. Absolute and relative thyroid weights in males and uterine weights in females were non-statistically significantly reduced in all treatment groups; however, a clear dose–response pattern was absent. Absolute and relative adrenal weights were statistically significantly increased by 24% and 15% in males and by 21% and 19% in females, respectively, at 5000 ppm. Macroscopy showed no abnormalities. Histopathology showed diffuse vacuolation of adrenocortical cells in 1/6 males and 5/6 females at 5000 ppm, compared with 1/6 males and 0/6 females in the control group. Diffuse hypertrophy of adrenocortical cells was observed in 4/6 males and 6/6 females at 5000 ppm, compared with 0/6 males and 1/6 females in the control group. Additionally, 1/6 females exhibited subcapsular cell hyperplasia in the adrenals (versus 0/6 in controls and all other dose groups), which was, however, not observed in other short-term mouse studies.

Based on adrenocortical vacuolation in females and adrenocortical hypertrophy and increased adrenal weights in both sexes at 5000 ppm (equal to 663 mg/kg bw per day), the NOAEL was 1000 ppm (equal to 135 mg/kg bw per day) (Yoshida, 2004d).

Cyflumetofen (batch no. 01D1; purity 97.67%) was administered for 90 days in the diet to groups of 10 male and 10 female 5-week-old ICR (Crj:CD-1) mice. The concentrations in feed were 0, 300, 1000, 3000 and 10 000 ppm (equal to 0, 35.4, 117, 348 and 1200 mg/kg bw per day for males and 0, 45.0, 150, 447 and 1509 mg/kg bw per day for females, respectively) (Yoshida, 2004f). Clinical observations of all animals were recorded daily. Body weights and feed consumption were measured at initiation and weekly thereafter (feed consumption was measured on 4 consecutive days). Ophthalmology and urine analysis were not performed. Haematological analysis and clinical chemistry were performed on all animals after 13 weeks of treatment. At study termination, organ weights and macroscopy were investigated in all dose groups. Histological analysis of adrenals, ovaries and organs and tissues that revealed gross lesions at macroscopy was performed in all animals at study termination; the remaining organs and tissues were investigated only in the control and highest-dose groups at study termination.

No treatment-related effects on mortality, clinical signs, body weight, feed consumption or haematology were noted. Clinical chemistry analysis demonstrated statistically significantly reduced aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities at 1000, 3000 or 10 000 ppm (at this concentration, statistically significant for AST only) in males. As the mean AST and ALT activities in concurrent controls were, however, 2-fold higher than the historical control group means and no clear dose–response relationship could be deduced from the data, a relationship with treatment is unlikely. Absolute and relative adrenal weights were statistically significantly increased by 32% and 27% in males and by 11% and 23% (not statistically significant) in females at

10 000 ppm. Macroscopy revealed enlargement of the adrenals in 9/10 males at 10 000 ppm (compared with 0/10 in controls and all other treatment groups). Histopathology showed diffuse vacuolation of adrenocortical cells at 3000 ppm (2/10) and 10 000 ppm (7/10) in females (all other groups: 0/10) and diffuse hypertrophy of adrenocortical cells in 1/10 males at 3000 and 10 000 ppm (all other groups: 0/10).

Based on vacuolation and hypertrophy of the adrenal cortex in females and males, respectively, at 3000 ppm (equal to 348 mg/kg bw per day), the NOAEL was 1000 ppm (equal to 117 mg/kg bw per day) (Yoshida, 2004f).

Rats

Cyflumetofen (batch no. HN0101004; purity 98%) was administered for 14 days in the diet to groups of six male and six female 5-week-old Fischer (F344/DuCrj) rats. The concentrations in feed were 0, 1000 and 10 000 ppm (equal to 0, 101 and 981 mg/kg bw per day for males and 0, 105 and 1000 mg/kg bw per day for females, respectively) (Sakai, 2001). Clinical observations, body weights and feed consumption of all animals were recorded daily. No haematological analysis was done. Urine collection for analysis was performed overnight after 2 weeks of treatment. At study termination, clinical chemistry and organ weights were investigated in all dose groups. Histological analysis in all other organs was performed only in the control and high-dose groups.

No treatment-related effect on mortality was noted. No changes in clinical signs, body weight or feed consumption were noted at 1000 ppm. In females at 10 000 ppm, soiled perineal region, a statistically significant (≤ 10%) decrease in body weights (throughout the treatment period) and a statistically significant decrease in feed consumption (at the early and late treatment periods) were observed; in males at the same dose, a statistically significant decrease in feed consumption (in the absence of obvious body weight effects) was restricted to the first 2 days of treatment. Additionally, a statistically significant decrease in feed efficiency was noted in both sexes at the first day of treatment. There were no changes in urine analysis in any dose group of either sex.

Clinical chemistry revealed statistically significant decreases in alkaline phosphatase, AST, ALT and creatine phosphokinase activities and a significantly higher albumin/globulin ratio in males at 1000 and 10 000 ppm. Additionally, a statistically significant increase in total protein and albumin levels and a decrease in total cholesterol, triglyceride and total bilirubin levels were noted in males at 10 000 ppm. Females at 10 000 ppm showed a statistically significant decrease in creatine phosphokinase, creatine and globulin and a statistically significant increase in albumin and albumin/globulin ratio. In females at 1000 ppm, a statistically significant decrease in triglyceride and total bilirubin levels was observed, and a decreasing trend (although not statistically significant) was also detected in females at 10 000 ppm. In males, organ weight analysis revealed a statistically significant increase in relative liver and adrenal weights at 1000 and 10 000 ppm, which was paralleled by statistically significantly higher absolute liver and adrenal weights at 10 000 ppm. Furthermore, in males, a statistically significant decrease in absolute and relative thymus weights and a statistically significant increase in relative testis (right side only) and kidney weights were noted. In females, a statistically significant increase in relative and absolute adrenal weights at 1000 and 10 000 ppm and a statistically significant increase in relative kidney and liver weights were observed at 10 000 ppm. Moreover, in females, absolute spleen weight was statistically significantly decreased at 10 000 ppm. At macroscopy, hypertrophy of the adrenals was observed in all males and females at 10 000 ppm and in 5/6 females at 1000 ppm (versus 0/6 in controls).

Histopathological examination showed, in all males and females at 1000 and 10 000 ppm, diffuse vacuolation of cortical cells in adrenals and diffuse hypertrophy of hepatocytes in the liver (versus none in controls). Vacuolation of interstitial gland cells was observed in all females at 1000 and 10 000 ppm, and vacuolation of corpora lutea in the ovary was additionally noted in all females at 10 000 ppm only (compared with none in controls).

Based on changes in clinical chemistry, an increase in liver and adrenal weights, hypertrophy of the adrenals in females and histopathological alterations in the adrenal cortex in both sexes and in

the ovary at the lowest dose of 1000 ppm (equal to 101 mg/kg bw per day), no NOAEL was derived from this study (Sakai, 2001).

Cyflumetofen (batch no. 01C1; purity 99.3%) was administered for 28 days in the diet to groups of six male and six female 5-week-old Fischer (F344/DuCrj) rats. The concentrations in feed were 0, 100, 500, 1000 and 5000 ppm (equal to 0, 7.5, 37.6, 75.1 and 384 mg/kg bw per day for males and 0, 8.05, 40.8, 79.8 and 409 mg/kg bw per day for females, respectively) (Yoshida, 2004e). Clinical observations of all animals were made daily, whereas body weights and feed consumption of all animals were measured weekly. Urine collection for analysis was performed after 4 weeks of treatment. At study termination, haematology, clinical chemistry, organ weights and macroscopy were investigated in all dose groups. Histological analysis of selected organs (liver, kidneys, pituitary, adrenals, testes, ovaries) was performed in all animals at study termination. In order to characterize vacuolations observed in the adrenals of both sexes and in the ovaries of females, some animals of the control and highest-dose groups were stained with Oil Red O.

There were no deaths or treatment-related changes in general clinical observations, body weights, feed consumption or haematology in any dose groups. In females, urine analysis revealed a statistically significant increase in ketones at 5000 ppm. Clinical chemistry showed statistically significant increases in blood urea nitrogen (+15% compared with controls) and potassium (+7% compared with controls) at 5000 ppm in males, statistically significant decreases in total cholesterol and triglycerides at 1000 and 5000 ppm in males and in total cholesterol at 5000 ppm in females, and a statistically significant decrease in sodium (−5% compared with controls) at 5000 ppm in females. Additionally, a slight, statistically significant decrease in alkaline phosphatase activity was noted in males at 5000 ppm. Absolute and relative liver weights were statistically significantly increased at 1000 ppm in males (+10% and +8% compared with controls, respectively) and at 5000 ppm in both sexes (males: +29% and +31% compared with controls, respectively; females: +16% compared with controls for both parameters). Absolute and relative kidney weights were statistically significantly increased at 5000 ppm in both sexes (≤ 14% increase compared with controls for both sexes and parameters). Relative adrenal weight was elevated by 13% and 15% at 1000 ppm in males and females, respectively (statistically significant in females only). Both absolute and relative adrenal weights were statistically significantly increased at 5000 ppm in males (+36% and +33% compared with controls, respectively) and females (+100% compared with controls for both parameters). Macroscopy exhibited enlarged and discoloured adrenals in all animals in both sexes at 5000 ppm (versus none in controls). Histopathology revealed diffuse hypertrophy of hepatocytes in all males at 1000 ppm and in all animals of both sexes at 5000 ppm (compared with 0/6 in controls). Diffuse vacuolation of adrenocortical cells was observed in all animals of both sexes at 1000 and 5000 ppm and was paralleled by diffuse hypertrophy in all females at 1000 and 5000 ppm (compared with 0/6 in controls). Vacuolation of interstitial cells in the ovaries was observed in 2/6 females at 1000 ppm and 6/6 females at 5000 ppm compared with 0/6 in controls. The vacuolation in the adrenals of rats of both sexes and in the ovaries of female rats was demonstrated to be lipid deposition.

Based on decreased serum cholesterol and triglyceride concentrations and increased absolute and relative liver weights in males, increased relative adrenal weights in both sexes and histopathology in the liver (males), adrenals (both sexes) and ovaries at 1000 ppm (equal to 75.1 mg/kg bw per day), the NOAEL was 500 ppm (equal to 37.6 mg/kg bw per day) (Yoshida, 2004e).

Cyflumetofen (batch number and purity not provided) was administered for 26 days in the diet to groups of five male and five female CRL:WI(Han) rats. The concentrations in feed were 0, 500, 1500, 4000 and 12 000 ppm (equal to 0, 43, 128, 339 and 1028 mg/kg bw per day for males and 0, 46, 132, 351 and 1039 mg/kg bw per day for females, respectively) (Buesen, 2010). No details on clinical observations were reported. Feed consumption and body weight were determined on study days 0 (body weight only), 3, 7, 14, 21 and 26 for all animals. Clinical pathology (urine analysis, clinical chemistry, haematology) and ophthalmology were not performed. At study termination, organ

weights and histopathology of adrenal, ovary and liver were measured or investigated in all animals. Macroscopy was performed on all animals; however, details were not reported. This range-finding study was conducted to determine the susceptibility of Wistar rats to cyflumetofen toxicity.

Body weight was statistically significantly decreased in males (maximum of −9% on day 26) and in females (maximum of −10% on day 26) at 12 000 ppm. Moreover, body weight change was statistically significantly decreased in males during the treatment period (maximum −27% on day 3) and in females (maximum −59% on day 3), reaching statistical significance towards the end of the study at 12 000 ppm. Body weight gain was also statistically significantly decreased in males on day 3 (−22%) at 4000 ppm. Feed consumption was decreased in females (statistically significantly at the beginning and end of the study) and males at 12 000 ppm during the treatment period. Statistically significant increases in absolute and relative adrenal weights were observed starting from 500 ppm in females and from 4000 ppm in males, reaching maximal increases relative to controls in females (absolute: +82%; relative: +101%) and males (absolute: +69%; relative: +87%) at 12 000 ppm. Additionally, statistically significant increases in absolute and relative liver weights in males were noted starting from 4000 ppm onwards and in females at 12 000 ppm. In females, statistically significant increases in absolute and relative ovary weights at 4000 ppm (absolute: +29%; relative: +34%) and 12 000 ppm (not statistically significant) were observed. Enlarged and/or discoloured adrenals were seen at 4000 and 12 000 ppm in both sexes. Additionally, discoloration of the ovaries was noted in all females, and a focus (not specified in detail) in the liver was detected in one female at 12 000 ppm.

Centrilobular, hepatocellular hypertrophy in the liver of all males and in one female at 4000 ppm and in all males and three females at 12 000 ppm was noted. Adrenocortical cell vacuolation in males (3/5) and females (3/5) at 1500 ppm and in all animals at 4000 and 12 000 ppm was identified. Finally, vacuolation of luteal cells in the ovary was detected at 4000 ppm (4/5) and 12 000 ppm (5/5) in females.

Based on the increased incidence of adrenal cell vacuolation in both sexes and an increase in absolute and relative adrenal weights in females at 1500 ppm (equal to 128 mg/kg bw per day), the NOAEL was 500 ppm (equal to 43 mg/kg bw per day) (Buesen, 2010).

Cyflumetofen (batch no. 01D1; purity 97.67%) was administered for 90 days in the diet to groups of 10 male and 10 female 6-week-old Fischer (F344/DuCrj) rats. The concentrations in feed were 0, 100, 300, 1000 and 3000 ppm (equal to 0, 5.4, 16.5, 54.5 and 167 mg/kg bw per day for males and 0, 6.28, 19.0, 62.8 and 193 mg/kg bw per day for females, respectively) (Yoshida, 2004g). Detailed clinical observations, body weights and feed consumption were recorded for all animals prior to initiation of treatment and weekly thereafter. A functional observational battery analysis was performed on all animals at week 11. Ophthalmology, urine analysis, haematology and clinical chemistry were performed at 13 weeks. Macroscopic and organ weight investigations were done in all test groups at termination. Histological analysis of selected organs (liver, kidneys, adrenals, ovaries and gross lesions identified during macroscopy) was performed for all animals, and the remaining tissues and organs were investigated in the control and highest-dose groups only at study termination. Additionally, electron microscopic analysis of the left adrenal (both sexes) and left ovary (females) from a single animal per dose group was performed.

No treatment-related effects on mortality, body weight, feed consumption, functional observations or ophthalmoscopy were noted. Clinical observations revealed a statistically significant decrease in rearing in males: 6/10 at 100 ppm (not statistically significant), 3/10 at 300 ppm, 2/10 at 1000 ppm and 0/10 at 3000 ppm, compared with 9/10 in controls at 1 week of treatment. However, as the decreased incidences were observed only in the first week of treatment and not thereafter, this was not considered to be an adverse effect. Haematology showed a statistically significantly elongated prothrombin time in males at 3000 ppm (+17%) and a statistically significantly decreased total leukocyte count in females receiving 1000 or 3000 ppm. Clinical chemistry exhibited a slight, statistically significant reduction in globulin and a slight, statistically significant increase in albumin/globulin ratio at 1000 and 3000 ppm in females. Absolute liver weight was statistically

significantly increased at 3000 ppm in males (+8%), and relative liver weight was statistically significantly increased at 1000 ppm in males (+4%) and at 3000 ppm in both sexes (+12% in males; +6% in females). Relative kidney weight was slightly, but statistically significantly, increased at 3000 ppm in both sexes. Relative adrenal weight was statistically significantly increased at 1000 ppm (+8%) and 3000 ppm (+42%) in females and was paralleled by a statistically significant increase in absolute adrenal weight (+42%) compared with controls at 3000 ppm in the same sex. Macroscopy and histopathology confirmed the toxicological effects on the adrenals; enlarged and discoloured adrenals were observed at 3000 ppm in all females (compared with 0/10 in all other groups), and diffuse hypertrophy of adrenocortical cells was noted at 1000 and 3000 ppm in all females (compared with 0/10 in all other groups). Mild to moderate diffuse vacuolation of adrenocortical cells was observed in males at 1000 ppm (6/10) and 3000 ppm (10/10), compared with 0/10 in all other groups. Additionally, mild to moderate vacuolation of interstitial gland cells in the ovaries was seen at 1000 ppm (1/10) and 3000 ppm (8/10), compared with 0/10 in all other groups.

Based on increased relative adrenal weights in females, vacuolation and hypertrophy of the adrenal cortex in males and females, respectively, and vacuolation of ovarian interstitial cells at 1000 ppm (equal to 54.5 mg/kg bw per day), the NOAEL was 300 ppm (equal to 16.5 mg/kg bw per day) (Yoshida, 2004g).

Dogs

Cyflumetofen (batch no. 01H1; purity 98.4%) was administered for 28 days in gelatine capsules to groups of three male and three female 6-month-old Beagle dogs at a dose level of 0, 100, 300 or 1000 mg/kg bw per day (Nagashima, 2003a). Detailed clinical observations were performed at initiation of dosing and 3 times a day thereafter, body weights were measured before initiation of treatment and weekly thereafter and feed consumption of all animals was measured prior to initiation of treatment and daily thereafter. Ophthalmology was investigated before initiation of dosing and at week 3, and urine analysis, haematology and clinical chemistry were performed after 4 weeks of treatment. Organ weights, macroscopy and histopathology were performed in all groups at termination.

No treatment-related effects on mortality, body weight, feed consumption, ophthalmology, haematology, urine analysis or macroscopy were observed. Clinical observations revealed faeces with whitish appearance (probably test substance) in all animals at 1000 mg/kg bw per day during the treatment period and occasionally in two males (weeks 3 and 4) and two females (week 2) at 300 mg/kg bw per day. Clinical chemistry showed a slight increase in potassium and calcium levels at 300 and 1000 mg/kg bw per day in males, with a doubtful dose–response correlation. Increases in absolute and relative adrenal weights were noted in one male and in one female (absolute weight only was increased in a second female) at 300 mg/kg bw per day and in two animals of each sex at 1000 mg/kg bw per day. Macroscopy indicated dark red foci (designated as capillary dilatation) on the right atrioventricular valve of the heart in one female at 1000 mg/kg bw per day. Histopathology revealed fine vacuoles in adrenocortical cells (zona fasciculata and zona reticularis) in two males and three females at 300 mg/kg bw per day and in all animals at 1000 mg/kg bw per day. Fine vacuoles were also observed in the zona glomerulosa in one male at 300 mg/kg bw per day and in two animals of each sex at 1000 mg/kg bw per day.

Based on increased absolute and relative adrenal weights, fine vacuolation of adrenocortical cells in both sexes and dark red foci on the right atrioventricular valve of the heart in one female at 300 mg/kg bw per day, the NOAEL was 100 mg/kg bw per day (Nagashima, 2003a).

Cyflumetofen (batch no. 01H1; purity 98.4%) was administered for 90 days (males) and 91 days (females) in gelatine capsules to groups of four male and four female 6-month-old Beagle dogs at a dose level of 0, 30, 300 or 1000 mg/kg bw per day (Nagashima, 2003b). Detailed clinical observations were performed before initiation of dosing and 3 times a day thereafter, body weights were measured before initiation of treatment and weekly thereafter and feed consumption of all

animals was measured prior to initiation of treatment and daily thereafter. Ophthalmology was investigated before initiation of dosing and at weeks 7 and 12, and urine analysis, haematology and clinical chemistry were performed prior to treatment and at weeks 7 and 13 of treatment. Organ weights, macroscopy and histopathology were performed in all groups at termination of the study.

No treatment-related effects on mortality, feed consumption, ophthalmology, clinical chemistry or urine analysis were noted. Clinical observations indicated faeces with whitish appearance (probably test substance) 1–7 times per week in all animals at 1000 mg/kg bw per day during the treatment period and occasionally in one male and all females at 300 mg/kg bw per day. Body weight gain, from day 1 to day 90, was reduced by 64% and 36% of control values at 1000 mg/kg bw per day in males and females, respectively. Urine analysis exhibited occult blood (accompanied by the observation of erythrocytes in urinary sediment) in 1/4 females at 300 mg/kg bw per day, 1/4 females at 1000 mg/kg bw per day at week 7 and 1/4 females at the highest dose at week 13. It was assumed that the blood finding in urine was attributed to estrous bleeding, which was observed around the same time. Haematology showed a statistically significant increase in monocyte ratios relative to the control group in males at 30 mg/kg bw per day (+61%), 300 mg/kg bw per day (+57%; not statistically significant at this dose) or 1000 mg/kg bw per day (+67%) at week 13. However, as values of treatment groups were similar to pretest values on an individual level and as a clear dose–response correlation was not apparent, findings might be considered to be incidental. A statistically significant increase in blood urea nitrogen (+42%) relative to the control group was observed at 1000 mg/kg bw per day in females, and a statistically significant increase in γ-globulin was noted at 30 mg/kg bw per day (+22%) and 1000 mg/kg bw per day (+22%) in males. As the latter (γ-globulin increase) did not show a clear dose–response relationship and as the values were already considerably elevated at pretest compared with controls, a relationship with treatment was questionable. Macroscopy indicated dark red foci (designated as capillary dilatation) on the right atrioventricular valve of the heart, dark red foci in the mucosa of the urinary bladder and enlargement of the adrenals in one male at 1000 mg/kg bw per day. Absolute and relative adrenal weights were increased (right/left: +26%/+37% and +30%/+40%, respectively) relative to controls in males at 1000 mg/kg bw per day. Additionally, absolute and relative pituitary weights were statistically significantly increased at 300 mg/kg bw per day (+40% for both) and 1000 mg/kg bw per day (+28% and +40%, respectively) relative to controls in females. A non-statistically significant increase in absolute and relative testis weights (pronounced in the right testis: about 130% of control values for both parameters) was also observed at 1000 mg/kg bw per day. High incidences of fine (all three zonae) and large (zona fasciculata only) vacuoles of adrenocortical cells (categorized as slight to mild) were observed in females (fine vacuoles: 2/4; large vacuoles: 3/4) and males (fine vacuoles: 4/4; large vacuoles: 3/4) at 1000 mg/kg bw per day. Large vacuoles (slight in the degree of severity) within the zona fasciculata were also noted in lower-dose groups (30 and 300 mg/kg bw per day): 1/4, 2/4, 2/4 and 3/4 in females and 0/4, 1/4, 0/4 and 3/4 in males at 0, 30, 300 and 1000 mg/kg bw per day, respectively.

Based on reduced body weight gain in both sexes, increased absolute and relative adrenal and testis weights in males and pituitary weights in females, high incidences of vacuolation of the adrenal cortex in both sexes and dark red foci on the right atrioventricular valve of the heart in one male at 1000 mg/kg bw per day, the NOAEL was 300 mg/kg bw per day (Nagashima, 2003b).

Cyflumetofen (batch no. 01H1; purity 98.4%) was administered for 1 year in gelatine capsules to groups of four male and four female 6-month-old Beagle dogs at a dose level of 0, 30, 300 or 1000 mg/kg bw per day (Nagashima, 2008). Detailed clinical observations were performed before initiation of dosing and 3 times a day thereafter. Body weights were measured before initiation of treatment, then weekly until week 14 and every fortnight thereafter. Feed consumption of all animals was measured prior to initiation of treatment and daily thereafter. Ophthalmology was investigated before initiation of dosing and at weeks 26 and 52, and urine analysis, haematology and clinical chemistry were performed before initiation of the study and at weeks 26 and 52 of treatment. Organ weights, macroscopy and histopathology were performed in all groups at termination of the study.

No treatment-related effects on mortality, body weight or feed consumption were noted. Clinical observations indicated faeces with white-yellowish appearance (probably test substance) 1–7 times per week in all animals during the treatment period at 1000 mg/kg bw per day and sporadically in all animals during the treatment period at 300 mg/kg bw per day. Additionally, soft faeces were frequently observed from week 2 to week 4 in one male at 1000 mg/kg bw per day. Urine analysis showed occult blood in one female at 300 and 1000 mg/kg bw per day and erythrocytes in urinary sediment at 1000 mg/kg bw per day at week 26, which are considered to be due to estrous bleeding. Haematology indicated a statistically significant transient increase (+66%) in leukocyte counts in females at 1000 mg/kg bw per day at week 26. Clinical chemistry showed a decrease in triglyceride concentrations in males (−40% and −31% relative to controls at weeks 26 and 52, respectively) and females (−43% relative to controls at week 52 only) at 1000 mg/kg bw per day. Absolute and relative adrenal weights were increased relative to controls in males (right/left: +40%/+43% and +47%/+51%, respectively) and females (right/left: +68%/+64% and +68%/+66%, respectively) at 1000 mg/kg bw per day. Additionally, absolute and relative pituitary weights were increased at 1000 mg/kg bw per day (+22% and +21%, respectively) relative to controls in females. Absolute and relative liver weights were decreased by 19% (statistically significantly) and 15%, respectively, in males, and absolute and relative prostate weights were reduced by 40% (statistically significantly) and 37%, respectively, at 1000 mg/kg bw per day. Enlargement of the adrenals was observed in one female at 300 mg/kg bw per day and in one male and all females at 1000 mg/kg bw per day. Additionally, dark red foci on the right atrioventricular valve of the heart of one male were observed at 30 and 1000 mg/kg bw per day. Histopathology revealed slight swelling of interstitial cells in the testes in one male at 1000 mg/kg bw per day. Fine and/or large vacuolation of adrenocortical cells, degeneration of cortical cells, interstitial fibrosis and infiltration of brown pigment-laden macrophages or cell infiltration (mainly lymphocytes) in the adrenals were observed in both sexes at 300 and 1000 mg/kg bw per day (see Table 4). Fine and large vacuolations (designated as slight and occasionally mild) in adrenocortical cells were also noted in both sexes at 30 mg/kg bw per day and in females of the control group (large vacuolations categorized as slight only). However, vacuolations were of a lower degree of severity and not accompanied by degenerative processes, compared with the 300 and 1000 mg/kg bw per day dose groups. Whereas fine vacuolations were observed in all zonae of the adrenals, large vacuolations and degenerative reactions were confined to the zona fasciculata and zona reticularis (not to the zona glomerulosa).

Based on increased incidences of vacuolations accompanied by degenerative processes (e.g. interstitial fibrosis and infiltration of brown pigment–laden macrophages) at 300 mg/kg bw per day, the NOAEL was 30 mg/kg bw per day (Nagashima, 2008).

(b) Dermal application

Cyflumetofen (batch no. 01H1; purity 98.4%) was administered for 28 days by the dermal route (6 hours/day, 5 days/week, semi-occlusive dressing) to groups of 10 male and 10 female 9-week-old CRL:WI(Han) rats at a dose level of 0, 100, 300 or 1000 mg/kg bw per day (Buesen et al., 2010b). Detailed clinical observations of all animals were performed and feed consumption of all animals was measured prior to initiation of treatment and weekly thereafter. Body weight was measured prior to and at the start of treatment and twice weekly thereafter. A functional observational battery analysis was performed on all animals at the end of the study. Ophthalmology was performed at the end of the study on the control and highest-dose groups, and urine analysis, haematology and clinical chemistry of all animals were performed at the end of treatment. Macroscopy and organ weight investigations were done in all animal groups at termination. Histological analysis of selected organs was performed in the control and highest-dose groups at study termination.

No treatment-related effects on mortality, clinical signs, body weight, feed consumption, functional observational battery or clinical pathology were noted. In addition, no local dermal irritation was observed. The only (minor) finding in organ pathology was confined to a slight, statistically significant increase in absolute kidney weight in males at 300 mg/kg bw per day (+10%)

and 1000 mg/kg bw per day (+8%) relative to controls and a slight, statistically significant increase in relative liver weight in males at 1000 mg/kg bw per day (+8%) relative to controls.

As no adverse systemic (or local) treatment-related effects were observed, the NOAEL was 1000 mg/kg bw per day, the highest dose tested, for male and female rats (Buesen et al., 2010b).

Table 4. Histopathology of the adrenals in the 1-year dog study (four dogs of each sex per dose)

	0 mg/kg bw per day		**30 mg/kg bw per day**		**300 mg/kg bw per day**		**1 000 mg/kg bw per day**	
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Focal cell infiltration (total)	–	–	–	–	1	2	1	3
Slight	–	–	–	–	1	1	1	3
Mild	–	–	–	–	–	1	–	–
Interstitial fibrosis (total)	–	–	–	–	–	2	1	3
Slight	–	–	–	–	–	2	1	3
Fine vacuolation in cortical cells (total)	–	–	1	2	4	4	3	4
Slight	–	–	–	1	1	2	–	–
Mild	–	–	1	1	3	2	1	–
Moderate	–	–	–	–	–	–	2	4
Large vacuolation in cortical cells (total)	–	4	1	3	2	4	4	4
Slight	–	4	1	2	2	1	2	1
Mild	–	–	–	1	–	3	2	2
Moderate	–	–	–	–	–	–	–	1
Degeneration in cortical cells[a] (total)	–	–	–	–	1	2	2	1
Slight	–	–	–	–	1	2	1	–
Mild	–	–	–	–	–	–	1	1
Pigment-laden macrophages (slight)	–	–	–	–	1	–	1	–

bw: body weight; F: female; M: male

[a] Enlarged cortical cells filled with cytoplasmic vacuoles, brown pigment in cytoplasm and karyorrhexis-like figure of the cortical cells.

Source: Nagashima (2008)

2.3 Long-term studies of toxicity and carcinogenicity

Mice

Cyflumetofen (batch no. 01D1; purity 97.67%) was administered for 78 weeks in the diet to groups of 52 male and 52 female 5-week-old ICR (Crj:CD-1) mice. The concentrations in feed were 0, 150, 500, 1500 and 5000 ppm (equal to 0, 15.5, 54.3, 156 and 537 mg/kg bw per day for males and 0, 14.3, 48.1, 144 and 483 mg/kg bw per day for females, respectively) (Yoshida, 2004a). All animals were checked for mortality and subjected to general clinical observations (at least twice a day) and body weight and feed consumption measurements (at initiation of treatment, once weekly from week 1 to week 13 and once every 4 weeks from week 16 onwards). Detailed clinical observations were recorded at least weekly during treatment. Blood sampling was performed at week 52, on surviving animals at week 78 (haematological analysis was not performed at week 52, as no pronounced effects were observed at week 78) and on all animals killed in extremis during treatment. No urine analysis or clinical chemistry investigations were done. Selected organ weight analysis (10 animals of each sex per dose) and macroscopy were performed in all dose groups (including all animals killed or found dead). Histopathology was analysed in the control and highest-dose groups at terminal kill after 78 weeks or in all animals (regardless of the dose group) killed in extremis during treatment. Histopathological analysis was additionally performed on the adrenal glands and on all gross lesions in the 150, 500 and 1500 ppm groups.

No effects on mortality, clinical signs, body weight or feed consumption were noted. Haematology revealed a statistically significant decrease in large unstained cells in males (−34% relative to controls) at 5000 ppm. Large unstained cells are normally considered to include immature cells or tumour cells; therefore, reductions in cell count are considered to be of unclear toxicological relevance. A non-statistically significant increase in absolute adrenal weights by 20% relative to the control group was noted. Macroscopy in males killed in extremis or found dead showed a statistically significant increase in ascites of the abdominal cavity (4/15 versus 0/15) at 5000 ppm. Histopathology indicated a statistically significant increase in the incidence of diffuse vacuolation of cortical cells in the adrenals at 5000 ppm in males (8/52 versus 1/52 in controls) and females (26/52 versus 7/52 in controls). The incidences of diffuse/focal hypertrophy of adrenals were similar to those in the control group. There were no treatment-related neoplastic changes.

Cyflumetofen showed no carcinogenic potential. Based on an increase in adrenal weights (most predominant in females) and vacuolation in the adrenal cortex in both sexes at 5000 ppm (equal to 483 mg/kg bw per day), the NOAEL for systemic toxicity was 1500 ppm (equal to 144 mg/kg bw per day) (Yoshida, 2004a).

Cyflumetofen (batch no. 09/0510-3 (009025); purity 97.82%) was administered for 78 weeks in the diet to groups of 52 male and 52 female 5-week-old ICR (Crj:CD-1) mice. The concentrations in feed were 0 and 10 000 ppm (equal to 0 and 1143 or 1132 mg/kg bw per day for males and females, respectively) (Yoshida, 2013). All animals were checked for mortality and subjected to general clinical observations (at least twice a day) and body weight and feed consumption measurements (at initiation of treatment, once weekly from week 1 to week 13 and once every 4 weeks from week 16 onward). Detailed clinical observations were recorded at least weekly during treatment. Blood sampling was performed at week 52, on surviving animals at week 78 (haematological analysis was not performed at week 52, as no pronounced effects were observed at week 78) and on all animals killed in extremis during treatment. No urine analysis or clinical chemistry investigations were done. Selected organ weight analysis, macroscopy and haematology were performed in all animals (including all animals killed or found dead) at study termination.

The following treatment-related effects were observed at 10 000 ppm:

- Mortality was statistically significantly higher in treated females than in control females at weeks 59 and 64–78 (3/52 and 17/52 for females in the 0 and 10 000 ppm groups, respectively, at week 78). However, the increased mortality rates observed for females were

within the historical control range (range: 9/52–23/52; mean: 13/52; number of studies at the Institute of Environmental Toxicology [IET] laboratory in Ibaraki, Japan: 6).

- Clinical signs were confined to a statistically significant increase in the incidences of decreased spontaneous motor activity, bradypnoea, pale-coloured skin and pale-coloured eye/eyelid in females.
- Haematologically, males showed a statistically significant increase in total leukocyte count (+35% relative to the control group).
- Females exhibited statistically significant increases in absolute and relative weights of the adrenals (+34% and 42% relative to controls, respectively) and statistically significant increases in the incidences of soiled fur in the external genital region (5/52 versus 0/52 in controls), enlargement of the spleen (10/52 versus 3/52 in controls) and spot(s) in the liver (5/52 versus 0/52 in controls).

No treatment-related neoplastic lesions were observed.

Non-neoplastic histopathology showed a statistically significant increase in the incidences of diffuse vacuolation of cortical cells in males (21/52 versus 0/52 in controls) and females (41/52 versus 2/52 in controls) and of deposition of brown pigment in the corticomedullary junction of the adrenal gland in males (14/52 versus 6/52 in controls) and females (44/52 versus 27/52 in controls). No treatment-related increase in diffuse/focal hypertrophy of adrenals was observed.

Additionally, a statistically significant increase in the incidence of extramedullary haematopoiesis in the spleen was noted in females (14/52 versus 5/52 in controls).

In conclusion, there was no evidence of carcinogenic potential at 10 000 ppm. Cyflumetofen-induced treatment-related findings were restricted to clinical signs in females and effects on the adrenals (e.g. increased adrenal weights, diffuse vacuolation of cortical cells and brown pigment depositions in both sexes) and the spleen (enlargement and haematopoiesis in females) (Yoshida, 2013).

Rats

Cyflumetofen (batch no. 01D1; purity 97.67%) was administered for 52 weeks in the diet to groups of 50 male and 50 female 5- or 6-week-old Fischer (F344/DuCrj) rats. The concentrations in feed were 0, 50, 150, 500 and 1500 ppm (equal to 0, 1.9, 5.6, 18.8 and 56.8 mg/kg bw per day for males and 0, 2.3, 6.9, 23.3 and 69.2 mg/kg bw per day for females, respectively) (Yoshida, 2004b,c). A main group of 20 animals of each sex per dose was terminated after 52 weeks, and 10 animals of each sex per dose from a satellite group of 30 animals of each sex per dose were terminated after 4, 13 and 26 weeks of administration. All animals in the main and satellite groups were checked for mortality (twice a day) and subjected to general clinical observations (daily) and body weight measurements (at initiation of treatment, once weekly from week 1 to week 13 and once every 4 weeks from week 16 onwards). Detailed clinical observations were recorded in the main study before study initiation and weekly during treatment, and feed consumption of the main group was measured once weekly from week 1 to week 13 and once every 4 weeks from week 16 onwards. The animals in the main group were subjected to functional observations at 49 weeks of treatment (10 animals of each sex per group), and ophthalmology was performed before study initiation and in week 52 in all animals in the control and 1500 ppm groups. Haematology, clinical chemistry, urine analysis, organ weight measurements, macroscopy and histopathology were performed at termination on 10 animals of each sex per dose of the satellite group after 4, 13 and 26 weeks and on 10 animals of each sex per dose (except for macroscopy and histopathology: 20 animals of each sex per dose) of the main group in week 52. Histopathology was performed on selected organs (liver, kidneys, adrenals, ovaries and all gross lesions) of animals in all groups and on the remaining organs of all animals of the control and highest-dose groups. (Organ pathology generally also included those animals found dead or killed during the treatment period.)

No effects on mortality, body weight, feed consumption, ophthalmoscopy or macroscopy were noted. Haematological examinations showed a slight, statistically significant decrease in platelet count in males at 1500 ppm after 4 and 26 weeks of treatment and in females after 13 weeks.

Additionally, a slight, statistically significant decrease in fibrinogen concentration was detected in males at 1500 ppm after 13 weeks and in females from 150 ppm onwards after 52 weeks (however, a clear dose–response relationship was not apparent). Clinical chemistry revealed statistically significant decreases in blood urea nitrogen at 50 ppm and higher in males (a maximum 11% reduction relative to controls), in total protein and albumin concentrations in females from 150 ppm onwards (a maximum 7% reduction relative to controls) and in globulin concentrations at 500 ppm and higher (a maximum 7% reduction relative to controls) in females after 13 weeks, without a clear correlation with dose. Additionally, total cholesterol and triglyceride concentrations were statistically significantly decreased from 150 ppm onwards (except for the decrease in triglyceride concentration at 1500 ppm, which was not statistically significant), with highest reductions of 12% (at 150 ppm) and 33% (at 500 ppm) relative to controls, respectively, in females after 13 weeks. The only statistically significant, pronounced change in electrolytes in several dose groups was a transient decrease in calcium levels from 150 ppm and higher in females (–9% at most) after 13 weeks (no clear dose–response relationship was apparent, however). Slight, statistically significant increases in absolute and relative liver weights in males at 1500 ppm after 4 weeks and in relative liver weight also at 13 and 26 weeks were observed. Relative kidney weight was slightly but statistically significantly increased in males after 13 weeks and in females after 52 weeks at 1500 ppm. In addition, females in this group showed statistically significant increases in absolute and relative adrenal weights at almost all time points (with maximal increases of 15% and 17%, respectively). Finally, a statistically significant decrease in absolute weight of epididymides was observed in males at 1500 ppm after 26 weeks. Non-neoplastic histopathological findings in males were restricted to an increased incidence of hepatocellular diffuse hypertrophy in the liver after 4 weeks, high incidences of diffuse vacuolation (incidence range over all time points: 42–100%) of adrenocortical cells at all time points at 1500 ppm and diffuse hypertrophy of adrenocortical cells in 1/19 males after 52 weeks at the same dose. Additionally, all females examined at 1500 ppm revealed diffuse hypertrophy of adrenocortical cells at all time points.

Vacuolation of interstitial gland cells in the ovaries was observed in 10–30% of females at 1500 ppm at each time point except after 4 weeks.

Based on an increase in absolute and relative adrenal weights in females, an increase in liver weights in males, a reduction in total cholesterol and triglyceride concentrations in both sexes, adrenocortical vacuolations in males and adrenocortical hypertrophy (pronounced in females), and vacuolations in ovary cells at 1500 ppm (equal to 56.8 mg/kg bw per day), the NOAEL for systemic toxicity was 500 ppm (equal to 18.8 mg/kg bw per day) (Yoshida, 2004b,c).

Cyflumetofen (batch no. 09/0510-3 (009025); purity 97.82%) was administered for 52 weeks in the main group (20 animals of each sex per dose) and for up to 26 weeks in the satellite group (30 animals of each sex per dose) in the diet to 5-week-old Fischer (F344/DuCrj) rats. The concentrations in feed were 0 or 6000 ppm (equal to 0 and 250 or 319 mg/kg bw per day for males and females, respectively) (Yoshida, 2012). At each dose, a main group of 20 animals of each sex was terminated after 52 weeks, and satellite groups of 10 animals each were subjected to interim kill after 4, 13 and 26 weeks of treatment. All animals were checked daily for mortality and general clinical conditions, and body weights were recorded at initiation of treatment, once weekly from week 1 to week 13 and once every 4 weeks from week 16 onwards. Detailed clinical observations were checked in the main group once prior to the initiation of treatment and weekly thereafter, and feed consumption of the main group was measured once weekly from week 1 to week 13 and once every 4 weeks from week 16 onwards. The animals in the main group were subjected to functional observations at 49 weeks of treatment (10 animals of each sex per group), and ophthalmology was performed before study initiation and in week 52 in all control animals and animals in the 6000 ppm group. Haematology, clinical chemistry, urine analysis, organ weights, macroscopy and histopathology were performed at termination from 10 animals of each sex per dose of the satellite group after 4, 13 and 26 weeks and from 10 animals of each sex per dose (except for macroscopy and histopathology: 20 animals of each

sex per dose) of the main group after 52 weeks. Histopathology was performed on all test animals. (Organ pathology generally included those found dead or killed during the treatment period.)

No treatment-related effects on feed consumption, mortality or ophthalmoscopy were noted.

The following effects were observed at 6000 ppm:

- General clinical signs included a statistically significant increase in soiled fur (external genital region) in 8/20 females of the main group.
- Body weight was statistically significantly decreased in females during the majority of the study period in the main group (maximally −6% towards the end of treatment).
- Haematology revealed a statistically significant transient decrease in platelet count (maximally −9% relative to controls in females) and fibrinogen concentration (maximally −12% relative to controls in females) and a significant elongation in prothrombin time (maximally +37% relative to controls in males) in both sexes after 4 weeks. Males also showed statistically significant decreases in platelet count and fibrinogen concentration after 13 weeks and a statistically significant elongation in prothrombin time after 13 and 52 weeks (with the tendency to recover with time) as well as a statistically significant increase in activated partial thromboplastin time after 4 (+11%), 13 (+4%) and 52 (+4%) weeks. Reticulocyte counts were statistically significantly decreased in males (−8%) after 52 weeks and in females (−15%) after 26 weeks. Finally, a statistically significant increase in lymphocyte counts was observed in females after 26 and 52 weeks (+23% relative to controls).
- Clinical chemistry findings included significant decreases in total cholesterol and triglyceride levels after 4 weeks (maximal reduction occurring in males: −39% and −9%, respectively) and after 26 weeks (females only), and triglyceride levels were additionally reduced at 52 weeks in males. Males also showed slight, statistically significant increases in albumin and total protein levels and albumin/globulin ratio after 13, 26 or 52 weeks. Finally, blood urea nitrogen was statistically significantly increased in both sexes at several time points (maximal value of +14% relative to control group in females at 52 weeks).
- Macroscopy revealed significant increases in the incidence of white-coloured adrenal glands in both sexes and in the incidence of enlarged adrenal glands in females at all time points.
- Statistically significant increases in absolute and relative adrenal weights were observed in both sexes (maximally +73% relative to control group in females) at each time point. Both sexes showed a statistically significant increase in absolute (males only) and relative liver weights (maximally +13% and +15% in males, respectively, relative to control group) and statistically significant increases in absolute (males only) and relative kidney and thyroid weights (at each time point for kidney only). Finally, statistically significant decreases in absolute (−17%) and relative uterine weights (−12%) were observed after 52 weeks.
- Histopathologically, all males at each time point (except 19/20 males at 52 weeks) showed diffuse vacuolation of adrenocortical cells, whereas all females exhibited diffuse hypertrophy of adrenocortical cells at each examination period. Males also showed significant increases in the incidences of focal atrophy of acinar cells in the pancreas (14/20 versus 4/20 in controls) and hyperplasia of Leydig cells (19/20 versus 6/20 in controls) after 52 weeks, whereas females indicated high increases in the incidences of vacuolation of interstitial gland cells in the ovary at all time periods.

In conclusion, the treated animals showed pronounced effects on body weight, clinical signs and several organs, including adrenals, liver, pancreas, testis and ovary, at 6000 ppm (equal to 250 mg/kg bw per day) (Yoshida, 2012).

Cyflumetofen (batch no. 01D1; purity 97.67%) was administered in the diet to groups of 50 male and 50 female 5- or 6-week-old Fischer (F344/DuCrj) rats for 104 weeks. The concentrations in feed were 0, 150, 500 and 1500 ppm (equal to 0, 4.92, 16.5 and 49.5 mg/kg bw per day for males and 0, 6.14, 20.3 and 61.9 mg/kg bw per day for females, respectively) (Yoshida, 2004b). All animals

were checked for mortality and subjected to general clinical observations (at least twice a day) and body weight and feed consumption measurements (at or before initiation of treatment, once weekly from week 1 to week 13 and once every 4 weeks from week 16 onwards). Detailed clinical observations were recorded prior to and weekly during treatment. Haematology was performed at weeks 53, 78 and 104 and on all animals killed in extremis. Selected organ weight analysis (10 animals of each sex per dose) and macroscopy were performed on animals from all dose groups. Histopathology was performed in the control and highest-dose groups at terminal kill after 104 weeks or in all animals (regardless of the dose group) killed in extremis during treatment. Histopathological analysis was additionally performed on selected organs (liver, kidneys, adrenals, ovaries, uterine horn and all gross lesions) in the 150 and 500 ppm dose groups.

Clinical chemistry, urine analysis, ophthalmology and functional observational battery examination were performed in a concurrent chronic rat study (Yoshida, 2004b).

No effects on mortality, clinical signs, body weight or feed consumption were noted. Haematologically, eosinophil count was statistically significantly decreased in males at 1500 ppm (−17%).

Because an adrenal tumour (complex phaeochromocytoma) was observed in one male of the control group (leading to a decrease in adrenal weights in all treatment groups), reanalysis of adrenal weights was conducted after excluding this animal; this resulted in statistically significant increases in absolute and relative adrenal weights in males at 500 ppm and higher (maximally +21% and +23% at 500 ppm, respectively) and females at 1500 ppm (+9% and +18%, respectively). Macroscopy showed a statistically significant increase in the incidence of mass(es) in the testis in all treated groups killed in extremis or found dead (see Table 5), which corresponded partly to histopathological lesions, such as testicular interstitial cell tumours (Leydig cell adenoma [LCA]), and were associated with increased incidences of atrophies in testis and epididymis. The six males with LCA died between week 84 and week 104 (two control males with LCA died at week 85 and week 88). At study termination, however, the incidences of masses or LCA in males in the treated group were similar to those of the control group, resulting in overall non-statistically significant increases of 14% and 6% for incidences of masses in the testis and LCA, respectively, at 1500 ppm. Moreover, the incidence of Leydig cell hyperplasia was statistically significantly decreased at the highest dose. Increases in the incidence of epididymal atrophy were apparent at 500 ppm (+18%; statistically significant) and 1500 ppm (+14%; not statistically significant), and statistically significant decreases in seminiferous tubule atrophy were observed at the top dose. Furthermore, an increase in luminal dilatation of the gland in the uterine horn was observed in all treatment groups at 0 (14%), 150 (17%), 500 (22%) and 1500 ppm (31%), with statistical significance at the highest dose. However, as this lesion was not elevated relative to controls in a second carcinogenicity study at 6000 ppm, the increase in incidence with dose can be considered incidental. In addition, the incidence of diffuse hypertrophy of cortical cells in the adrenals was statistically significantly increased in both sexes of the 1500 ppm group.

In this study, cyflumetofen showed increased incidences of testicular masses and LCA in animals that died or were terminated ad interim in all treatment groups towards the end of the study. The overall incidences of LCA were, however, similar to those of the control group at study termination (incidence of surviving and killed/dead animals together). The NOAEL for systemic toxicity was 500 ppm (equal to 16.5 mg/kg bw per day), based on increased adrenal weights accompanied by histopathological changes in the adrenals at 1500 ppm (equal to 49.5 mg/kg bw per day in males), and the NOAEL for carcinogenicity was 1500 ppm (equal to 49.5 mg/kg bw per day), the highest dose tested (Yoshida, 2004b).

Cyflumetofen (batch no. 09/0510-3 (009025); purity 97.82%) was administered in the feed to 5-week-old Fischer (F344/DuCrj) rats (50 of each sex per dose) at a dose of 0 or 6000 ppm (equal to 0 and 220 or 287 mg/kg bw per day for males and females, respectively) for 104 weeks (Takahashi, 2013). All animals were checked for mortality and subjected to general clinical observations (at least twice a day) and body weight and feed consumption measurements (at or before initiation of

treatment, once weekly from week 1 to week 13 and once every 4 weeks from week 16 onwards). Detailed clinical observations were recorded prior to and weekly during treatment. Haematology was performed at weeks 53, 78 and 104 and on all animals killed in extremis during treatment. Selected organ weight analysis, macroscopy and histopathology were performed in all animals after 104 weeks or in all animals killed in extremis during treatment. Clinical chemistry, urine analysis, ophthalmology and functional observational battery examination were performed in a concurrent carcinogenicity study in rats.

Table 5. Selected macroscopic/histopathological findings in testis and associated tissues in male rats in a carcinogenicity study

	0 ppm	150 ppm	500 ppm	1 500 ppm
Animals killed in extremis or found dead				
Testis: Mass(es)	0/8	5/10* (50%)	4/7* (57%)	6/9** (66%)
Testis: Atrophy	1/8	6/10	4/7	5/9
Epididymis: Atrophy	0/8	5/10*	4/7*	2/9
Testis: Leydig cell adenoma	2/8 (25%)	5/10 (50%)	4/7 (57%)	6/9 (66%)
All animals examined (terminal kills + ad interim deaths/kills)				
Testis: Mass(es)	38/50 (76%)	41/50 (82%)	39/49 (79%)	45/50 (90%)
Testis: Atrophy	21/50	26/50	29/49	14/50
Epididymis: Atrophy	36/50	40/50	44/49*	43/50
Seminiferous tubule: Atrophy	23/50	13/49	20/48	10/50**
Testis: Hyperplasia, interstitial cell	23/50	12/49	16/48	10/50**
Testis: Leydig cell adenoma	43/50 (86%)	42/49 (86%)	43/48 (89%)	46/50 (92%)
Historical control data for Leydig cell adenoma (IET laboratory)	**Mean**	**Range**		
1998–2003 (7 studies)	78%	68–86%		
2005–2009 (5 studies)	76.4	62–84%		

IET: Institute of Environmental Toxicology (Japan); ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Fisher's exact probability test)

Source: Yoshida (2004b)

No treatment-related effects on mortality or feed consumption were noted.

The following effects were observed at 6000 ppm:

- General clinical signs included statistically significant increases in the incidences of tactile hair loss and soiled fur (mainly observed in the external genital region) in females.
- Body weights were statistically significantly decreased in weeks 76–104 in the males (7% reduction at week 104) and consistently throughout treatment in the females (maximally 3% reduction at week 104).
- Haematological findings included statistically significant decreases in lymphocytes (−18%), neutrophils (−20%), monocytes (−19%) and eosinophils (−14%) in males after 104 weeks.
- Slight, statistically significant increases in absolute and relative weights of the kidneys and liver (non-statistically significant changes in absolute liver weight in males) were observed in both sexes. After exclusion of one male at 6000 ppm and two males in the control group because of spontaneous adrenal tumours, absolute and relative adrenal weights were statistically significantly increased in males (+34% and 43% relative to controls, respectively) and females (+68% and +73% relative to controls, respectively). Statistically significant

increases in absolute and relative weights of ovaries (+29% and +35%, respectively) were noted in females, and statistically significant increases in absolute and relative testis weights (201% and 212% of control values, respectively) were observed in males.

- Males showed statistically significant increases in the incidences of mass(es) in the testis (above the historical control range), which correlated well histopathologically with the increased incidence of LCA (see Table 6) and the increased testis weights mentioned above. The incidence of LCA at 6000 ppm was clearly above the historical control range from the IET laboratory (2005–2012) and above the mean of the historical control data from the same laboratory for 1993–2005. The increase in LCA tumours was accompanied by significant decreases in the incidences of atrophy, softening and hyperplasia in testis and by an increase in atrophy in the epididymis and seminiferous tubule. Additionally, a non-statistically significant increase in C-cell thyroid adenoma (within the historical control range of the IET laboratory) and C-cell carcinoma (outside the historical control range of the IET laboratory) was observed in males (see Table 7). The incidence of the C-cell carcinoma in the control males was, however, at the upper historical control range limit. Further histopathological lesions included a statistically significant increase in focal atrophy of the pancreatic acinar cells in males. Additionally, a statistically significant increase in diffuse adrenocortical cell hypertrophy and a statistically significant increase in focal and diffuse (females only) adrenocortical cell vacuolation were observed in both sexes. In contrast to the preceding carcinogenicity study (Yoshida, 2004b), no treatment-related increase in luminal dilatation of the glands in the uterine horn was noted.

In conclusion, cyflumetofen induced changes in body weight, clinical signs and pathology (primary effects on the adrenal gland and testis) at 6000 ppm (equal to 220 mg/kg bw per day in males). Neoplastic findings included a statistically significantly increased incidence of LCA and a non-statistically significant increase in the incidence of C-cell carcinoma of the thyroid gland in males. Both lesions were above the incidences of the historical control range of recent years for Fischer rats (Takahashi, 2013).

2.4 Genotoxicity

(a) In vitro studies

Cyflumetofen was tested for genotoxicity in five in vitro assays. The results of the in vitro genotoxicity tests are summarized in Table 8. With the exception of the mouse lymphoma thymidine kinase (TK) gene mutation assay, which is summarized in more detail below, all of the other in vitro tests were negative.

In the mouse lymphoma gene mutation assay (Verspeek-Rip, 2007), cyflumetofen concentrations from 1 to 333 µg/mL used in a range-finding test revealed high cytotoxicity at 100 µg/mL, with reduction of relative suspension growth by 97% and 31% in the absence and presence of S9 mix, respectively. At 333 µg/mL, little cell survival was observed. In the mutagenicity test, the following concentrations were chosen for the gene mutagenicity assay: 0, 20, 30, 40, 50, 60, 70, 80 and 90 µg/mL for treatment without S9 mix and 0, 10, 20, 40, 60, 80, 100, 120 and 140 µg/mL for treatment with S9 mix. Mutation frequency was approximately 2-, 2.7- and 3.3-fold increased relative to controls at 70, 80 and 90 µg/mL, respectively, in the experiment without S9 mix and at a mean of 2.5- and 6.2-fold relative to controls at 120 and 140 µg/mL, respectively, in the experiment with S9 mix. Moreover, cyflumetofen revealed an up to 4.5- and 1.6-fold increase (in the absence of S9 mix) and an up to 6.9- and 2.8-fold increase (in the presence of S9 mix) in the mutation frequency of small and large colonies, respectively, which might suggest that cyflumetofen could mainly induce chromosomal aberration; this was, however, not confirmed by the in vitro chromosomal aberration studies in hamsters and the micronucleus assay in mice (see Table 8). The increase in mutation frequency was accompanied by cytotoxicity, reflected by a dose-related 64–91% reduction in relative total growth of cells relative to controls at 70 µg/mL and above in the experiment without S9 mix and a dose-related 56% and 90% reduction in relative total growth of cells relative to controls at 120 and 140 µg/mL in the experiment with S9 mix. Moreover, precipitation of cyflumetofen was observed at

and above 90 µg/mL (without S9 mix) and 100 µg/mL (with S9 mix). A statistical evaluation was not performed in this study.

Table 6. Selected macroscopic/histopathological findings in testis and associated tissues in male rats in a carcinogenicity study

	0 ppm	6 000 ppm	IET laboratory: historical control data	
Animals killed in extremis or found dead				
Number of animals examined	9	7		
Testis: Mass(es)	3 (33%)	7* (100%)		
Testis: Atrophy	5	0*		
Testis: Softening	3	0		
Epididymis: Atrophy	6	6		
Seminiferous tubule: Atrophy	5	0*		
Testis: Hyperplasia, interstitial cell	4	0		
Leydig cell adenoma	5 (55%)	7 (100%)		
All animals examined (terminal kills + ad interim deaths/kills)			Historical control data (2005–2012); *n* = 7; testis masses	
Number of animals examined	50	50	Mean	Range
Testis: Mass(es)	34 (68%)	48** (96%)	65%	56–76%
Testis: Atrophy	12	1**		
Testis: Softening	19	2**		
Epididymis: Atrophy	40	48*		
Seminiferous tubule: Atrophy	27	5**	Historical control data (2008–2012); *n* = 5; Leydig cell adenoma	
Testis: Hyperplasia, interstitial cell	19	3**	Mean	Range
Leydig cell adenoma	38 (76%)	48** (96%)	69%	56–82%

IET: Institute of Environmental Toxicology (Japan); ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Fisher's exact probability test)

Source: Takahashi (2013)

Table 7. Incidences of C-cell tumours in the thyroid of rats in a carcinogenicity study

	Males		Females		IET laboratory: Historical control data (2008–2012), males, *n* = 5	
	0 ppm	6 000 ppm	0 ppm	6 000 ppm		
	n = 50	*n* = 49	*n* = 49	*n* = 50	Mean	Range
C-cell adenoma	11 (22%)	15 (30%)	9 (18%)	5 (10%)	34%	2–44%
C-cell adenocarcinoma	9 (18%)	15 (30%)	2 (4%)	4 (8%)	12.40%	6–18%

IET: Institute of Environmental Toxicology (Japan); ppm: parts per million

Source: Takahashi (2013)

Table 8. Results of genotoxicity studies with cyflumetofen

End-point	Test system	Concentration	Batch no.; purity	Result	Reference
Reverse mutation (Ames)	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 *Escherichia coli* WP2 uvrA	20.6–5 000 µg/plate (±S9)	01D1; 97.67%	Negative[a]	Matsumoto (2001)
Mouse lymphoma TK	L5178Y cell line	10–140 µg/mL (±S9)[b]	01H1; 98.4%	Positive[b]	Verspeek-Rip (2007)
Chromosomal aberration	Chinese hamster lung cells	6.25–200 µg/mL (±S9)[c]	01D1; 97.67%	Negative	Matsumoto (2003a)
Chromosomal aberration	V79 Chinese hamster cells	0.31–320 µg/mL (±S9)[d]	01H1; 98.4%	Negative	Schulz (2011)
Micronucleated bone marrow cells	Male ICR (Crj: CD-1) mice	500, 1 000, 2 000 mg/kg bw in 5 males per dose	01D1; 97.67%	Negative	Matsumoto (2003b)
In vivo DNA repair assay (UDS)	Male Wistar (Han) rats	1 000 and 2 000 mg/kg bw in 3 males per dose	01H1; 98.4%	Negative	Buskens (2007)

bw: body weight; DNA: deoxyribonucleic acid; S9: 9000 × *g* supernatant fraction from rat liver homogenate; TK: thymidine kinase; UDS: uscheduled DNA synthesis

[a] Precipitation of test substance without S9.
[b] More details are provided in the text.
[c] Additionally to a 6-hour exposure ±S9, a continuous treatment test was performed for 24 and 48 hours −S9.
[d] Besides four tests with 4-hour exposure times ±S9, two tests with 18 hours of exposure −S9 were performed.

In conclusion, cyflumetofen was mutagenic in the mouse lypmphoma L5178Y in vitro study. However, mutagenicity was observed only at cytotoxic and precipitating concentrations.

(b) In vivo studies

Cyflumetofen was tested for genotoxicity in two in vivo assays. The results of the in vivo genotoxicity tests are presented in Table 8. Neither the micronucleus assay in mice nor the deoxyribonucleic acid (DNA) repair assay in rats (unscheduled DNA synthesis [UDS]) revealed evidence of a genotoxic potential of cyflumetofen.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Rats

In a range-finding one-generation reproductive toxicity study, cyflumetofen (batch no. 01D1; purity 97.67%) was administered in the diet to groups of eight male and eight female at least 7-week-old Wistar Hannover (BrlHan:WIST@Jcl[GALAS]) rats (Takahashi, 2002). The concentrations in feed were 0, 100, 300, 1000 and 3000 ppm. Feed was administered during a 3-week premating period and the subsequent mating period (F_0) until weaning of the F_1 pups to evaluate the potential effects on parental rats and their offspring. Overall group mean intakes of test substance in the treated groups throughout the premating phase and mating period were as follows (in the respective order of parental males and females): 5.89 and 10.59 mg/kg bw per day in the 100 ppm group; 17.4 and 31.1 mg/kg bw

per day in the 300 ppm group; 57.1 and 107.8 mg/kg bw per day in the 1000 ppm group; and 177 and 306 mg/kg bw per day in the 3000 ppm group. Clinical observations, body weight development and feed consumption were recorded regularly (weekly or at least once a day in the case of general clinical observations). In all parental animals, selected organs were weighed, and adrenals were examined microscopically. Reproductive performance was assessed based on successful mating (litter with at least one pup alive), duration of gestation, precoital interval and number of implantation sites. F_1 pups were investigated for clinical conditions and survival (at least daily), body weight development (at least weekly during lactation) and sex ratio. Pups were killed at postnatal days (PNDs) 25–27, the brain, adrenals, spleen, thymus and uterus (females only) were weighed in one male and one female per litter in each dose group, and the adrenals were examined microscopically.

The following treatment-related effects were observed in parental animals: At necropsy, a statistically significant increase in white-coloured adrenals in males (3/8 versus 0/8 in controls) and a statistically significant increase in enlarged and white-coloured adrenals in females (5/8 versus 0/8 in controls) were observed at 3000 ppm. Organ weight measurements revealed an increase in absolute (+7%) and relative kidney weights (+13%, statistically significant relative to controls) in males at 3000 ppm. Moreover, females showed a statistically significant increase in absolute (+27%) and relative (+25%) adrenal weights, relative to controls. Histopathology indicated a statistically significant increase in vacuolation of adrenocortical cells in males (3/8 versus 0/8 in controls) at 1000 ppm (3/8 versus 0/8 in controls) and 3000 ppm (7/8 versus 0/8 in controls). Additionally, statistically significant increases in hypertrophy of adrenocortical cells were observed at 1000 ppm (6/8) and 3000 ppm (8/8) in females (versus 1/8 in controls).

The following treatment-related effects (relative to controls) were observed in F_1 pups: A statistically significant decrease in body weights of male (−7%) and female (−8%) pups on lactation day 21 at 3000 ppm was noted. Organ weight analysis revealed statistically significantly elevated absolute and relative adrenal weights in male (+33% and +43%, respectively) and female (+28% and +36%, respectively) pups at 3000 ppm. Histopathology revealed a statistically significant increase in incidences of hypertrophy of adrenocortical cells in males (6/8 versus 0/0 in controls) and females (5/8 versus 0/8 in controls) at 1000 ppm, and all pups showed this finding at 3000 ppm.

The NOAEL for parental toxicity was 300 ppm (equal to 17.4 mg/kg bw per day), based on increased incidences of vacuolation (males) and hypertrophy (females) of adrenocortical cells at 1000 ppm (equal to 57.1 mg/kg bw per day). The NOAEL for offspring toxicity was also 300 ppm (equal to 17.4 mg/kg bw per day), based on increased incidences of hypertrophy of adrenocortical cells in both sexes at 1000 ppm (equal to 57.1 mg/kg bw per day). The NOAEL for reproductive toxicity was 3000 ppm (equal to 177 mg/kg bw per day), the highest dose tested (Takahashi, 2002).

Cyflumetofen (batch no. 01D1; purity 97.67%) was administered in the diet to groups of 24 male and 24 female 5-week-old Wistar Hannover (BrlHan:WIST@Jcl[GALAS]) rats in a two-generation reproductive toxicity study (Takahashi, 2004). The concentrations in feed were 0, 150, 500 and 1500 ppm. Feed was administered from a 10-week premating period (F_0) over two generations until weaning of the F_2 pups. After a 10-week premating phase under exposure, F_0 animals were mated and allowed to rear F_1 litters. Twenty-four male and 24 female F_1 weanlings (one or two per litter) 22–25 days of age were selected from each group to become F_1 parents and were mated after a 10-week premating period to produce the F_2 generation. The doses achieved are depicted in Table 9. Clinical observations, body weight development and feed consumption were recorded regularly (weekly or at least once a day in the case of general clinical observations) in parental animals. Reproductive performance was assessed based on successful mating (litter with at least one pup alive), duration of gestation, precoital interval, sperm parameters and number of implantation sites. After weaning of offspring, all parental animals (F_0, F_1) were terminated. In all parental animals, selected organs were weighed. Adrenals were examined microscopically in all animals of every dose group, whereas all other organs were analysed from 10 females and 10 males in the control and highest-dose groups. Serum samples from 10 animals of each sex were selected from F_1 parental animals (at termination) in each dose group and analysed for concentrations of luteinizing hormone

(LH), follicle stimulating hormone (FSH), testosterone for males and progesterone and l7β-estradiol for females. Offspring (F_1 and F_2 pups) were investigated for clinical conditions and survival (at least daily), body weight development (days 0, 7, 14 and 21 during lactation), sexual development (preputial separation analysed daily from PND 35 onward and vaginal opening examined daily from PND 26 onward in F_1 pups; anogenital distance examined on PND 4 in F_2 pups) and sex ratio. Pups were terminated after weaning (on PND 26), the brain, adrenals, spleen, thymus, ovaries and uterus (females only) were weighed for one male and one female per litter and dose group, and the adrenals were examined microscopically.

Table 9. Doses achieved in the multigeneration study in rats

	Doses (mg/kg bw per day)					
	Males			Females		
	150 ppm	500 ppm	1 500 ppm	150 ppm	500 ppm	1 500 ppm
F_0 parents premating	10.4	34.6	100.3	12	39.7	121.6
F_1 parents premating	11.5	38.0	114	12.6	44.1	128.6
F_0 females during gestation	–	–	–	9.2	30.6	95
F_1 females during gestation	–	–	–	8.7	31.4	92
F_0 females during lactation	–	–	–	24.7	85.3	252.4
F_1 females during lactation	–	–	–	24.3	84.4	232

bw: body weight; F_0: parental generation; F_1: first filial generation; ppm: parts per million

Source: Takahashi (2004)

Parental animals: No treatment-related effects on mortality, clinical signs, body weight, feed consumption, mating, fertility, gestation or sperm evaluation were observed. The mean estrous cycle length in the F_0 (4.1 days versus 4.0 days in controls and all other treatment groups) and F_1 generations (4.2 days versus 4.0 in controls and all other treatment groups with statistical significance) was slightly longer at 1500 ppm. The values were within the range, but above the means, of the historical control data of the IET laboratory (nine studies between 2004 and 2009; F_0: mean: 4.04 [range: 4.0–4.1 days]; F_1: mean: 4.15 [range: 4.0–4.3 days]). A statistically significant prolongation of completion of preputial separation in F_1 males at 1500 ppm and of vaginal opening in F_1 females at 500 ppm and higher was noted. All values were around the mean and within the historical control range of the same findings in the IET laboratory (see Table 10). At necropsy, increased incidences of white-coloured and enlarged adrenals were observed in females at 500 ppm and above in F_0 (statistically significant for enlargement of the adrenals at 1500 ppm) and F_1 females (statistically significant for both findings at 1500 ppm). Organ weight analysis revealed a statistically significant increase in absolute and relative adrenal weights in F_0 and F_1 females at 500 ppm and higher and in F_0 and F_1 males at 1500 ppm. Additionally, statistically significant increases in absolute and relative pituitary and ovary weights were observed in F_0 females at 1500 ppm (only relative weights of both organs were statistically significantly increased in F_1 females). Moreover, absolute prostate weights were statistically significantly decreased in F_1 males. Histopathology revealed a statistically significantly increased incidence of hypertrophy of the zona glomerulosa in F_0/F_1 females and F_1 males only (not statistically significant, 9/24 versus 4/24 in controls) at 500 ppm and in both sexes and generations at 1500 ppm. There was a statistically significant increased incidence of hypertrophy of the zona fasciculata in F_0/F_1 females at 1500 ppm. Additionally, a statistically significant increase in interstitial vacuolation of ovaries was observed in females. The following concentrations of hormones were statistically significantly reduced in females: progesterone at 150 ppm and higher, FSH at 500 ppm and higher and 17β-estradiol at 1500 ppm. Testosterone levels were

non-statistically significantly reduced in males in all treatment groups, without a clear dose–response correlation (see Table 11). No treatment-related change in LH levels was observed.

Table 10. Sexual development parameters in F_2 pups (anogenital distance) and F_1 parental rats (preputial separation, vaginal opening)

	Anogenital distance in F_2 males (PND 4)			Preputial separation in F_1 males		Vaginal opening in F_1 females		Historical control data for F_1 (9 studies from IET laboratory; 2004–2009)	
Dietary concentration (ppm)	**BW (g)**	**Absolute AGD (mm)**	**Relative AGD**	**Days of age**	**BW (g)**	**Days of age**	**BW (g)**	**Preputial separation**	**Vaginal opening**
0	11.2	6.12	0.274	40.9	178	29.5	92	**Mean (days):**	
150	10.9	6.49	0.294**	40.5	179	30	95	41.7	30.8
500	11.3	6.4	0.286	41.2	179	30.7*	96	**Range (days):**	
1 500	10.3	6.32	0.291**	41.9*	182	31.0**	95	40.7–42.5	28.2–31.9

AGD: anogenital distance; BW: body weight; F_1: first filial generation; IET: Institute of Environmental Toxicology (Japan); PND: postnatal day; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

Source: Takahashi (2004)

Table 11. Group mean serum hormone concentrations in F_1 parental rats[a]

Sex	Dietary concentration (ppm)	FSH (ng/mL)	Testosterone (ng/mL)	Progesterone (ng/mL)	17β-Estradiol (pg/mL)
Male	0	9.63 ± 1.67	1.2 ± 0.92	–	–
	150	9.97 ± 1.44	0.81 ± 0.55	–	–
	500	8.39 ± 1.97	0.53 ± 0.47	–	–
	1 500	10.06 ±1.46	0.8 ± 0.89	–	–
Female	0	7.58 ± 0.87	–	15.7 ± 4.1	19.3 ± 4.9
	150	9.32 ± 1.06	–	10.9 ± 4.7*	18.6 ± 6.1
	500	3.70 ± 2.09*	–	9.9 ± 3.5**	17.4 ± 4.8
	1 500	3.69 ± 1.66*	–	8.3 ± 2.4**	12.7 ± 4.9*

–: not measured; FSH: follicle stimulating hormone; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

[a] Values represent mean ± standard deviation.

Source: Takahashi (2004)

Pups: Male and female F_2 pups revealed a statistically significant decrease in body weight (maximally −8% in relation to controls) on lactation days 7, 14 and 21 at 1500 ppm. A statistically significant elevation in relative anogenital distance was observed at 150 and 1500 ppm in F_2 males only. A statistically significant increase in absolute and relative adrenal weights in F_1/F_2 male and female pups (except for the increase in absolute adrenal weight in F_2 pups, which was not statistically significant) was noted at 500 ppm. Additionally, relative brain weights were statistically significantly increased in male and female F_2 pups. Histopathology showed an increase in incidences of hypertrophy in the zona glomerulosa and zona fasciculata of the adrenals in F_1 and F_2 males at 500 ppm (statistically significant for either zona glomerulosa or zona fasciculata in F_1 and F_2 males, respectively) and in both sexes at 1500 ppm.

The NOAEL for parental toxicity was 150 ppm (equal to 10.4 mg/kg bw per day), based on increased incidences of white-coloured and enlarged adrenals in females, increased adrenal weights in females, elevated incidence of hypertrophy of adrenocortical cells in both sexes, and delayed sexual maturation and sexual hormonal changes in females at 500 ppm (equal to 34.6 mg/kg bw per day). The NOAEL for offspring toxicity was also 150 ppm (equal to 10.4 mg/kg bw per day), based on increased adrenal weights and increased incidences of hypertrophy of adrenocortical cells in males at 500 ppm (equal to 34.6 mg/kg bw per day). The NOAEL for reproductive toxicity was 1500 ppm (equal to 100.3 mg/kg bw per day), the highest dose tested (Takahashi, 2004).

(b) Developmental toxicity

Rats

In a dose range–finding developmental toxicity study, cyflumetofen (batch no. 01C1; purity not provided) was administered by oral gavage from gestation day (GD) 6 to GD 19 to groups of eight female time-mated BrlHan:WIST@Tac(GALAS)Wistar rats (York, 2001b). The dose levels were 0, 100, 500 and 1000 mg/kg bw per day. General clinical observations, body weights and feed consumption were recorded. All rats terminated at day 20 of gestation were given a macroscopic examination, including external observation and an examination of the thoracic, abdominal and pelvic viscera. The following developmental parameters were examined: number of corpora lutea, gravid uterine weight, number and distribution of implantation sites and uterine contents, number of live fetuses, number of early and late intrauterine deaths, weight, gross external alterations and sex of fetuses.

There were no treatment-related effects on mortality, clinical signs, body weights, feed consumption, developmental parameters or macroscopy in maternal animals and no treatment-related effects on litter parameters or gross external alterations in fetuses under the conditions of this range-finding study.

Therefore, the NOAEL for maternal and developmental toxicity was 1000 mg/kg bw per day, the highest dose tested, under the conditions of this range-finding study (York, 2001b).

In a developmental toxicity study, cyflumetofen (batch no. 01D1; purity 97.67%) was administered by oral gavage from GD 6 to GD 19 to groups of 25 female time-mated BrlHan:WIST@Tac(GALAS)Wistar rats (York, 2001a). The dose levels were 0, 50, 250 and 1000 mg/kg bw per day. General clinical observations, body weights and feed consumption were recorded. All rats terminated at day 20 of gestation were given a macroscopic examination, including external observation and an examination of the thoracic, abdominal and pelvic viscera. The placenta, liver and kidneys were weighed, and the adrenals were weighed and examined microscopically. The following developmental parameters were examined: number and distribution of corpora lutea, gravid uterine weight, number and distribution of implantation sites and uterine contents, number of early and late intrauterine deaths, number of live fetuses, and weight, gross external changes, soft tissue and skeletal alterations and sex of fetuses.

There were no treatment-related effects on mortality, clinical signs or feed consumption in dams. Corrected body weight gains on GDs 6–20 were statistically significantly reduced at 1000 mg/kg bw per day. Organ weight analysis revealed a statistically significant increase in absolute and relative weights of the left adrenal at 250 mg/kg bw per day and of the right and left adrenals at 1000 mg/kg bw per day, which were accompanied by a statistically significant increase in slight diffuse hypertrophy of the adrenocortical cells at 1000 mg/kg bw per day and slight or moderate bilateral diffuse cytoplasmic vacuolation of cortical cells (2/25 versus 0/25 in controls) at 250 mg/kg bw per day (non-statistically significant) and at 1000 mg/kg bw per day (24/25). There was no effect on distribution and number of implantations, corpora lutea or resorptions.

There were no treatment-related effects on number of live fetuses or external or visceral alterations. Skeletal changes were confined to an increase in the incidence of wavy ribs, which were

observed in 3.9% (0 mg/kg bw per day), 3.4% (50 mg/kg bw per day), 5.5% (250 mg/kg bw per day) and 10.2% (1000 mg/kg bw per day) of fetuses, with statistical significance at the highest dose, and in 20.8% (0 mg/kg bw per day), 13% (50 mg/kg bw per day), 17.4% (250 mg/kg bw per day) and 28% (1000 mg/kg bw per day) of litters. Moreover, an increased incidence of incompletely ossified sternal centra was observed in 3.3% (0 mg/kg bw per day), 2.7% (50 mg/kg bw per day), 7.9% (250 mg/kg bw per day) and 8.3% (1000 mg/kg bw per day) of fetuses, with statistical significance at the highest dose, and in 16.7% (0 mg/kg bw per day), 8.7% (50 mg/kg bw per day), 39.1% (250 mg/kg bw per day) and 36% (1000 mg/kg bw per day) of litters, with statistical significance at the two highest doses.

The NOAEL for maternal toxicity was 50 mg/kg bw per day, based on increased adrenal weights accompanied by an increased incidence of vacuolation of adrenocortical cells at 250 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 50 mg/kg bw per day, based on an increased incidence of incompletely ossified sternal centra at 250 mg/kg bw per day (York, 2001a).

Rabbits

In a dose range–finding developmental toxicity study, cyflumetofen (batch no. 01D1; purity not provided) was administered by oral gavage to groups of five female 5- to 7-month-old time-mated New Zealand White (Hra:(NZW) SPF) rabbits from GD 6 to GD 28 (York, 2003a). The dose levels were 0, 10, 100, 500 and 1000 mg/kg bw per day. General clinical observations, body weights and feed consumption were recorded. All rabbits terminated on day 29 of gestation were given a macroscopic examination, including external observation and an examination of the thoracic, abdominal and pelvic viscera. The following developmental parameters were examined: number of corpora lutea, gravid uterine and placental weights, number and distribution of implantation sites, number of live fetuses, number of early and late intrauterine deaths, and weight, gross external alterations and sex of fetuses.

There were no treatment-related effects on mortality, feed consumption, developmental parameters or macroscopy in maternal animals and no treatment-related effects on litter parameters or gross external alterations in fetuses under the conditions of this range-finding study. The only adverse clinical sign, which may have been test related, was ungroomed coat in one rabbit at 1000 mg/kg bw per day. Moreover, body weight gains were reduced in the 100, 500 and 1000 mg/kg bw per day groups during the entire dosing period, but not in a treatment-related fashion (78%, 90% and 90% of the control group value, respectively).

Therefore, the NOAEL for maternal and developmental toxicity was 1000 mg/kg bw per day, the highest dose tested, under the conditions of this range-finding study (York, 2003a).

In a developmental toxicity study, cyflumetofen (batch no. 01D1; purity 97.67%) was administered by oral gavage to groups of 25 female time-mated (5–7 months old) New Zealand White (Hra:(NZW) SPF) rabbits from GD 6 to GD 28 (York, 2003b). The dose levels were 0, 50, 250 and 1000 mg/kg bw per day. General clinical observations, body weights and feed consumption were recorded. All rats terminated on day 29 of gestation were given a macroscopic examination, including external observation and an examination of the thoracic, abdominal and pelvic viscera. The placenta, liver, kidneys and adrenals were weighed. Placentae were examined for size, colour and shape. The following developmental parameters were examined: number and distribution of corpora lutea, gravid uterine weight, number and distribution of implantation sites, number of early and late intrauterine deaths, number of live fetuses, and weight, gross external changes, soft tissue and skeletal alterations and sex of fetuses.

There were no treatment-related effects on mortality or clinical signs in dams. A non-statistically significant decrease in body weight gain during GDs 6–29 at 250 and 1000 mg/kg bw per day (76% and 68% of control value, respectively) was observed, which partly correlated with a decrease in feed consumption (93% and 78% of control value, respectively) during GDs 18–21 at the same doses. Organ weights revealed a (non-statistically significant) decrease in absolute and relative liver weights at 250 mg/kg bw per day (by 7.8% and 4%, respectively) and 1000 mg/kg bw per day

(by 8.3% and 6%, respectively). Additionally, the absolute weights of the left and right adrenals were reduced by 11.9% and 6.3%, respectively, at 1000 mg/kg bw per day.

Male, female and combined fetal weights were statistically significantly reduced (by approximately 12% relative to controls) at 1000 mg/kg bw per day. Moreover, placental weights combined by sex were significantly reduced (−12% relative to the control group) at 1000 mg/kg bw per day. A non-statistically significant increase in fetal and litter incidences in flexed downward forepaws at 1000 mg/kg bw per day (three fetuses [1.4%] in two litters [8%] versus 0 in all other treatment groups and controls) was noted. The incidences were within the historical control range of the same laboratory (range in fetuses: 0–4%; mean: 0.12%; range in litters: 0–25%; mean: 0.89%; studies dated from 2002 to 2004). An increased incidence of fetuses (5% versus 1.6% in controls) and statistically significantly increased incidence of litters (40% versus 8.3% in control) with angulated hyoid alae were noted at 1000 mg/kg bw per day; the latter was above the historical control range of litter incidences. Additionally, a statistically significant increase in litter (32% versus 0% in controls) and fetal (7.3% versus 0% in controls) incidences of incompletely ossified sternal centra was seen (above the historical control range) at 1000 mg/kg bw per day. Moreover, the number of ossification sites per fetus per litter was statistically significantly increased in the thoracic and caudal vertebrae and in the ribs (above the historical control range) or was statistically significantly decreased in the case of lumbar vertebrae and of the xiphoid in the sternum (both below the historical control range) at the two highest doses. Overall, a statistically significant increase in the fetal incidence of variations (16.5% versus 7.6%; calculated per litter: 18.6% versus 6.3%) was noted at 1000 mg/kg bw per day.

The NOAEL for maternal toxicity was 50 mg/kg bw per day, based on slight body weight loss at GDs 6–9 and decreased body weight gain during the entire treatment period, which partly correlated with decreased feed consumption at 250 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was also 50 mg/kg bw per day, based on changes in the number of ossification sites in the vertebrae, ribs and sternum at 250 mg/kg bw per day (York, 2003b).

2.6 Special studies

(a) Neurotoxicity

In an acute neurotoxicity study, a single dose of cyflumetofen (batch no. 01H1; purity 97.08%) was administered by gavage to groups of 10 male and 10 female approximately 5-week-old Wistar Crl:WI(Han) rats (Buesen et al., 2010a). The dose levels were 0, 125, 500 and 2000 mg/kg bw. Animals were observed for 14 days. General clinical observations and feed consumption were recorded daily during the dosing period, and body weights were measured prior to treatment and weekly thereafter. Functional observational battery and locomotor activity were recorded prior to treatment and at days 0, 7 and 14. At day 15, animals were killed, and five of each sex per dose were used for neuropathological analysis: brain weights were measured and a gross examination of the nervous system was performed in all animals, and microscopic analysis was performed on the nervous system in the control and highest-dose groups. To show the responsiveness of the test, the applicant conducted positive control studies for acute neurotoxicity in Wistar rats with triethyltin bromide (batch no. 00206DS; purity 97%), methylphenidate hydrochloride (batch no. 1032818; purity 100%) and diazepam (batch no. H06379; purity not provided). All studies revealed clinical and neuropathological effects that were characteristic for each substance (Mellert, Kaufmann & van Ravenzwaay, 2008; Kaspers, 2009).

Clinical examinations, functional observational battery as well as motor activity measurement revealed no test substance–related effects in male or female Wistar rats at any dose level or time point during treatment. Brain weight determination and neuropathological examinations (macroscropy and microscopy) did not reveal any treatment-related neuropathological findings.

The NOAEL for acute systemic and neurotoxicological effects was 2000 mg/kg bw per day, the highest dose tested (Buesen et al., 2010a).

In a subchronic neurotoxicity study, cyflumetofen (batch no. 01H1; purity 97.08%) was administered in the diet to groups of 10 male and 10 female approximately 7-week-old Wistar Crl:WI(Han) rats over a period of 90 days (Buesen et al., 2011a). The dose levels were 0, 500, 1500 and 5000 ppm (equal to 0, 30, 89 and 293 mg/kg bw per day for males and 0, 41, 99 and 353 mg/kg bw per day for females, respectively). Detailed clinical observations were performed prior to treatment and weekly during the dosing period. Body weight and feed consumption were measured prior to treatment and weekly during the dosing period. (Additionally, body weights were recorded on days when the functional observational battery was performed.) Functional observational battery and locomotor activity were recorded prior to study initiation and at days 1, 22, 50 and 85. At day 91, animals were killed, and five animals of each sex per group were used for neuropathological analysis: brain weights were measured and a gross examination of the nervous system was performed in all animals, and microscopic analysis was performed on the nervous system in the control and highest-dose groups. Additionally, adrenal glands were weighed and examined histologically in all treatment groups.

No treatment-related effects on mortality, clinical signs or feed consumption were seen. Overall body weight gain (days 0–91) was non-statistically significantly decreased at 5000 ppm in both sexes. No treatment-related effects on functional observational battery or locomotor activity were observed, and no neuropathological effects were noted. Pathological examination of the adrenals revealed a statistically significant increase in absolute and relative adrenal weights at 5000 ppm in males (141% and 148% of control value, respectively) and at 1500 and 5000 ppm in females (193% and 198% of control value, respectively, at the highest dose). All animals at 5000 ppm showed a light beige discoloration of the adrenals (concomitantly with a moderate enlargement of the adrenals in females). A clear increase in the incidence and severity of diffuse vacuolation in the adrenocortical cells was detected at 1500 ppm and higher compared with the controls and the lowest dose (500 ppm) in both sexes. Generally, diffuse vacuolation was seen in all zonae in the adrenals, but predominantly in the zona fasciculata at 1500 ppm and higher. Increased degrees of severity of vacuolation (microvesicular to macrovesicular patterns) were mainly observed in the zona fasciculata and zona reticularis.

Based on increased adrenal weights in females and increased incidences of histopathological findings in adrenals (vacuolation of adrenocortical cells) in both sexes at 1500 ppm (equal to 89 mg/kg bw per day), the NOAEL for systemic toxicity was 500 ppm (equal to 30 mg/kg bw per day). No treatment-related changes in neurobehaviour or neuropathology were noted. Therefore, the NOAEL for subchronic neurotoxicity was 5000 ppm (equal to 293 mg/kg bw per day), the highest dose tested (Buesen et al., 2011a).

(b) Immunotoxicity

Cyflumetofen (batch no. 01H1; purity 97.08%) was administered in the diet to groups of eight female 6-week-old Crl:WI (Han) rats for 28 days to investigate immunotoxicity. The concentrations in feed were 0, 500, 1500 and 5000 ppm (equal to 0, 33, 107 and 349 mg/kg bw per day, respectively). Cyclophosphamide monohydrate (batch no. 1362353; purity 100%) as immunosuppressant was administered by gavage at 4.5 mg/kg bw per day to a positive control group of eight female Wistar Han rats (Buesen et al., 2011b). All animals were immunized 6 days before blood sampling and necropsy using sheep red blood cells (sRBCs) administered intraperitoneally. Detailed clinical observations were performed before treatment and weekly during treatment, and body weight development was investigated at the start of treatment and twice weekly during treatment. Feed intake was recorded weekly. Blood samples were collected at the scheduled termination, and the primary T cell–dependent antibody response (anti-sRBC immunoglobulin M [IgM] enzyme-linked immunosorbent assay [ELISA]) was measured. Adrenals, spleen and thymus were weighed, macroscopy was performed on all animals and the adrenals were examined microscopically.

There were no treatment-related effects on clinical signs, body weight or feed consumption. Six days after immunization, no changes in the sRBC IgM titres were found in any cyflumetofen-treated groups. Regarding pathology, immune-relevant organs such as thymus and spleen were

without findings. Statistically significant increases in the incidence of absolute and relative weights of the adrenals were noted at 500 ppm (+11% and +12%, respectively) and above (maximally +87% for both parameters). The increases in weight correlated with light discoloration and enlargement of the adrenals and microscopic changes, such as an increased incidence and degree of severity of vacuolation in the adrenocortical cells of the zona fasciculata and zona reticularis at 1500 and 5000 ppm. The oral administration of the positive control substance cyclophosphamide monohydrate led to findings indicative of immunotoxicity, which was reflected by significantly lower sRBC IgM antibody titres as well as reduced spleen and thymus weights.

The NOAEL for the immunotoxicologically relevant effects was 5000 ppm (equal to 349 mg/kg bw per day), the highest dose tested, and the NOAEL for systemic toxicity was 500 ppm (equal to 33 mg/kg bw per day), based on increases in adrenal weight, enlargement and discoloration of adrenals and microscopic changes, such as vacuolation of adrenocortical cells, at 1500 ppm (equal to 107 mg/kg bw per day) (Buesen et al., 2011b).

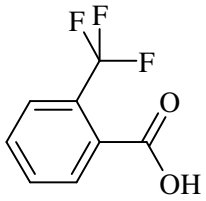
(c) Toxicity of metabolites

B-1 (2-(trifluoromethyl)benzoic acid with CAS no. 433-97-6), a goat and plant metabolite and food processing hydrolysis product, B-3 (2-(trifluoromethyl)benzamide with CAS no. 360-64-5), a soil metabolite, and the impurity AB-13 were tested for acute oral toxicity and potential genotoxic properties. B-1 is also a major metabolite of cyflumetofen in rats (urine: < 10%; faeces: 17%; and bile: < 0.8% of low dose).

Studies with metabolite B-1

Metabolite B-1 (Fig. 3) (batch no. FHE01; purity 99%) was tested in an acute oral toxicity study and in one in vivo and three in vitro genotoxicity tests (see Table 12).

Fig. 3. Structure of B-1 (2-(trifluoromethyl)benzoic acid)



Acute toxicity: The oral LD_{50} in female Wistar (Crl@WI) BR rats was greater than 2000 mg/kg bw. Lethargy, hunched posture, uncoordinated movements and/or piloerection were noted among the animals between days 1 and 3 (Beerens-Heijnen, 2004).

Genotoxicity: With the exception of the mouse lymphoma TK gene mutation assay, which is described in more detail below, the other two in vitro assays and one in vivo DNA repair assay (UDS) were negative. In addition, a toxicity prediction assessment of B-1 (Barentsen, 2008b) revealed no structural alerts (genotoxicity, mutagenicity, chromosome damage and carcinogenicity as major end-points examined) in a DEREK for Windows program (version 10.0.2) analysis.

In the first mouse lymphoma gene mutation assay (Verspeek-Rip, 2004b), B-1 concentrations from 1 to 1900 µg/mL revealed slight cytotoxicity at 1000 µg/mL and above, with reduction of relative total growth by up to 44% with or without S9 mix; a maximally 3-fold increase in mutation frequency (above the historical control range) was observed at the two highest concentrations (1000 and 1900 µg/mL) with and without S9 mix after 3-hour exposure times. In the second experiment, mutation frequency was increased 8-fold relative to controls at the highest concentration without S9 mix after a 24-hour exposure (1901 µg/mL) and above the historical control range, accompanied by marked cytotoxicity (reduction of relative total growth by 91%). No significant increase in mutation

frequency occurred in the second experiment with S9 mix treatment (no cytotoxicity up to the highest dose), and the positive results of the first experiment were therefore not confirmed. As the plates of the positive control without S9 mix addition could not be evaluated, the second experiment was repeated with an additional concentration of 1500 µg/mL, resulting in 2.7-fold and 7.6-fold increases in mutation frequency (again above the historical control range) at 1500 and 1900 µg/mL, respectively, without S9 mix treatment (two highest concentrations) after a 24-hour exposure, which was again accompanied by marked cytotoxicity (82% and 92% reduction in relative total growth). No precipitation occurred up to the highest concentration. In general, the increased mutation frequencies were reflected by an increase in the fraction of small revertant colonies relative to controls (whereas the fractions of large revertant colonies were only marginally increased).

Table 12. Results of genotoxicity studies with B-1

End-point	Test system	Concentration	Batch no.; purity	Result	Reference
Reverse mutation (Ames)	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 *Escherichia coli* WP2 uvrA	3–5 000 µg/plate (±S9)	FHE01; 99%	Negative[a]	Verspeek-Rip (2004c)
Mouse lymphoma TK	L5178Y cell line	1–1 901 µg/mL (±S9)	FHE01; 99%	Positive[b]	Verspeek-Rip (2004b)
Chromosomal aberration	Peripheral human lymphocytes	33–1 901 µg/mL (±S9)	FHE01; 99%	Negative	Buskens (2004b)
In vivo DNA repair assay (UDS)	Male Wistar (Han) rats	1 000 and 2 000 mg/kg bw in 3 males per dose	FHE01; 99%	Negative	Buskens (2009b)

bw: body weight; DNA: deoxyribonucleic acid; S9: 9000 × *g* supernatant fraction from rat liver homogenate; TK: thymidine kinase; UDS: unscheduled DNA synthesis

[a] Precipitation at 3330 and 5000 µg/plate ±S9.
[b] More details are provided in the text.

In conclusion, metabolite B-1 was mutagenic in the mouse lymphoma L5178Y in vitro study in experiments without S9 mix treatment predominantly at prolonged exposure times, at high concentrations and occasionally accompanied by pronounced cytotoxicity.

Studies with metabolite B-3

Metabolite B-3 (Fig. 4) (batch no. MA080115; purity 99.1%) was tested in one in vivo and three in vitro genotoxicity tests (see Table 13). No acute oral toxicity test has been performed with B-3. The acute toxicity of B-3 relative to cyflumetofen was, however, estimated from the UDS assay in rats. Additionally, a 28-day rat study was performed with B-3, and a position paper on the genotoxicity of B-3 and derivation of a metabolite-specific acceptable daily intake (ADI) was provided by the applicant (Meurer, 2014).

Genotoxicity: The results of the in vitro genotoxicity tests are summarized in Table 13. One tester strain of Ames tests, *Salmonella typhimurium* TA100, gave a positive response. B-3 induced 1.7- and 1.9-fold increases in the number of revertants at 3330 and 5000 µg/plate (two highest doses with and without S9 mix), respectively. In a repeat experiment, B-3 induced 1.6- and 2.1-fold increases at the two highest concentrations (3330 and 5000 µg/plate), respectively, without S9 mix pretreatment. As the numbers were at or above the historical control range and concentration

dependent, B-3 was considered to be mutagenic in the TA100 tester strain at high concentrations. No precipitation or bacteriotoxicity was noted. In addition, in a mouse lymphoma TK gene mutation assay, B-3 was positive without S9 mix at high concentrations: B-3 induced an up to 2-fold increase in the mutation frequency at the highest concentration of 1891 µg/plate (without S9 mix and with a 3-hour exposure). In a repeat experiment (with a 24-hour exposure), B-3 induced 2- and 4.4-fold increases in mutation frequency above the historical control range at 1000 and 1891 µg/mL (two highest concentrations without S9 mix), accompanied by moderate cytotoxicity (reduction of relative total growth by 47%) at the highest dose. No positive response was obtained with S9 mix. In conclusion, B-3 was mutagenic in the mouse lymphoma test system in the absence of S9 mix at high concentrations.

Fig. 4. Structure of B-3 (2-(trifluoromethyl)benzamide)

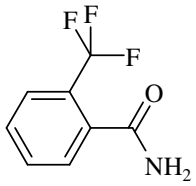


Table 13. Results of genotoxicity studies with B-3

End-point	Test system	Concentration	Batch no.; purity	Result	Reference
Reverse mutation (Ames)	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 *Escherichia coli* WP2 uvrA	3–5 000 µg/plate (±S9)	MA080115; 99.1%	Positive in TA100	Verspeek-Rip (2009b)
Mouse lymphoma TK	L5178Y cell line	1–1 891 µg/mL (±S9)	MA080115; 99.1%	Positive	Verspeek-Rip (2009a)
Chromosomal aberration	Peripheral human lymphocytes	33–1 891 µg/mL (±S9)	MA080115; 99.1%	Negative	Buskens (2009c)
In vivo DNA repair assay (UDS)	Male Wistar (Han) rats	175 and 350 mg/kg bw in 3 males per dose	MA080115; 99.1%	Negative	Buskens (2009a)

bw: body weight; DNA: deoxyribonucleic acid; S9: 9000 × *g* supernatant fraction from rat liver homogenate; TK: thymidine kinase; UDS: unscheduled DNA synthesis

An in vitro chromosomal aberration assay on peripheral human lymphocytes and an in vivo UDS assay did not reveal clastogenic effects or an increased DNA repair rate. Based on the results of the in vivo UDS test in rats, a more acutely toxic effect with B-3 compared with cyflumetofen is expected, as 1/2 males died at 2000 mg/kg bw within 3.5 hours after dosing, and 1/1 male at either 500 or 1000 mg/kg bw had to be terminated for humane reasons in the range-finding study. Moreover, animals at 250 mg/kg bw and higher exhibited clinical signs, such as ataxia and lethargy. A toxicity prediction assessment of B-3 (Barentsen, 2008c) revealed no structural alerts (genotoxicity, mutagenicity, chromosome damage and carcinogenicity as major end-points examined) in a DEREK for Windows program (version 10.0.2) analysis.

Short-term study of toxicity: B-3 (Reg. No. 4288294; batch no. L84-230; purity 99.4%) was administered for 28 days in the diet to groups of five male and five female 6-week-old CRL:WI(Han) rats. The concentrations in feed were 0, 75, 300 and 1200 ppm (equal to 0, 6.1, 24.1 and 85.3 mg/kg bw per day for males and 0, 6.2, 23.9 and 88.2 mg/kg bw per day for females, respectively) (Stark, 2014). General clinical signs were conducted daily. Detailed clinical signs, feed consumption and body weights were recorded weekly. Functional observational battery and motor activity observations were conducted at the end of the study. Clinical pathology (clinical chemistry, urine analysis, haematology) was performed towards the end of the study. At study termination, organ weights, macroscopy and histopathology were performed. Histopathological analysis was performed in the highest-dose and control group animals. The Mallory Heidenhain's stain was performed to assess eosinophilic droplets, and two males of each of the control and highest-dose groups were immunostained with an antibody to α_{2u}-globulin.

Body weight was statistically significantly decreased from study days 7 and 14 onwards in males and females, respectively, reaching maximal reductions of −23.1% and −16.8% on study day 28 in males and females, respectively, at 1200 ppm.

Body weight gain was statistically significantly decreased in males by 16.5% at 300 ppm and in males and females up to −45.7% and −55.4% after 28 days, respectively, and correlated partly with decreases in feed consumption at 1200 ppm. In the functional observational battery, a dose-related reduction in exploration of the area was observed at 300 ppm (one female) and at 1200 ppm (one female and three males versus 0 in the control groups). Additionally, hindlimb grip strength was statistically significantly decreased (−15.8%) in males and forelimb grip strength was decreased by 16.8% in females at 1200 ppm. Clinical chemistry revealed a statistically significant increase in γ-glutamyltransferase activities and potassium levels in both sexes and a statistically significant decrease in chloride (both sexes) and calcium (males only) levels at 1200 ppm. Total protein and albumin levels were statistically significantly increased at 300 ppm (by 5%) and at 1200 ppm (by 11% and 8%, respectively) in both sexes, and globulins were statistically significantly increased in females at 300 and 1200 ppm (~12%) and in males at 1200 ppm (~17%). Cholesterol values were statistically significantly increased by 40% at 300 ppm in females and by 63% and 50% in females and males at 1200 ppm, respectively, whereas triglyceride levels were reduced in males at 1200 ppm. Urine analysis revealed blood in urine in males in the two highest dose groups, and transitional epithelial cells and granulated and epithelial cell casts were found in the urine sediment of animals in all treated groups. Additionally, a decrease in urinary pH (also at 300 ppm) and a statistically significant increase in urine volume were noted in males at 1200 ppm. Organ weight analysis revealed a statistically significant decrease in absolute brain weight in males at 300 ppm and in both sexes at 1200 ppm, a statistically significant decrease in absolute heart weight in both sexes at 1200 ppm and statistically significant increases in relative kidney weight in males at 300 ppm and relative testis and thyroid weights in males at 1200 ppm. Furthermore, a statistically significant increase in absolute liver weight was noted in males at 1200 ppm, and statistically significant increases in relative liver weights were seen in both sexes at 300 ppm and above (maximally by 25% and 64% in males, respectively); in females, statistically significant decreases in absolute spleen weight at 300 ppm and relative spleen weight at 300 ppm and above and a statistically significant decrease in absolute ovary weight at 1200 ppm were observed. Organ pathology showed an increased incidence of liver discoloration in both sexes and focal constriction (males only) at 1200 ppm, partly accompanied by an increased incidence of centrilobular hypertrophy in both sexes at 300 ppm and above. Moreover, an increased incidence of discoloration of kidneys at 300 ppm and above was noted, which was partly associated with increased incidences of tubular degeneration, granular and hyaline casts and higher degrees of eosinophilic droplets in all treated male groups. Immunohistochemical staining for α_{2u}-globulin showed that these effects were due to an α_{2u}-globulin nephropathy, which is considered a male rat–specific effect with no relevance to humans. Additionally, an increased incidence of decreased size of prostate, seminal vesicles and coagulating glands (1/5 males each) and increased incidences of uterine, cervical and vaginal atrophy (3/5 females each) and of decreased cellularity in bone marrow (2/5) were observed.

Based on body weight changes in males, reduced exploration of the area in females, slight changes in clinical chemistry, increases in liver weights and centrilobular hypertrophy in the liver in

both sexes at 300 ppm (equal to 23.9 mg/kg bw per day), the NOAEL was 75 ppm (equal to 6.1 mg/kg bw per day) (Stark, 2014).

Position paper from the applicant: The applicant stressed that the negative in vivo UDS assay in rat liver adequately covered the need for any in vivo follow-up assay for point mutations with the metabolite B-3 (Meurer, 2014). As no elevated DNA synthesis was observed in the UDS assay in rat liver and the liver is supposed to be one of the major target organs of B-3 (as shown in the 28-day rat study), B-3 can be considered not genotoxic in vivo. To underline this conclusion, BASF is performing an in vivo comet assay with metabolite B-3 (final report scheduled for early 2015). An ADI for B-3 of 0.01 mg/kg bw was derived by BASF based on a NOAEL of 6.1 mg/kg bw per day, using a safety factor of 600. An additional safety factor of 6 was considered in the calculation to cover the extrapolation from a subacute to a chronic exposure.

Studies with impurity AB-13

AB-13 (Fig. 5) (batch no. HN0305021; purity 99.2%) was tested in an acute oral toxicity study (Teunissen, 2004) with female Wistar (Crl@WI) BR rats and in three in vitro genotoxicity tests (see Table 14).

Fig. 5. Structure of AB-13

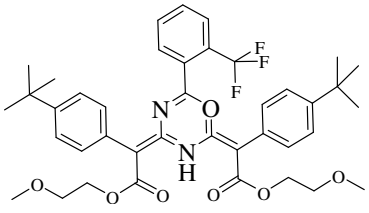


Table 14. Results of genotoxicity studies with AB-13

End-point	Test system	Concentration	Batch no.; purity	Result	Reference
Reverse mutation (Ames)	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 *Escherichia coli* WP2 uvrA	3–5 000 µg/plate (±S9)	HN0305021; 99.2%	Negative	Buskens (2004c)
Mouse lymphoma TK	L5178Y cell line	0.03–100 µg/mL (±S9)[a]	HN0305021; 99.2%	Negative	Verspeek-Rip (2004a)
Chromosomal aberration	Peripheral human lymphocytes	3–333 µg/mL (±S9)	HN0305021; 99.2%	Negative	Buskens (2004a)

S9: 9000 × *g* supernatant fraction from rat liver homogenate; TK: thymidine kinase

[a] Precipitation occurred at 100 µg/mL ±S9.

Acute toxicity: The LD_{50} value was greater than 2000 mg/kg bw in female Wistar rats. Hunched posture, uncoordinated movements and shallow respiration were noted among the animals between days 1 and 2.

Genotoxicity: All in vitro genotoxicity assays were negative. In addition, a toxicity prediction assessment of AB-13 (Barentsen, 2008a) revealed no structural alerts for genotoxicity, mutagenicity, chromosome damage or carcinogenicity in a DEREK for Windows program (version 10.0.2) analysis.

(d) Mode of action for Leydig cell adenoma

Five in vitro mechanistic studies were performed to identify a possible mode of action for LCA via perturbation of the estrogen or androgen system.

To determine the potential of cyflumetofen to inhibit aromatase (CYP19) activity (Foster, 2011), the United States Environmental Protection Agency (USEPA)–recommended aromatase assay was performed, as described in the Endocrine Disruptor Screening Program Test Guideline OPPTS 890.1200 (2009). In short, the aromatase human CYP19 + P450 reductase Supersomes™ kit purchased from BD Biosciences was used to investigate the impact of cyflumetofen (batch no. 009020; purity 99.5%) on the conversion of [1β-^{3}H]androstenedione to estradiol by measuring the production of ^3H_2O (representing aromatase activity) by liquid scintillation counting. Differing concentrations of cyflumetofen (10^{-10}–$10^{-4.5}$ mol/L) were compared with a positive control, 4-hydroxyandrostenedione (formestane; 10^{-10}–10^{-5} mol/L) and a negative control (test substance replaced by ethanol), to determine the extent of inhibition of aromatase activity. Incubations were performed in triplicate (except negative controls: quadruplicate) for 15 minutes, and aromatase activities from three independent experiments were calculated.

The results revealed that cyflumetofen was not an inhibitor of CYP19 aromatase activity. The maximum extent of inhibition of CYP19 aromatase activity by cyflumetofen was 8% (92% of full activity control), 9% (91% of full activity control) and 4% (96% of full activity control), in three independent experiments, without any clear concentration-dependent patterns. 4-Hydroxyandrostenedione (the positive control) caused up to a 99% inhibition of the aromatase activity in all experiments in a concentration-dependent fashion (Foster, 2011).

To determine the potential of cyflumetofen to function as an estrogen receptor (ER) agonist, an in vitro human ERα-transcriptional activation system (hERα-HeLa-9903 cell line purchased from JCRB Cell Bank) was used, in which ER activation is reflected by luciferase activity (Steady-Glo® Luciferase Assay System from Promega), as described in the USEPA Endocrine Disruptor Screening Program Test Guideline OPPTS 890.1300 and in Organisation for Economic Co-operation and Development (OECD) Test Guideline 455 (2009). In short, cyflumetofen (batch no. 009020; purity 99.5%) was applied to hERα-HeLa-9903 cells at concentrations of 10^{-11}–10^{-5} mol/L in triplicate in three independent experiments (Akhurst, 2011a). The responsiveness of the test system was assessed in parallel during each test using appropriate concentrations of a strong estrogen (E2, 17β-estradiol), a weak estrogen (17α-estradiol), a very weak agonist (17α-methyltestosterone) and a negative chemical (corticosterone). As a positive and negative control, E2 (10^{-9} mol/L) and dimethyl sulfoxide (DMSO) (0.1% volume per volume [v/v]) were used, respectively (six replicates each). Cytotoxicity was assessed in all cyflumetofen-treated cells and negative control cells. The test chemical was considered to produce a positive response if the RPC_{Max} (the maximum level of response induced by a test chemical concentration relative to the response induced by E2 at 10^{-9} mol/L) was at least 10% and if this exceedance occurred in at least two out of three independent assays.

Cyflumetofen was not cytotoxic at the highest test concentration used. In all three assays, highest RPC_{Max} values were achieved at the maximal cyflumetofen concentration (10^{-5} mol/L). In the first, second and third assays, RPC_{Max} values were 7.8%, 10.4% and 4.3%, respectively. Therefore, cyflumetofen was negative in two out of three assays and only marginally positive (RPC_{Max}: 10.4%) in the remaining assay.

In conclusion, cyflumetofen was considered negative in the hERα-transcriptional activation assay (Akhurst, 2011a).

To determine the potential of cyflumetofen to interact with ER, an in vitro ER-binding assay using rat uterine cytosol was conducted in compliance with the USEPA Endocrine Disruptor Screening Program Test Guideline OPPTS 890.1250 (2009). In short, cytosol protein preparations from uteri (isolated from up to 100-day-old ovariectomized Sprague-Dawley (CD (Crl:CD(SD))) rats) were obtained and pooled, and the final protein concentration suitable for the competitive binding assay was determined (Matthews, 2012). The competitive binding assay was carried out as follows: 1 nmol/L [^{3}H]17β-estradiol, various concentrations of cyflumetofen (10^{-10}–10^{-4} mol/L) and solvents were incubated overnight at 4 °C with 0.3 mg/mL cytosolic uterine protein concentrations (containing ER). After an extensive washing procedure, samples were subjected to liquid scintillation counting (measuring of bound [^{3}H]17β-estradiol). The responsiveness of the test system was assessed in parallel during each test using appropriate concentrations of non-radiolabelled 17β-estradiol, a weak positive control (19-norethindrone) and a negative control (octyltriethoxylsilane). Moreover, a solvent control (ethanol) was incubated as well. Assays were performed in triplicate for each concentration and in three runs. From the fitted protein binding curves, median inhibitory concentrations (IC_{50} values) and relative binding affinities were determined for the test substances. A positive response in this assay is defined as greater than 50% displacement of radiolabelled 17β-estradiol from the receptor at one or more of the test concentrations used.

As cyflumetofen did not compete with 17β-estradiol in protein binding, no [^{3}H]17β-estradiol protein binding curve could be defined in any runs. A maximum of 14% displacement of [^{3}H]17β-estradiol from proteins in the cytosolic uterine preparations was observed at 10^{-4} mol/L (100 µmol/L) on run 1 only. Therefore, cyflumetofen was considered to be "not interactive" for ER binding. The reference standard and the positive and negative control compounds gave results that were consistent with the USEPA criteria for assay performance (Matthews, 2012).

To determine the potential of cyflumetofen to interact with androgen receptors (AR), an in vitro AR binding assay using rat prostate cytosol was conducted as outlined in the USEPA Test Guideline OPPTS 890.1150 (2009). In short, cytosol protein preparations from prostate (isolated from up to 90-day-old castrated Sprague-Dawley rats) were obtained and pooled, and the final protein concentration suitable for the competitive binding assay was determined (Willoughby, 2012). In a preliminary validation assay, it was shown that the AR was present in reasonable concentrations in protein preparations and revealed appropriate affinity for [^{3}H]R1881 (a synthetic androgen, also called methyltrienolone). The competitive binding assay was carried out as follows: 1 nmol/L [^{3}H]R1881, various concentrations of cyflumetofen (10^{-10}–10^{-3} mol/L in the first run and 10^{-11}–10^{-4} mol/L in the remaining runs) and solvents were incubated overnight at 4 °C with 8.8 mg/mL cytosolic prostate protein concentrations. After an extensive washing procedure, samples were subjected to liquid scintillation counting (measuring bound [^{3}H]R1881). All concentrations were tested in replicates of three, and three independent runs were performed. The responsiveness of the test system was assessed in parallel during each run using appropriate concentrations of non-radiolabelled R1881 and a weak positive (dexamethasone) control. Moreover, a solvent control (DMSO) was incubated as well. From the fitted protein binding curves, IC_{50} values and relative binding affinities were determined for the test substances and controls. A positive response in this assay is defined as greater than 50% displacement of radiolabelled R1881 from the receptor at one or more of the test concentrations.

As precipitation was observed at 10^{-3} and 10^{-4} mol/L in the first run, the concentration range of cyflumetofen was shifted to lower concentrations. A maximum of 16.4% and 13.4% displacement of [^{3}H]R1881 was observed from proteins in the cytosolic prostate preparations at the top two concentrations (10^{-3} and 10^{-4} mol/L), respectively, accompanied by precipitation of the test substance in the first run only. In the second and third runs, 15% and 16% displacement of the ligand were noted at the highest dose of 10^{-4} mol/L, respectively, without significant changes at lower concentrations.

As only marginal displacements of the ligand at concentrations at or near precipitation of cyflumetofen were observed, cyflumetofen was considered to be "not interactive" for AR binding.

The reference standards and the solvent control compounds gave results that were consistent with the USEPA criteria for assay performance (Willoughby, 2012).

To determine the potential of cyflumetofen to interact with steroidogenesis, an in vitro H295R steroidogenesis assay was performed, as outlined in the USEPA Test Guideline OPPTS 890.1550 (2009). The objective of the H295R steroidogenesis assay is to detect substances that affect production of 17β-estradiol (E2) and testosterone. H295R cells are human adenocarcinoma cells that express genes encoding all the key enzymes in the steroidogenesis pathway, beginning with the sequence of reactions from cholesterol to the production of E2 and testosterone. The aim of this study was to determine the fold change in testosterone and E2 concentrations in cell culture after treatment with cyflumetofen (Akhurst, 2011b). In short, 24-well plates with H295R cells were incubated with various concentration of cyflumetofen (10^{-11}–10^{-5} mol/L in the first run and 10^{-10}–10^{-5} mol/L in the second and third runs) at 37 °C for 48 hours. All concentrations were tested in triplicate, and three independent runs were performed. After incubation, the supernatant cell culture medium was analysed by ELISA (based on the principle of competitive binding) to measure E2 and testosterone concentrations. The ELISA methods were based on commercially available kits (DRG, catalogue no. EIA-1559 [testosterone] and EIA-2693 Huntingdon Life Sciences LBA002716 [E2]). The responsiveness of the test system was assessed in parallel during each run using 10 µmol/L forskolin, an inducer of steroidogenesis, and 1 µmol/L prochloraz, an inhibitor of steroidogenesis. Moreover, a solvent control (DMSO) was incubated as well. A chemical was judged to be positive if the fold increases or decreases were statistically significantly ($P \leq 0.05$) different from the solvent control.

In the second run, cyflumetofen was cytotoxic at 10 µmol/L, with a viability value of 61% of the solvent control, and therefore this value was excluded from the statistical analysis. In the first run, cyflumetofen caused a statistically significant increase in E2 concentration to 1.50 times the solvent control value at the highest concentration of 10 µmol/L (see Table 15). In the second and third runs, an additional concentration, 5 µmol/L, was included in the dose range in order to further investigate this response. In these remaining runs, cyflumetofen caused a statistically significant increase in E2 concentration to maximally 1.64 times the solvent control value at this highest analysable concentration of 5 µmol/L. In addition, in the first and second runs, cyflumetofen caused a statistically significant decrease in testosterone concentration to maximally 0.63 times the solvent control value at the statistically analysable top concentrations of 1, 5 and 10 µmol/L. In the third run, cyflumetofen caused a statistically significant decrease in testosterone concentration to maximally 0.75 times the solvent control value at 5 and 10 µmol/L.

In conclusion, cyflumetofen demonstrated an influence on the steroidogenesis pathway, acting as an inducer of E2 production at concentrations of 5 µmol/L and above (maximally 1.64-fold) and an inhibitor of testosterone production at concentrations of 1 µmol/L and above (maximally 0.63-fold) (Akhurst, 2011b).

In a position paper from the applicant (van Cott & Meurer, 2013), to elucidate a possible mode of action for the induction of LCA by cyflumetofen, the applicant concluded that a possible treatment-related carcinogenic effect on Leydig cells by cyflumetofen at the highest dose (6000 ppm) in the second carcinogenicity study (Takahashi, 2013) cannot be disregarded; the suggested mode of action included a decrease in testosterone levels (as indicated in the in vitro steroidogenesis assay) and/or enhanced biliary excretion of testosterone due to liver enzyme induction (not mechanistically investigated, however), which could result in a transient compensatory higher LH production along the hypothalamic–pituitary–testicular axis and Leydig cell proliferation and LCA formation in the testis. Fischer rats have a high background incidence of LCA formation. Therefore, as Fischer rats are extremely sensitive to changes in LH levels and subsequent LCA formation compared with humans, as increased incidences of LCA were observed only at high doses in rats and as testicular lesions were not observed in short-term studies in rats or any other species, lifetime exposure risk assessment based on the chronic reference dose will be protective of human health; therefore, cyflumetofen can be considered “not likely to be carcinogenic to humans”, according to the applicant.

Table 15. Mean fold change in estradiol and testosterone concentrations relative to solvent controls after treatment with cyflumetofen (top four/five concentrations shown)

Cyflumetofen concentration	Estradiol mean (±SD) fold change relative to controls			Testosterone mean (±SD) fold change relative to controls		
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
10 µmol/L	1.5 ± 0.07***	1.93 ± 0.09[#]	1.57 ± 0.06***	0.71 ± 0.03***	0.47 ± 0.05[#]	0.76 ± 0.04**
5 µmol/L	–	1.64 ± 0.06***	1.29 ± 0.02***	–	0.63 ± 0.09***	0.75 ± 0.07***
1 µmol/L	0.91 ± 0.05	1.12 ± 0.02	0.98 ± 0.03	0.84 ± 0.07*	0.77 ± 0.02***	0.87 ± 0.05
100 nmol/L	0.89 ± 0.01	0.84 ± 0.02	0.89 ± 0.05	0.95 ± 0.05	0.98 ± 0.07	0.89 ± 0.1
10 nmol/L	0.85 ± 0.05*	0.83 ± 0.11	0.87 ± 0.08	0.98 ± 0.06	0.92 ± 0.05	0.98 ± 0.01

SD: standard deviation; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; [#]: not included in the statistical analysis, as it produced only 61% cell viability

Source: Akhurst (2011b)

(e) Other special studies

Cyflumetofen (batch no. 01D1; purity 97.7%) was administered for up to 28 days in the diet to groups of male and female 6- to 8-week-old Fischer (F344/DuCrlCrj) rats, to investigate the mechanism(s) for the effects on adrenal gland and ovary in a non-GLP and non-guideline study (Takeda, 2006). The concentrations in feed were 0 ppm (10 animals of each sex), 100 ppm (eight animals of each sex) and 5000 ppm (10 animals of each sex), which are equal to 0, 7.44 and 378 mg/kg bw per day for males and 0, 7.59 and 347 mg/kg bw per day for females, respectively. Clinical observations were performed at least daily. Body weights and feed consumption were recorded prior to treatment and once weekly thereafter. Serum hormone levels were measured, including adrenocorticotrophic hormone (ACTH) and corticosterone (main glucocorticosteroid), after 4 weeks using a radioisotope method. Necropsy in males was performed after 4 weeks (females were selected for necropsy on the day of diestrus within an additional 5-day period of treatment). All gross findings and adrenal and ovary weights were recorded in all animals. Two animals of each sex of the 0 and 5000 ppm groups were subjected to ultrastructural examination of the adrenal gland and/or ovary. From the remaining eight animals of each sex per dose, half of the left adrenal gland was used for quantitative analysis of gene expression by reverse transcription and real-time quantitative polymerase chain reaction (PCR) of CYP11A1 (cholesterol monooxygenase, a cholesterol side-chain cleavage enzyme), CYP11B1 (steroid 11β-hydroxylase), neutral cholesteryl ester hydrolase (NCEH) and hormone-sensitive lipase (HSL) genes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard to normalize gene expression levels. The other half of the left adrenals and the left ovaries were subjected to histopathological examination, and the right adrenals were additionally used for quantitative cholesterol measurements.

There were no treatment-related clinical signs or changes in body weight or feed consumption. In addition, no treatment-related differences in serum concentrations of ACTH or corticosterone were observed. At necropsy, all animals revealed enlarged and whitish adrenal glands at 5000 ppm. Absolute and relative adrenal weights were statistically significantly increased in males (+26% and +20% relative to controls, respectively) and females (+65% and 64% relative to controls, respectively). Moreover, relative ovary weight was statistically significantly increased (+10% relative to controls) and absolute ovary weight was non-statistically significantly elevated (+11% relative to controls) at 5000 ppm. However, it was suspected that the effects on the ovary were incidental, because, after excluding two females with spontaneous large ovarian cysts, there were only marginal,

non-statistically significant increases in ovary weights. Histopathologically, diffuse cytoplasmic vacuolation of adrenocortical cells and of the ovary (females only) was noted in all animals at 5000 ppm. Ultrastructurally, the vacuolations turned out to be lipid droplets within the adrenocortical and ovary cells. Gene expression analysis by reverse transcription and real-time quantitative PCR showed a statistically significant increase in absolute and relative (compared with GAPDH) CYP11A1 expression in females (+33% and +36% compared with controls, respectively) and males (+24% and +25% compared with controls, respectively) at 5000 ppm. In addition, a statistically significant decrease in absolute and relative HSL expression in females (−31% compared with controls for both parameters) and males (−21% and −22% compared with controls, respectively) was noted, and a statistically significant decrease (by 30%) in absolute and relative CYP11B1 expression in females was observed at 5000 ppm. Total cholesterol, free cholesterol and cholesteryl ester concentrations in adrenals were increased by 24% (statistically significantly), 20% and 54% in males, respectively, and by 54% (statistically significantly), 39% (statistically significantly) and 126% in females, respectively, at 5000 ppm.

In conclusion, cyflumetofen revealed an increase in total cholesterol (predominantly cholesteryl ester) concentrations in adrenals, which correlated with vacuolation by lipid deposition of the adrenocortical cells and of ovarian interstitial cells at 5000 ppm (equal to 7.44 mg/kg bw per day) in both sexes. Furthermore, cyflumetofen may have an inhibitory effect on HSL expression at 5000 ppm in both sexes; as HSL is involved in cholesterol catabolism, its inhibition might result in cholesterol and other lipid deposition. Catabolism of lipids might be inhibited between 7.44 and 347 mg/kg bw per day, leading to the formation of lipid droplets in adrenals and ovarian cells. As a secondary response, CYP11A1 expression may be enhanced in both sexes at 5000 ppm by the elevated supply of lipids (Takeda, 2006).

To investigate pharmacological safety aspects in dogs, respiratory rate, blood pressure, heart rate and electrocardiogram were examined in conscious dogs. After vehicle treatment of four approximately 14-month-old male Beagle dogs and a withdrawal period of 6 days, the same animals were administered a single dose of cyflumetofen (batch no. 01D1; purity 97.67%) of 0 or 2000 mg/kg bw per day by gelatine capsule (Inui, 2003). A blood pressure–electrocardiogram transmitter was implanted subcutaneously in the flank, and a catheter from a pressure sensor connected to the transmitter was introduced into the femoral artery. Electrocardiographic electrodes were implanted subcutaneously on the right outer side of the chest and left outer side of the abdominal area. Moreover, a respiration pickup was attached at the thorax region, connected to a multitelemeter transmitter (which was placed in a jacket worn by the animal). The respiratory rate, blood pressure (systolic blood pressure, diastolic blood pressure and mean blood pressure), heart rate and electrocardiogram parameters (PQ interval, QRS duration, QT interval and QTc) were measured before administration and at 0.5, 1, 3, 6 and 24 hours after administration telemetrically and analysed with a data acquisition and real-time analysis software. Electrocardiographic parameters were analysed from 20 stable waveforms at the time point of measurements.

Systolic blood pressure, heart rate, electrocardiogram (PQ interval, QRS duration, QT interval, QTc) and respiratory rate in the 2000 mg/kg bw group showed no statistically significant differences when compared with controls at 0.5, 1, 3, 6 and 24 hours after administration. Diastolic blood pressure and mean blood pressure at 1 hour after administration were slightly, but statistically significantly, higher in the treated group; however, values were within the range of variation of post-administration measurements in the control group.

In conclusion, cyflumetofen did not reveal treatment-related adverse effects on the respiratory or cardiovascular system in dogs (Inui, 2003).

3. Observations in humans

Medical examination (including clinical signs, blood pressure, chest X-ray, haematological and clinical chemistry findings and urine analysis) of workers engaged in the production of

cyflumetofen at Kyoyu Agri Co., Ltd and Sugai Chemical Co., Ltd was conducted (16 workers in total) from 2007 to 2008. No remarkable abnormal findings were reported in any workers (Higashida, 2009; Okamoto, 2009a,b).

Comments

Biochemical aspects

The specific metabolism or degradation of the individual enantiomers of the racemic mixture was not investigated.

In a pharmacokinetic study performed in mice, peak concentrations of radiolabel in plasma were reached rapidly (0.5–1 hour after dosing) and were 2.4-fold higher in females than in males. Moreover, in females, a second C_{max} was observed at 2 or 8 hours after dosing, suggesting enterohepatic recirculation. Terminal half-lives were in the range of 22–60 hours. The absorption in mice represented by the AUC was up to 2-fold higher in females than in males and increased sublinearly with increasing doses.

Absorption of cyflumetofen in rats was approximately 69–78% at the low dose (3 mg/kg bw), based on urinary and biliary excretion, with saturation at the high dose (approximately 35–46% absorption at 250 mg/kg bw). There were no remarkable differences in absorption according to sex or the position of the radiolabels used. Saturation of oral absorption was also noted after repeated daily administration at the low dose. Peak concentrations in plasma after a single high-dose application occurred after 1–4 hours. Radioactivity in plasma decreased biexponentially, with a terminal half-life of 12–22 hours. The AUC increased less than proportionally with the dose, confirming saturation of absorption. The AUC was up to 2-fold higher in females than in males at the high dose.

The major route of elimination at the low dose was the urine (58–67%), with lower amounts in faeces (25–33%). At the high dose, the major route of excretion was the faeces (68–80%), with lower amounts in urine (14–26%). Studies in bile duct–cannulated rats showed that at least 30% and 18% were excreted in bile in males and females, respectively, regardless of dose level. A decrease in urinary excretion in cannulated compared with non-cannulated rats suggests that some reabsorption from the intestinal tract after biliary excretion might occur at low doses. Within 72 hours after administration, at least 95% of the absorbed dose was excreted.

In rats, the half-lives for elimination from tissues were in the range of 9–24.5 hours at the low dose and 14–42.5 hours at the high dose, with the longest half-life in adipose tissue, followed by bone marrow. Highest residues were identified in the liver, followed by the kidney, regardless of sex, dose, label position or time point of measurement. Levels of radioactivity in tissues appeared to have reached steady state after 4 days of repeated daily dosing. Residues in erythrocytes and skin declined relatively slowly.

Cyflumetofen was extensively metabolized. The predominant metabolic pathway was cleavage of the *tert*-butylphenyl (A-ring) and trifluorotolyl (B-ring) moieties. Major reactions on the A-ring were cleavage of the methoxyethyl group, hydroxylation at the butyl group, and decarboxylation and glucuronidation at the butyl group. Major reactions on the B-ring were glutathione conjugation at the carboxyl group and further metabolism to mercapturic acid or thiolactic acid. In addition, hydroxylation and oxidation reactions at the butyl group and cleavage of the carboxylic ester moiety were observed on the parent structure (intact A- and B-ring).

Toxicological data

The oral LD_{50} was greater than 2000 mg/kg bw in female rats. The dermal LD_{50} was greater than 5000 mg/kg bw in rats, and the LC_{50} in an inhalation study in rats was greater than 2.65 mg/L. Cyflumetofen was not irritating to the skin of rabbits and was slightly irritating to the eyes of rabbits. Skin sensitization was observed in a maximization assay in guinea-pigs.

In repeated-dose toxicity studies in mice, rats or dogs, the main effects were on the adrenals (vacuolation and hypertrophy of cortical cells) and liver (e.g. hepatocellular hypertrophy). LCA occurred at the highest doses in the rat carcinogenicity study.

In a 4-week mouse feeding study with dietary concentrations of 0, 100, 500, 1000 and 5000 ppm (equal to 0, 13.1, 67.2, 135 and 663 mg/kg bw per day for males and 0, 14.5, 74.9, 150 and 763 mg/kg bw per day for females, respectively), absolute and relative adrenal weights were increased at 5000 ppm. Histopathology showed an increased incidence of diffuse vacuolation and hypertrophy of adrenocortical cells at 5000 ppm. The NOAEL was 1000 ppm (equal to 135 mg/kg bw per day), based on increased adrenal weights and histopathological changes in the adrenals at 5000 ppm (equal to 663 mg/kg bw per day).

In a 13-week mouse feeding study with dietary concentrations of 0, 300, 1000, 3000 and 10 000 ppm (equal to 0, 35.4, 117, 348 and 1200 mg/kg bw per day for males and 0, 45.0, 150, 447 and 1509 mg/kg bw per day for females, respectively), the NOAEL was 1000 ppm (equal to 117 mg/kg bw per day), based on histopathological changes in adrenocortical cells (e.g. increased incidence of diffuse vacuolation in females and of diffuse hypertrophy in males) at 3000 ppm (equal to 348 mg/kg bw per day).

In a 2-week rat feeding study with dietary concentrations of 0, 1000 and 10 000 ppm (equal to 0, 101 and 981 mg/kg bw per day for males and 0, 105 and 1000 mg/kg bw per day for females, respectively), the lowest-observed-adverse-effect level (LOAEL) was 1000 ppm (equal to 101 mg/kg bw per day), the lowest dose tested, based on changes in clinical chemistry, an increase in absolute and/or relative weights of liver and adrenals, hypertrophy of the adrenals in females and histopathological alterations, such as diffuse vacuolation of adrenocortical cells, at all doses. Moreover, all females showed vacuolation of interstitial cells in the ovary; vacuolation of corpora lutea was additionally observed at 10 000 ppm.

In a 4-week rat feeding study with dietary concentrations of 0, 100, 500, 1000 and 5000 ppm (equal to 0, 7.5, 37.6, 75.1 and 384 mg/kg bw per day for males and 0, 8.05, 40.8, 79.8 and 409 mg/kg bw per day for females, respectively), the NOAEL was 500 ppm (equal to 37.6 mg/kg bw per day), based on clinical chemistry changes (decreases in total cholesterol and triglycerides) and increases in organ weights (liver and adrenal) at 1000 ppm (equal to 75.1 mg/kg bw per day). In addition, histopathological effects, such as diffuse hypertrophy of hepatocytes (in all males at 1000 ppm and in both sexes at 5000 ppm), diffuse vacuolation of adrenocortical cells in both sexes accompanied by diffuse hypertrophy in all females and an increased incidence of vacuolation of interstitial cells in the ovaries, were observed at 1000 ppm and above. The results of lipid staining performed on the adrenals of both sexes and ovaries of females indicated that the vacuolation was due to the presence of lipid.

In a 4-week (26 days) rat feeding study with dietary concentrations of 0, 500, 1500, 4000 and 12 000 ppm (equal to 0, 43, 128, 339 and 1028 mg/kg bw per day for males and 0, 46, 132, 351 and 1039 mg/kg bw per day for females, respectively), the NOAEL was 500 ppm (equal to 43 mg/kg bw per day), based on increased adrenal weight in females and elevated incidences of adrenocortical cell vacuolation in both sexes at 1500 ppm (equal to 128 mg/kg bw per day).

In a 13-week rat feeding study with dietary concentrations of 0, 100, 300, 1000 and 3000 ppm (equal to 0, 5.4, 16.5, 54.5 and 167 mg/kg bw per day for males and 0, 6.28, 19.0, 62.8 and 193 mg/kg bw per day for females, respectively), the NOAEL was 300 ppm (equal to 16.5 mg/kg bw per day), based on increased adrenal weights and diffuse hypertrophy of adrenocortical cells in females, mild to moderate diffuse vacuolation of adrenocortical cells in males and vacuolation of ovarian interstitial cells at 1000 ppm (equal to 54.5 mg/kg bw per day).

In a 4-week dog study, cyflumetofen was administered in gelatine capsules at a dose of 0, 100, 300 or 1000 mg/kg bw per day. The NOAEL was 100 mg/kg bw per day, based on increases in adrenal weights in both sexes and increased incidences of fine vacuoles in adrenocortical cells (predominantly in the zona fasciculata and zona reticularis) at 300 mg/kg bw per day. Dark red foci (designated as capillary dilatation) on the right atrioventricular valve of the heart were observed in one female at 1000 mg/kg bw per day.

In a 13-week dog study, cyflumetofen was administered in gelatine capsules at a dose of 0, 30, 300 or 1000 mg/kg bw per day. The NOAEL was 300 mg/kg bw per day, based on a reduction in body weight gain (from day 1 to day 90), elevated absolute and relative adrenal weights in males, increased absolute and relative pituitary weights in females and an increase in absolute and relative testis weights at 1000 mg/kg bw per day. In addition, dark red foci (designated as capillary dilatation) on the right atrioventricular valve of the heart and dark red foci in the mucosa of the urinary bladder in one male and increased incidences of vacuolation of adrenocortical cells in females and males were observed at 1000 mg/kg bw per day.

In a 52-week dog study, cyflumetofen was administered in gelatine capsules at a dose of 0, 30, 300 or 1000 mg/kg bw per day. The NOAEL was 30 mg/kg bw per day, based on increased incidences of vacuolation in the adrenal cortex in both sexes, accompanied by degenerative processes (e.g. interstitial fibrosis and infiltration of brown pigment–laden macrophages) at 300 mg/kg bw per day.

In two 18-month feeding studies in mice, dietary concentrations were 0, 150, 500, 1500 and 5000 ppm (equal to 0, 15.5, 54.3, 156 and 537 mg/kg bw per day for males and 0, 14.3, 48.1, 144 and 483 mg/kg bw per day for females, respectively) in the first study and 0 and 10 000 ppm (equal to 0 and 1143 or 1132 mg/kg bw per day for males and females, respectively) in the second study. The overall NOAEL was 1500 ppm (equal to 144 mg/kg bw per day), based on increased adrenal weight (predominantly in females) and an increase in the incidence of diffuse vacuolation of adrenocortical cells in both sexes at 5000 ppm (equal to 483 mg/kg bw per day). There was no increased incidence of tumours in these studies.

In two 52-week feeding studies in rats, dietary concentrations were 0, 50, 150, 500 and 1500 ppm (equal to 0, 1.9, 5.6, 18.8 and 56.8 mg/kg bw per day for males and 0, 2.3, 6.9, 23.3 and 69.2 mg/kg bw per day for females, respectively) in the first study and 0 and 6000 ppm (equal to 0 and 250 or 319 mg/kg bw per day for males and females, respectively) in the second study. The overall NOAEL for systemic toxicity was 500 ppm (equal to 18.8 mg/kg bw per day), based on a reduction in total cholesterol and triglyceride concentrations in both sexes at several time points, increased liver weight (most pronounced early during the study period) in both sexes and increased adrenal weight in females at 1500 ppm (equal to 56.8 mg/kg bw per day). In addition, histopathology revealed an increased incidence of diffuse vacuolation of adrenocortical cells in males and an increased incidence of diffuse hypertrophy of adrenocortical cells in females at 1500 ppm. Moreover, vacuolation of interstitial gland cells in the ovaries was observed at 1500 ppm and above. In the second study, statistically significant increases in the incidence of hyperplasia of Leydig cells (19/20 versus 6/20 in controls) were observed at 6000 ppm at study termination.

In two 104-week studies in Fischer rats, dietary concentrations were 0, 150, 500 and 1500 ppm (equal to 0, 4.92, 16.5 and 49.5 mg/kg bw per day for males and 0, 6.14, 20.3 and 61.9 mg/kg bw per day for females, respectively) in the first study and 0 and 6000 ppm (equal to 0 and 220 or 287 mg/kg bw per day for males and females, respectively) in the second study. The overall NOAEL for systemic toxicity was 500 ppm (equal to 16.5 mg/kg bw per day), based on an increase in adrenal weights in both sexes, an increase in the incidence of epididymal atrophy and an increased incidence of diffuse hypertrophy and/or vacuolation of adrenocortical cells in both sexes at 1500 ppm (equal to 49.5 mg/kg bw per day). The overall NOAEL for carcinogenicity was 1500 ppm (equal to 49.5 mg/kg bw per day), the highest dose tested in the first study, based on a statistically significantly increased incidence of LCA at 6000 ppm (equal to 220 mg/kg bw per day) in the second study, which was higher than the historical control incidence range. The increase in the incidence of LCA was associated with a statistically significant increase in mass of the testis, with an increase in absolute and relative testis weights at 6000 ppm. There was a slight, non-significant increase in the incidence of C-cell adenomas and adenocarcinomas of the thyroid in male rats. As these tumours are common in rats and there were no accompanying preneoplastic lesions, it was concluded that their occurrence was incidental.

The Meeting concluded that cyflumetofen is carcinogenic in male rats but not in female rats or male or female mice.

Cyflumetofen was tested for genotoxicity in an adequate range of assays, both in vitro and in vivo. A mouse lymphoma gene mutation assay was positive with and without liver enzyme activation at concentrations close to those at which precipitation occurred. Cyflumetofen was not genotoxic in an Ames test or in an in vitro chromosomal aberration assay. There was no evidence of genotoxicity in an in vivo micronucleus assay or in an in vivo UDS assay in rat liver.

The Meeting concluded that cyflumetofen is unlikely to be genotoxic in vivo.

Five in vitro mechanistic studies were performed to identify a possible mode of action for the LCA via perturbation of the estrogen or androgen system. Cyflumetofen was not a significant aromatase inhibitor in a human recombinant cell system, did not significantly interact with AR or ER binding using rat prostate or uterine cytosol protein preparations, respectively, and was not an agonist in a human ER transcriptional activation system. In a steroidogenesis assay, cyflumetofen induced estrogen production at and above 5 µmol/L (maximally 1.64-fold) and inhibited testosterone production at and above 1 µmol/L (maximally 0.63-fold) in human adenocarcinoma cells. Fischer rats are very sensitive to decreased testosterone levels, which lead to a compensatory increase in LH production and subsequent Leydig cell proliferation and LCA progression. It cannot be excluded that this mode of action is relevant to humans. However, as an increased incidence of LCA was observed only at high doses in rats, cyflumetofen can be considered as not likely to be carcinogenic to humans at levels occurring in the diet.

In view of the lack of genotoxicity, the absence of carcinogenicity in mice and the fact that only LCA was observed in a particularly sensitive strain of rat at the highest dose tested, the Meeting concluded that cyflumetofen is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation study of reproductive toxicity in rats at dietary concentrations of 0, 150, 500 and 1500 ppm (equal to 0, 10.4, 34.6 and 100.3 mg/kg bw per day for males and 0, 12, 39.7 and 121.6 mg/kg bw per day for females, respectively), the NOAEL for parental toxicity was 150 ppm (equal to 10.4 mg/kg bw per day), based on increased incidences of white/enlarged adrenals and increased adrenal weights in females and an elevated incidence of hypertrophy of adrenocortical cells in both sexes at 500 ppm (equal to 34.6 mg/kg bw per day). In addition, delayed vaginal opening and decreased FSH and progesterone concentrations in serum were noted in females at 500 ppm. In offspring, the NOAEL was also 150 ppm (equal to 10.4 mg/kg bw per day), based on increased adrenal weights in both sexes and increased incidences of hypertrophy of adrenocortical cells in males at 500 ppm (equal to 34.6 mg/kg bw per day). The NOAEL for reproductive toxicity was 1500 ppm (equal to 100.3 mg/kg bw per day), the highest dose tested.

In a range-finding study on the developmental toxicity of cyflumetofen in rats administered 0, 100, 500 or 1000 mg/kg bw per day, no treatment-related developmental or maternal toxicity was observed. In the main study on the developmental toxicity of cyflumetofen in rats at dose levels of 0, 50, 250 and 1000 mg/kg bw per day, the NOAEL for maternal toxicity was 50 mg/kg bw per day, based on increased adrenal weights accompanied by an increased incidence of vacuolation of adrenocortical cells at 250 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 50 mg/kg bw per day, based on an increased incidence of incompletely ossified sternal centra at 250 mg/kg bw per day.

In a range-finding study on the developmental toxicity of cyflumetofen in rabbits at dose levels of 0, 10, 100, 500 and 1000 mg/kg bw per day, no treatment-related developmental or maternal toxicity was observed. In the main study on the developmental toxicity of cyflumetofen in rabbits at dose levels of 0, 50, 250 and 1000 mg/kg bw per day, the NOAEL for maternal toxicity was 50 mg/kg bw per day, based on slight body weight loss at GDs 6–9 and decreased body weight gain relative to controls during the entire treatment period, which partly correlated with a decrease in feed consumption at 250 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was also 50 mg/kg bw per day, based on changes in the number of ossification sites in the vertebrae, ribs and sternum at 250 mg/kg bw per day. An increased incidence of total variations (per fetus and per litter) was noted

at 1000 mg/kg bw per day. Skeletal variations were confined to increased fetal and litter incidences of incompletely ossified sterna centra (above the historical control range of the laboratory) and an increase in fetal and litter incidences of angulated hyoid alae at 1000 mg/kg bw per day.

The Meeting concluded that cyflumetofen is not teratogenic.

In an acute neurotoxicity study in rats administered cyflumetofen at a dose of 0, 125, 500 or 2000 mg/kg bw, the NOAEL for acute toxicity and neurotoxicity was 2000 mg/kg bw, the highest dose tested.

In a 13-week neurotoxicity study in rats administered cyflumetofen in the diet at a concentration of 0, 500, 1500 or 5000 ppm (equal to 0, 30, 89 and 293 mg/kg bw per day for males and 0, 41, 99 and 353 mg/kg bw per day for females, respectively), the NOAEL for systemic toxicity was 500 ppm (equal to 30 mg/kg bw per day), based on an increase in adrenal weights and an increased incidence and higher degree of severity of diffuse vacuolation of the adrenocortical cells (predominantly in the zona fasciculata) in both sexes at 1500 ppm (equal to 89 mg/kg bw per day). The NOAEL for subchronic neurotoxicity was 5000 ppm (equal to 293 mg/kg bw per day), the highest dose tested.

The Meeting concluded that cyflumetofen is not neurotoxic.

In a 4-week mouse immunotoxicity study with dietary concentrations of 0, 500, 1500 and 5000 ppm (equal to 0, 33, 107 and 349 mg/kg bw per day, respectively), the NOAEL for immunotoxicity was 5000 ppm (equal to 349 mg/kg bw per day), the highest dose tested. The NOAEL for systemic toxicity was 500 ppm (equal to 33 mg/kg bw per day), based on an increase in adrenal weights correlating with discoloration and enlargement of the adrenals and microscopic changes, such as vacuolation of adrenocortical cells, at 1500 ppm (equal to 107 mg/kg bw per day).

The Meeting concluded that cyflumetofen is not immunotoxic.

In a mechanistic study to further elucidate the effects on adrenal gland and ovary, male and female rats were fed diets containing 0, 100 or 5000 ppm (equal to 0, 7.44 and 378 mg/kg bw per day for males and 0, 7.59 and 347 mg/kg bw per day for females, respectively) for up to 28 days. Cyflumetofen caused enlarged and discoloured adrenals with increased weights. An increase in total cholesterol concentration, predominantly cholesteryl esters, in adrenals correlated with vacuolation by lipid deposition within the adrenocortical and ovarian interstitial cells at 5000 ppm in both sexes. Furthermore, a slight inhibitory effect on HSL expression, which is involved in cholesterol catabolism and therefore might result in lipid deposition, was noted at 5000 ppm in both sexes. In addition, CYP11A1 (cholesterol side-chain cleavage enzyme) expression was slightly enhanced in both sexes at 5000 ppm, probably due to the elevated supply of lipids. No effects were seen on ACTH or corticosterone levels in serum. In conclusion, catabolism of lipids might be inhibited at cyflumetofen doses above 7.44 mg/kg bw per day, leading to the formation of lipid droplets in adrenals and ovarian cells.

In a safety pharmacology study, respiratory rate, blood pressure and heart rate (including electrocardiogram) were measured in male dogs after a single-dose application of 0 or 2000 mg/kg bw. Cyflumetofen did not cause any treatment-related adverse effects on the respiratory or cardiovascular system in dogs under the conditions of the study.

Toxicological data on metabolites and/or degradates

Acute toxicity and genotoxicity studies were performed for B-1, a goat and plant metabolite and food processing hydrolysis product. B-1 is also a major metabolite in the rat (occurring at up to 28% of the applied dose).

B-1 was of low acute oral toxicity (LD_{50} > 2000 mg/kg bw per day).

The potential genotoxicity of B-1 was tested in an adequate range of in vitro and in vivo assays. A mouse lymphoma gene mutation assay was positive at 1000 µg/mL without liver enzyme

activation and in one of two experiments at 1000 µg/mL with enzyme activation. B-1 was not mutagenic in an Ames test and was not genotoxic in an in vitro chromosomal aberration assay. There was no evidence of genotoxicity in an in vivo UDS assay in rat liver.

The Meeting concluded that metabolite B-1 is unlikely to be genotoxic in vivo.

B-3, a soil metabolite, and AB-13, an impurity, have not been identified as food residues and are therefore not relevant for dietary risk assessment.

For AB-1, a goat metabolite and food processing hydrolysis product, no toxicological data were provided. AB-1 is an intermediate in the metabolism of rats and occurs at less than 1% of the applied dose in bile. It is further transformed to AB-3 and AB-2. In sum, AB-1, AB-2 and AB-3 and their glucuronidated derivatives account for greater than 20% of the applied dose in rats. As AB-1 is structurally similar to the parent and is transformed to metabolites, which represent a large portion of the metabolism in rats, its toxicity can therefore be considered to be covered by that of the parent.

Human data

In reports on manufacturing plant personnel, no adverse health effects were noted.

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

Toxicological evaluation

The Meeting established an ADI of 0–0.1 mg/kg bw per day on the basis of a NOAEL of 10.4 mg/kg bw per day in the two-generation rat feeding study, based on parental and offspring toxicity at 34.6 mg/kg bw per day. A safety factor of 100 was applied. The margin between the upper bound of the ADI and the LOAEL of 220 mg/kg bw per day for LCA in rats is 2200.

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

Levels relevant to risk assessment of cyflumetofen

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month studies of toxicity and carcinogenicity[a,b]	Toxicity	1 500 ppm, equal to 144 mg/kg bw per day	5 000 ppm, equal to 483 mg/kg bw per day
		Carcinogenicity	5 000 ppm, equal to 483 mg/kg bw per day[c]	–
Rat	Ninety-day study of toxicity[a]	Toxicity	300 ppm, equal to 16.5 mg/kg bw per day	1 000 ppm, equal to 54.5 mg/kg bw per day
	Two-year studies of toxicity and carcinogenicity[a,b]	Toxicity	500 ppm, equal to 16.5 mg/kg bw per day	1 500 ppm, equal to 49.5 mg/kg bw per day
		Carcinogenicity	1 500 ppm, equal to 49.5 mg/kg bw per day	6 000 ppm, equal to 220 mg/kg bw per day
	Two-generation study of reproductive	Reproductive toxicity	1 500 ppm, equal to 100.3 mg/kg bw per	–

Species	Study	Effect	NOAEL	LOAEL
	toxicity[a]		day[c]	
		Parental toxicity	150 ppm, equal to 10.4 mg/kg bw per day	500 ppm, equal to 34.6 mg/kg bw per day
		Offspring toxicity	150 ppm, equal to 10.4 mg/kg bw per day	500 ppm, equal to 34.6 mg/kg bw per day
	Developmental toxicity study[d]	Maternal toxicity	50 mg/kg bw per day	250 mg/kg bw per day
		Embryo/fetal toxicity	50 mg/kg bw per day	250 mg/kg bw per day
Rabbit	Developmental toxicity study[d]	Maternal toxicity	50 mg/kg bw per day	250 mg/kg bw per day
		Embryo/fetal toxicity	50 mg/kg bw per day	250 mg/kg bw per day
Dog	One-year study of toxicity[e]	Toxicity	30 mg/kg bw per day	300 mg/kg bw per day

[a] Dietary administration.
[b] Two studies combined.
[c] Highest dose tested.
[d] Gavage application.
[e] Gelatine capsules.

Estimate of acceptable daily intake (ADI)

0–0.1 mg/kg bw

Estimate of 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 cyflumetofen

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid, 68% at low dose (3 mg/kg bw), 35% at high dose (250 mg/kg bw)
Dermal absorption	20% at low concentration (0.2 g/L), 27% at high concentration (200 g/L)
Distribution	Widely distributed, highest levels in liver, followed by kidney and bone marrow
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	≥ 95% within 72 hours, mainly in urine, partly in bile

Metabolism in animals	Extensive, primarily cleavage between tolyl and phenyl moieties, hydroxylation and conjugation
Toxicologically significant compounds in animals and plants	Cyflumetofen, metabolite B-1 (goat, rat, plant and food processing hydrolysis product) and AB-1 (goat, rat and food processing hydrolysis product)
Acute toxicity	
Rat, LD_{50}, oral	> 2 000 mg/kg bw per day
Rat, LD_{50}, dermal	> 5 000 mg/kg bw per day
Rat, LC_{50}, inhalation	> 2.65 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Slightly irritating
Guinea-pigs, dermal sensitization	Sensitizing (maximization test)
Short-term studies of toxicity	
Target/critical effect	Adrenals: weight and histopathological changes (rat, mouse, dog)
Lowest relevant oral NOAEL	16.5 mg/kg bw per day (rat)
Lowest relevant dermal NOAEL	1 000 mg/kg bw per day, highest dose tested
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Adrenals: weight and histopathological changes; testis: LCA at highest dose (rats)
Lowest relevant NOAEL	16.5 mg/kg bw per day (rat)
Carcinogenicity	Unlikely to pose a carcinogenic risk from the diet
Genotoxicity	
	Unlikely to be genotoxic
Reproductive toxicity	
Target/critical effect	No reproductive toxicity
Lowest relevant parental NOAEL	10.4 mg/kg bw per day
Lowest relevant offspring NOAEL	10.4 mg/kg bw per day
Lowest relevant reproductive NOAEL	100.3 mg/kg bw per day, highest dose tested
Developmental toxicity	
Target/critical effect	Skeletal variations
Lowest relevant maternal NOAEL	50 mg/kg bw per day (rat and rabbit)
Lowest relevant embryo/fetal NOAEL	50 mg/kg bw per day (rat and rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	2 000 mg/kg bw per day, highest dose tested
Subchronic neurotoxicity NOAEL	293 mg/kg bw per day, highest dose tested
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Mechanistic studies	Inhibition of cholesterol catabolism possibly due to a decrease in hormone-sensitive lipase expression in the adrenals of rats
	No significant effect on respiratory/cardiovascular systems in

	dogs
	No significant effect in vitro on the aromatase and estrogen/adrenal receptor system
	Induction of 17β-estradiol synthesis and inhibition of testosterone synthesis in an in vitro steroidogenesis assay
	Possible mode of action of LCA: Testosterone level reduction and subsequent compensatory processes
Immunotoxicity NOAEL	349 mg/kg bw per day, highest dose tested
Studies on metabolites	B-1:
	LD_{50}: > 2 000 mg/kg bw
	Unlikely to be genotoxic in vivo
	Studies on B-3 and AB-13 were submitted, but these compounds are not relevant to a dietary risk assessment.
Medical reports	
	Three medical reports: No abnormal findings

Summary

	Value	Study	Safety factor
ADI	0–0.1 mg/kg bw	Two-generation reproductive toxicity study (rat)	100
ARfD	Unnecessary	–	–

References

Akhurst LC (2011a). Cyflumetofen – Estrogen receptor transcriptional activation (human cell line [HeLa-9903]). Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England, United Kingdom. OTSA-0578-FR; unpublished. Submitted to WHO by BASF.

Akhurst LC (2011b). Cyflumetofen – H295R steroidogenesis assay. Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England, United Kingdom. OTSA-0579-FR; unpublished. Submitted to WHO by BASF.

Barentsen HM (2008a). Consultancy report – Toxicity prediction assessment of AB-13. Notox BV, s-Hertogenbosch, the Netherlands. 2008/1098812; unpublished. Submitted to WHO by BASF.

Barentsen HM (2008b). Consultancy report – Toxicity prediction assessment of B-1. Notox BV, s-Hertogenbosch, the Netherlands. 2008/1098872; unpublished. Submitted to WHO by BASF.

Barentsen HM (2008c). Consultancy report – Toxicity prediction assessment of B-3. Notox BV, s-Hertogenbosch, the Netherlands. 2008/1098873; unpublished. Submitted to WHO by BASF.

Beerens-Heijnen (2004). Assessment of acute oral toxicity with B-1 in the rat (acute toxic class method). Notox BV, s-Hertogenbosch, the Netherlands. OTSA-0235; unpublished. Submitted to WHO by BASF.

Bowden AM (2003). OK-5101 – Acute (four-hour) inhalation study in rats. Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England, United Kingdom. OTSA-0077; unpublished. Submitted to WHO by BASF.

Buesen R (2010). Summary report – BAS 9210 I (cyflumetofen) – Test study in Wistar rats – Administration via the diet for 26 days. BASF SE, Ludwigshafen/Rhein, Germany. 2010/1034048; unpublished. Submitted to WHO by BASF.

Buesen R, Marxfeld H, Fabian E, van Ravenzwaay B (2010a). BAS 9210 I (cyflumetofen) – Acute oral neurotoxicity study in Wistar rats – Administration via gavage. BASF SE, Ludwigshafen/Rhein, Germany. 2010/1156035; unpublished. Submitted to WHO by BASF.

Buesen R, Strauss V, Küttler K, Fabian E, van Ravenzwaay B (2010b). BAS 9210 I (cyflumetofen) – Repeated dose 28-day dermal toxicity study in Wistar rats. BASF SE, Ludwigshafen/Rhein, Germany. 2010/1076322; unpublished. Submitted to WHO by BASF.

Buesen R, Gröters S, Becker M, van Ravenzwaay B (2011a). BAS 9210 I (cyflumetofen) – Repeated dose 90-day oral neurotoxicity study in Wistar rats – Administration via the diet. BASF SE, Ludwigshafen/Rhein, Germany. 2011/1178313; unpublished. Submitted to WHO by BASF.

Buesen R, Strauss V, Rey Moreno MC, Fabian E, van Ravenzwaay B (2011b). BAS 9210 I (cyflumetofen) – Immunotoxicity study in female Wistar rats – Administration via the diet for 4 weeks. BASF SE, Ludwigshafen/Rhein, Germany. 2010/1178745; unpublished. Submitted to WHO by BASF.

Buskens CAF (2004a). Evaluation of the ability of AB-13 (impurity of OK-5101) to induce chromosome aberrations in cultured peripheral human lymphocytes (with repeat experiment). Notox BV, s-Hertogenbosch, the Netherlands. OTSA-0193; unpublished. Submitted to WHO by BASF.

Buskens CAF (2004b). Evaluation of the ability of B-1 to induce chromosome aberrations in cultured peripheral human lymphocytes. Notox BV, s-Hertogenbosch, the Netherlands. OTSA-0173; unpublished. Submitted to WHO by BASF.

Buskens CAF (2004c). Evaluation of the mutagenic activity of AB-13 (impurity of OK-5101) in the *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay (with independent repeat). Notox BV, s-Hertogenbosch, the Netherlands. 2004/1038340; unpublished. Submitted to WHO by BASF.

Buskens CAF (2007). Evaluation of DNA repair inducing ability of OK-5101 in male rat hepatocytes (in vivo rat hepatocyte DNA-repair assay). Notox BV, s-Hertogenbosch, the Netherlands. 2007/1067386; unpublished. Submitted to WHO by BASF.

Buskens CAF (2009a). Evaluation of DNA repair inducing ability of 2-(trifluoromethyl)benzamide, B-3 in male rat hepatocytes (in vivo rat hepatocyte DNA-repair assay). Notox BV, s-Hertogenbosch, the Netherlands. 2009/1126684; unpublished. Submitted to WHO by BASF.

Buskens CAF (2009b). Evaluation of DNA repair inducing ability of B-1 in male rat hepatocytes (in vivo rat hepatocyte DNA-repair assay). Notox BV, s-Hertogenbosch, the Netherlands. 2009/1126680; unpublished. Submitted to WHO by BASF.

Buskens CAF (2009c). Evaluation of the ability of 2-(trifluoromethyl)benzamide, B-3 to induce chromosome aberrations in cultured peripheral human lymphocytes (with repeat experiment). Notox BV, s-Hertogenbosch, the Netherlands. 2009/1126682; unpublished. Submitted to WHO by BASF.

Corral E (2009). Determination of the dermal absorption of OK-5101 in human. Notox BV, s-Hertogenbosch, the Netherlands. 2009/1126688; unpublished. Submitted to WHO by BASF.

Fabian E, Landsiedel R (2011a). ^{14}C-BAS 9210 I (cyflumetofen) – Study on the plasmakinetics in mice (including amendment no. 1). BASF SE, Ludwigshafen/Rhein, Germany. 2011/7006265; unpublished. Submitted to WHO by BASF.

Fabian E, Landsiedel R (2011b). Study on the dermal penetration of ^{14}C-BAS 9210 I in BAS 9210 0 I in rats. BASF SE, Ludwigshafen/Rhein, Germany. 2011/1185892; unpublished. Submitted to WHO by BASF.

Foster JR (2011). Cyflumetofen – Aromatase (human recombinant) assay. Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England, United Kingdom. OTSA-0577-FR; unpublished. Submitted to WHO by BASF.

Hardwick T (2008). (Tolyl-U-^{14}C)-OK-5101: Excretion and tissue distribution in the rat. Covance Laboratories, Harrogate, North Yorkshire, England, United Kingdom. OTSA-0325-FR; unpublished. Submitted to WHO by BASF.

Higashida T (2009). Statements to medical examination for workers engaged in the production of cyflumetofen technical at Sugai Chemical Co. Ltd. Sugai Chemical Co. Ltd, Ishibashicho, Fukui City, Japan. 2009/1126685; unpublished. Submitted to WHO by BASF.

Hooiveld MJJ (2003). Assessment of contact hypersensitivity to OK-5101 in the albino guinea pig (maximisation-test). Notox BV, s-Hertogenbosch, the Netherlands. OTSA-0122; unpublished. Submitted to WHO by BASF.

Inui (2003). Pharmacology study of OK-5101 – Effects on the respiratory/cardiovascular system in dogs. Panapharm Laboratories Co. Ltd, Uto Kumamoto, Japan. 2003/1032459; unpublished. Submitted to WHO by BASF.

Kaspers U (2009). Positive control study concerning neurotoxicity in Wistar rats – Single administration by gavage or subcutaneously. BASF SE, Ludwigshafen/Rhein, Germany. 2009/7006730; unpublished. Submitted to WHO by BASF.

Matsumoto K (2001). OK-5101: Bacterial reverse mutation test. Institute of Environmental Toxicology, Mitsukaido, Ibaraki, Japan. OTSA-0009(EN)-FR; unpublished. Submitted to WHO by BASF.

Matsumoto K (2003a). OK-5101: In vitro mammalian chromosome aberration test. Institute of Environmental Toxicology, Mitsukaido, Ibaraki, Japan. OTSA-0011(EN)-FR; unpublished. Submitted to WHO by BASF.

Matsumoto K (2003b). OK-5101: Micronucleus test in mice. Institute of Environmental Toxicology, Mitsukaido, Ibaraki, Japan. OTSA-0019(EN)-FR; unpublished. Submitted to WHO by BASF.

Matthews A (2012). Cyflumetofen – Oestrogen receptor binding assay using rat uterine cytosol. Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England, United Kingdom. OTSA-0581-FR; unpublished. Submitted to WHO by BASF.

Mellert W, Kaufmann W, van Ravenzwaay B (2008). Triethyltin bromide – Positive control study for neurotoxicity in Wistar rats – single intraperitoneal administration. BASF SE, Ludwigshafen/Rhein, Germany. 2008/7020382; unpublished. Submitted to WHO by BASF.

Meurer K (2014). Cyflumetofen (BAS 9210 I) – Toxicological profile of metabolite B-3 (2-trifluoromethyl-benzamid). BASF SE, Ludwigshafen, Germany. 2014/1173574; unpublished. Submitted to WHO by BASF.

Moore EL (2003a). OK-5101 – Acute dermal toxicity to the rat. Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England, United Kingdom. OTSA-0069; unpublished. Submitted to WHO by BASF.

Moore EL (2003b). OK-5101 technical: Acute oral toxicity to the rat (fixed dose method). Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England, United Kingdom. OTSA-009; unpublished. Submitted to WHO by BASF.

Nagashima Y (2003a). 28-day repeated oral dose toxicity study of OK-5101 in Beagle dogs. Bozo Research Centre Ltd, Setagaya, Tokyo, Japan. OTSA-0027(EN); unpublished. Submitted to WHO by BASF.

Nagashima Y (2003b). 90-day repeated oral dose toxicity study of OK-5101 in Beagle dogs. Bozo Research Centre Ltd, Setagaya, Tokyo, Japan. OTSA-0047(EN)-FR; unpublished. Submitted to WHO by BASF.

Nagashima Y (2008). 1-year repeated oral dose toxicity study of OK-5101 in Beagle dogs (including amendment no. 1 and amendment no. 2). Bozo Research Centre Ltd, Setagaya, Tokyo, Japan. 2008/7021471; unpublished. Submitted to WHO by BASF.

Nakamura H (2004a). (^{14}C)OK-5101 (phenyl-ring-U- and tolyl-ring-U-label): Metabolic fate in rats (preliminary study). Institute of Environmental Toxicology, Joso-shi, Ibaraki, Japan. OTSA-0058(EN)-FR; unpublished. Submitted to WHO by BASF.

Nakamura H (2004b). (^{14}C)OK-5101 (phenyl-ring-U- and tolyl-ring-U-label): Metabolic fate in rats (biliary excretion study). Institute of Environmental Toxicology, Mitsukaido, Ibaraki, Japan. OTSA-0085(EN)-FR; unpublished. Submitted to WHO by BASF.

Nakamura H (2004c). (^{14}C)OK-5101 (phenyl-ring-U- and tolyl-ring-U-label): Metabolic fate in rats (elimination balance). Institute of Environmental Toxicology, Mitsukaido, Ibaraki, Japan. OTSA-0093(EN)-FR; unpublished. Submitted to WHO by BASF.

Nakamura H (2004d). (^{14}C)OK-5101 (phenyl-ring-U- and tolyl-ring-U-label): Metabolic fate in rats (pharmacokinetics). Institute of Environmental Toxicology, Mitsukaido, Ibaraki, Japan. OTSA-0071(EN)-FR; unpublished. Submitted to WHO by BASF.

Nakamura H (2004e). (^{14}C)OK-5101 (phenyl-ring-U- and tolyl-ring-U-label): Metabolic fate in rats (tissue distribution). Institute of Environmental Toxicology, Mitsukaido, Ibaraki, Japan. OTSA-0095(EN)-FR; unpublished. Submitted to WHO by BASF.

Ohyama K (2004a). (^{14}C)OK-5101 (phenyl-ring-U- and tolyl-ring-U-label): Metabolic fate in rats (quantitation and identification of metabolites in bile). Institute of Environmental Toxicology, Mitsukaido, Ibaraki, Japan. OTSA-0086(EN)-FR; unpublished. Submitted to WHO by BASF.

Ohyama K (2004b). (^{14}C)OK-5101 (phenyl-ring-U- and tolyl-ring-U-label): Metabolic fate in rats (quantitation and identification of metabolites in urine and feces). Institute of Environmental Toxicology, Mitsukaido, Ibaraki, Japan. OTSA-0094(EN)-FR; unpublished. Submitted to WHO by BASF.

Okamoto K (2009a). Statements to medical examination for workers engaged in the production of cyflumetofen at Kyoyu Agri Co., Ltd. Kyoyu Agri Co. Ltd, Inasatomachi, Nagano, Japan. 2009/1126687; unpublished. Submitted to WHO by BASF.

Okamoto K (2009b). Statements to medical examination for workers engaged in the production of cyflumetofen at Kyoyu Agri Co., Ltd. Kyoyu Agri Co., Ltd, Mamejima, Nagano, Japan. 2009/1126686; unpublished. Submitted to WHO by BASF.

Rees PB (2003a). OK-5101 – Eye irritation to the rabbit. Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England, United Kingdom. OTSA-0068RA2; unpublished. Submitted to WHO by BASF.

Rees PB (2003b). OK-5101 – Skin irritation to the rabbit. Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, England, United Kingdom. OTSA-0066; unpublished. Submitted to WHO by BASF.

Sakai S (2001). OK-5101: Repeated dose 2-week oral toxicity study in rats. Otsuka Chemical Co. Ltd, Naruto, Tokushima, Japan. OTSA-0010(EN)-FR; unpublished. Submitted to WHO by BASF.

Schulz M (2011). BAS 9210 I (cyflumetofen) – In vitro chromosome aberration assay in V79 cells. BASF SE, Ludwigshafen/Rhein, Germany. 2010/1151897; unpublished. Submitted to WHO by BASF.

Stark R (2014). Metabolite of BAS 9210 I, cyflumetofen, repeated-dose 28-day toxicity study in Wistar rats; administration via the diet, Reg. No. 4288294. BASF SE, Ludwigshafen, Germany. 2013/1399904; unpublished. Submitted to WHO by BASF.

Takahashi K (2002). OK-5101: Preliminary reproductive toxicity study in rats. Institute of Environmental Toxicology, Mitsukaido, Ibaraki, Japan. OTSA-0022(EN)-FR; unpublished. Submitted to WHO by BASF.

Takahashi K (2004). OK-5101: Reproductive toxicity study in rats. Institute of Environmental Toxicology, Mitsukaido, Ibaraki, Japan. OTSA-0059(EN)-FR; unpublished. Submitted to WHO by BASF.

Takahashi N (2013). BAS 9210 I (cyflumetofen): Carcinogenicity study in rats. Institute of Environmental Toxicology, Joso-shi, Ibaraki, Japan. 2013/1345430; unpublished. Submitted to WHO by BASF.

Takeda M (2006). OK-5101 technical: Mechanistic study on the mode of toxic effects in rats. Institute of Environmental Toxicology, Mitsukaido, Ibaraki, Japan. OTSA-0278(EN)-FR; unpublished. Submitted to WHO by BASF.

Teunissen MS (2004). Assessment of acute oral toxicity with AB-13 (analytical grade) in the rat (acute toxic class method). Notox BV, s-Hertogenbosch, the Netherlands. OTSA-0197; unpublished. Submitted to WHO by BASF.

van Cott AW, Meurer K (2013). Mode of action for Leydig cell tumors in a high dose 2-yr rat cancer study with cyflumetofen. BASF Corp., Research Triangle Park, NC, USA. 2013/7002798; unpublished. Submitted to WHO by BASF.

Verspeek-Rip CM (2004a). Evaluation of the mutagenic activity of AB13 (impurity of OK-5101) in an in vitro mammalian cell gene mutation test with L5178Y mouse lymphoma cells (with independent repeat). Notox BV, s-Hertogenbosch, the Netherlands. OTSA-0194; unpublished. Submitted to WHO by BASF.

Verspeek-Rip CM (2004b). Evaluation of the mutagenic activity of B-1 in an in vitro mammalian cell gene mutation test with L5178Y mouse lymphoma cells (with independent repeat). Notox BV, s-Hertogenbosch, the Netherlands. OTSA-0172; unpublished. Submitted to WHO by BASF.

Verspeek-Rip CM (2004c). Evaluation of the mutagenic activity of B-1 in the *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay (with independent repeat). Notox BV, s-Hertogenbosch, the Netherlands. OTSA-0171; unpublished. Submitted to WHO by BASF.

Verspeek-Rip CM (2007). Evaluation of the mutagenic activity of OK-5101 in an in vitro mammalian cell gene mutation test with L5178Y mouse lymphoma cells. Notox BV, s-Hertogenbosch, the Netherlands. OTSA-0362-FR; unpublished. Submitted to WHO by BASF.

Verspeek-Rip CM (2009a). Evaluation of the mutagenic activity of 2-(trifluoromethyl)benzamide, B-3 in an in vitro mammalian cell gene mutation test with L5178Y mouse lymphoma cells (with independent repeat). Notox BV, s-Hertogenbosch, the Netherlands. 2009/1126683; unpublished. Submitted to WHO by BASF.

Verspeek-Rip CM (2009b). Evaluation of the mutagenic activity of 2-(trifluoromethyl)benzamide, B-3 in the *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay (with independent repeat). Notox BV, s-Hertogenbosch, the Netherlands. 2009/112668; unpublished. Submitted to WHO by BASF.

Willoughby JA (2012). Cyflumetofen: Androgen receptor binding (rat prostate cytosol). CeeTox Inc., Kalamazoo, MI, USA. OTSA-0594-FR; unpublished. Submitted to WHO by BASF.

York RG (2001a). Oral (gavage) developmental toxicity study of OK-5101 in rats. Argus Research, Horsham, PA, USA. OTSA-0012-FR; unpublished. Submitted to WHO by BASF.

York RG (2001b). Oral (gavage) dosage-range developmental toxicity study of OK-5101 in rats. Argus Research Laboratories Inc., Horsham, PA, USA. OTSA-0006-FR; unpublished. Submitted to WHO by BASF.

York RG (2003a). Final pilot report: Oral (stomach tube) dosage-range developmental toxicity study of OK-5101 in rabbits. Argus Research, Horsham, PA, USA. OTSA-0073-FR; unpublished. Submitted to WHO by BASF.

York RG (2003b). Oral (stomach tube) developmental toxicity study of OK-5101 in rabbits. Argus Research Laboratories Inc., Horsham, PA, USA. OTSA-0074; unpublished. Submitted to WHO by BASF.

Yoshida T (2004a). OK-5101: Carcinogenicity study in mice. Institute of Environmental Toxicology, Mitsukaido, Ibaraki, Japan. OTSA-0061(EN)-FR; unpublished. Submitted to WHO by BASF.

Yoshida T (2004b). OK-5101: Carcinogenicity study in rats. Institute of Environmental Toxicology, Mitsukaido, Ibaraki, Japan. OTSA-0046(EN)-FR; unpublished. Submitted to WHO by BASF.

Yoshida T (2004c). OK-5101: Repeated dose 1-year oral toxicity study in rats. Institute of Environmental Toxicology, Mitsukaido, Ibaraki, Japan. OTSA-0045(EN)-FR; unpublished. Submitted to WHO by BASF.

Yoshida T (2004d). OK-5101: Repeated dose 28-day oral toxicity study in mice. Institute of Environmental Toxicology, Mitsukaido, Ibaraki, Japan. OTSA-0017(EN)-FR; unpublished. Submitted to WHO by BASF.

Yoshida T (2004e). OK-5101: Repeated dose 28-day oral toxicity study in rats. Institute of Environmental Toxicology, Mitsukaido, Ibaraki, Japan. OTSA-0002(EN)-FR; unpublished. Submitted to WHO by BASF.

Yoshida T (2004f). OK-5101: Repeated dose 90-day oral toxicity study in mice. Institute of Environmental Toxicology, Mitsukaido, Ibaraki, Japan. OTSA-0042(EN)-FR; unpublished. Submitted to WHO by BASF.

Yoshida T (2004g). OK-5101: Repeated dose 90-day oral toxicity study in rats. Institute of Environmental Toxicology, Mitsukaido, Ibaraki, Japan. OTSA-0014(EN)-FR; unpublished. Submitted to WHO by BASF.

Yoshida T (2012). BAS 9210 I (cyflumetofen): Repeated dose 1-year oral toxicity study in rats. Institute of Environmental Toxicology, Mitsukaido, Ibaraki, Japan. 2012/1343340; unpublished. Submitted to WHO by BASF.

Yoshida T (2013). BAS 9210 I (cyflumetofen): Carcinogenicitiy study in mice. Institute of Environmental Toxicology, Joso-shi, Ibaraki, Japan. 2013/1251154; unpublished. Submitted to WHO by BASF.