PYRACLOSTROBIN (addendum)

First draft prepared by
Katsuhiko Yoshizawa,[1] Midori Yoshida[2] and Angelo Moretto[3]

[1] Department of Food Sciences and Nutrition, Mukogawa Women's University, Hyogo, Japan
[2] Food Safety Commission, Cabinet Office, Tokyo, Japan
[3] Department of Biomedical and Clinical Sciences, University of Milan, and International Centre for Pesticides and Health Risk Prevention, ASST Fatebenefratelli Sacco, Milan, Italy

Explanation

Pyraclostrobin was evaluated previously by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 2003, when an acceptable daily intake (ADI) of 0–0.03 mg/kg body weight (bw) was established based on a no-observed-adverse-effect level (NOAEL) of 3.4 mg/kg bw per day in two 2-year studies in rats with application of a safety factor of 100. An acute reference dose (ARfD) of 0.05

mg/kg bw was established based on a NOAEL of 5 mg/kg bw per day for embryo and fetal toxicity in a developmental toxicity study in rabbits with application of a safety factor of 100. The 2003 Meeting noted that further information on the relationship between local irritation of the gastrointestinal tract and reduced body weight gains in pregnant rabbits and the effect of maternal nutritional deficit on fetal resorptions might allow the ARfD to be refined.

Following a request by the Codex Committee on Pesticide Residues for additional maximum residue levels and an evaluation of metabolites possibly relevant to these new uses, pyraclostrobin was placed on the agenda of the present Meeting, which assessed additional toxicological information available since the last review.

Additional studies on pyraclostrobin (inhalation toxicity in rats, carcinogenicity in rats and the mode of action for the induction of mucosal hyperplasia in the duodenum) and its metabolites (toxicity and genotoxicity studies) were evaluated by the present Meeting. In particular, the present Meeting reviewed the new studies on pyraclostrobin to determine whether they would allow its ARfD to be refined.

All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with relevant national or international test guidelines, unless otherwise specified. No additional information from a literature search was identified that complemented the toxicological information submitted for the current assessment.

Evaluation for acceptable intake

1. Biochemical aspects

1.1 Biotransformation

Five new studies on biotransformation were submitted to the present Meeting.

(a) Comparative in vitro metabolism among humans, rats, rabbits and dogs

A comparative in vitro metabolism study was performed using hepatocytes or microsomes from humans, rats, rabbits and dogs. The objective of this study was to compare the metabolism of pyraclostrobin in hepatocytes and microsomes from three animal species used in toxicological studies with the metabolism in human hepatocytes and microsomes in order to determine whether the metabolic profiles were similar among the species and whether a unique human metabolite occurs. To address these aims, pyraclostrobin radiolabelled on the chlorophenyl ([ch-^{14}C]) or tolyl ([tol-^{14}C]) ring at 3 or 10 µmol/L was incubated with hepatocytes from humans, rats or rabbits. Incubations with liver microsomes from humans, rats, rabbits or dogs were performed with the 10 µmol/L concentration only. The viability of the hepatocytes was determined after incubation for up to 180 minutes using a luminescent cell viability assay. After incubation for 10, 30, 60 or 180 minutes, the reaction was terminated. The resulting supernatant after concentration using a centrifugal evaporator was analysed. The pellet after centrifugal evaporation was extracted, and the radioactive residues in the resulting extract and final pellet were determined.

Twelve peaks were identified in human hepatocytes or human liver microsomes, which each represented more than 5% of the applied radioactivity. The peak representing pyraclostrobin and all 11 metabolite peaks were detected in either rat or rabbit hepatocytes. Four peaks each representing more than 5% of the applied radioactivity were found in human liver microsomes, and two additional peaks identified in human hepatocytes were also detected in human liver microsomes at lower concentrations. The peak representing pyraclostrobin and all five metabolite peaks found in human liver microsomes were detected in rat, rabbit or dog liver microsomes. The results indicated that there was no relevant unique human metabolite of pyraclostrobin.

The metabolic degradation of pyraclostrobin was faster in rat and rabbit hepatocytes than in human cells. Metabolites such as 500M04, 500M108, 500M103, 500M104 and 500M88 (see Appendix

1 for structures of metabolites of pyraclostrobin) were common to all species examined. In humans and rabbits, cleavage of the amide bond, resulting in the formation of metabolite 500M106, was a major degradation pathway; 500M106 was subsequently metabolized by conjugation with glucuronic acid to form metabolite 500M107. In humans and rabbits, metabolite 500M02, which is formed by dimerization, was also identified. Hydroxylation and conjugation to form metabolite 500M104 seemed to be more pronounced in rat hepatocytes than in human and rabbit hepatocytes. The investigation with liver microsomes, which included dog as an additional test species, confirmed the picture obtained with hepatocytes. After incubation of pyraclostrobin with dog liver microsomes, four biotransformation products were found, including low amounts of metabolite 500M102.

Table 1 provides an overview on all investigated test systems and incubation periods, including indications of the amounts of detected metabolites.

Table 1. Overview of the occurrence of metabolites of pyraclostrobin after incubation with hepatocytes or liver microsomes from humans, rats, rabbits or dogs

	Humans		**Rats**		**Rabbits**		**Dogs**
Metabolite code	**10 µmol/L**	**3 µmol/L**	**10 µmol/L**	**3 µmol/L[a]**	**10 µmol/L**	**3 µmol/L[a]**	**10 µmol/L**
Hepatocytes ([ch-^{14}C]pyraclostrobin)							
Pyraclostrobin	++	++	++[b]	nd	++[b]	nd	NA
500M03	+[c]	++[d]	nd	nd	nd	++	NA
500M108 (regioisomers)	++	++	+	++	++	++	NA
500M04	+	++	+[b]	nd	++	nd	NA
500M103	+	+	nd	nd	+	+	NA
500M104	++	++	++	+	+[b]	+	NA
500M107	+	++	nd	nd	+[b]	nd	NA
500M88	+	nd	+[b]	nd	+[b]	nd	NA
500M73	+	++	nd	nd	++	+	NA
500M106	++	++	nd	nd	++[b]	+	NA
500M02	++	++	nd	nd	++[b]	nd	NA
Hepatocytes ([tol-^{14}C]pyraclostrobin)							
Pyraclostrobin	++	++	++[b]	nd	++[b]	nd	NA
500M108 (regioisomers)	++	++	+[b]	nd	++[b]	++	NA
500M103	+	++	+[b]	nd	+[b]	++	NA
500M104	++	++	++	+	+[b]	nd	NA
500M107	nd	++	nd	nd	+[b]	nd	NA
500M88	+	nd	+[b]	nd	+[b]	nd	NA
500M73	++	++	nd	nd	++	nd	NA
500M106	++	++	nd	nd	++[b]	nd	NA
500M02	++	++	nd	nd	++	nd	NA
Liver microsomes ([ch-^{14}C]pyraclostrobin, 90 minutes)							
Pyraclostrobin	++	NA	++	NA	+	NA	++

Metabolite code	Humans		Rats		Rabbits		Dogs
	10 µmol/L	3 µmol/L	10 µmol/L	3 µmol/L[a]	10 µmol/L	3 µmol/L[a]	10 µmol/L
500M04	++	NA	++	NA	++	NA	++
500M88	++	NA	++	NA	+	NA	++
500M73	+	NA	+	NA	++	NA	+
500M106	+	NA	nd	NA	++	NA	nd
500M02	++	NA	nd	NA	++	NA	+

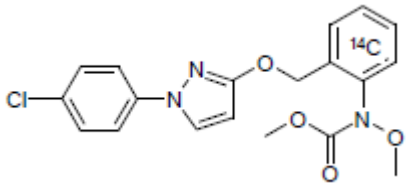
ch: chlorophenyl; NA: not applicable; nd: not detected; tol: tolyl
[a] Investigations performed at one incubation period (180 minutes).
[b] Detected at short incubation periods of 10 and 30 minutes.
[c] +: a mean portion of 0–5% of the applied radioactivity from at least one time interval.
[d] ++: a mean portion above 5% of the applied radioactivity from at least one time interval.
Source: Funk, Glaessgen & Kalyon (2014)

In conclusion, some species differences in metabolites were found. For example, 500M106, a major metabolite in humans and rabbits, was not found in rats. However, no human metabolite was identified that was not also found in rats, rabbits or dogs (Funk, Glaessgen & Kalyon, 2014).

(b) Influence of an inhibitor of human carboxylesterase on the in vitro metabolism of pyraclostrobin in human hepatocytes and human liver cytosol

A study was conducted to investigate the in vitro metabolism of pyraclostrobin labelled with ^{14}C at the tolyl ring (lot/batch no. 556-5501; purity 99.5%; Fig. 1) in human hepatocytes and human liver cytosol and the influence of an inhibitor of human carboxylesterase 1, WWL229 (lot/batch no. 104M4753V; purity 98.8%), on the biotransformation products. To address this issue, [tol-^{14}C]pyraclostrobin at 3 or 6 µmol/L with or without WWL229 at 20 µmol/L was incubated with human hepatocytes or human liver microsomes (both sexes mixed). All experiments were performed in triplicate. The viability of the hepatocytes was determined after an incubation for 180 minutes using a luminescent cell viability assay.

Fig. 1. Chemical structure of pyraclostrobin labelled with ^{14}C at the tolyl ring



Source: Funk & Bellwon (2016a)

For human hepatocytes incubated with pyraclostrobin without WWL229, pyraclostrobin and its biotransformation products 500M103, 500M104, 500M107, 500M73, 500M106 and 500M02 as well as an isomer of 500M02 were identified. The parent compound was time-dependently and completely metabolized after 180 minutes. In the presence of WWL229, biotransformation was slower, as pyraclostrobin was still present after 180 minutes, and metabolites 500M73, 500M107, 500M106 and 500M02 as well as an isomer of 500M02, formed after an initial cleavage of the ether and/or amide bond of the parent pyraclostrobin, were not detected at all. Instead, the formation of low levels of metabolite 500M88 was observed.

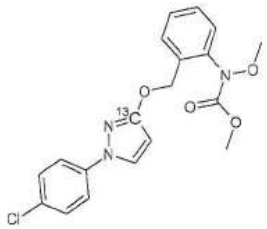
For human liver cytosol incubated with pyraclostrobin without WWL229, pyraclostrobin and its biotransformation products 500M73, 500M106 and 500M02 as well as an isomer of 500M02 were identified. Similar to the incubations performed on human hepatocytes, metabolites 500M73, 500M106 and 500M02 and an isomer of 500M02 were not detected in samples obtained after simultaneous incubation of human liver cytosol with pyraclostrobin and WWL229.

These results indicate an inhibition of the biotransformation of pyraclostrobin in the presence of WWL229. This compound especially inhibits the formation of metabolites 500M106, 500M73 and 500M02 (and its isomer) as well as their conjugates, showing the involvement of the enzyme human carboxylesterase 1 in the respective metabolic reactions (Funk & Bellwon, 2016a).

(c) Metabolism of pyraclostrobin in rat plasma

A study was conducted to clarify the fate and kinetic behaviour of pyraclostrobin after administration of a single oral dose of 50 mg/kg bw to rats. In particular, the purpose was to analyse rat plasma samples at different time points after dosing (especially at the time at which the maximum concentration [C_{max}] is reached, or T_{max}) to compare the results with those obtained in the previously evaluated rat metabolism study (Annex 1, reference *100*) and to specifically address metabolites present at short time intervals after dosing. For designated time points (0.25, 0.5, 1, 2, 3, 4, 8 and 16 hours), one female and one male Crl:WI(Han) rat (average body weights 312.1 g and 200.1 g for males and females, respectively) were dosed with a mixture of pyraclostrobin labelled with ^{14}C at the tolyl ring (tolyl-ring-U-^{14}C; batch no. 566-5011; purity 99.3%), pyraclostrobin labelled with ^{13}C at the pyrazole ring (pyrazole-3-^{13}C; batch no. 1026-1018; purity 99.8%; Fig. 2) and unlabelled pyraclostrobin (batch no. 1815-65; purity 95.7%). In addition, unlabelled metabolite 500M106 (batch no. L83-166; purity 98.9%) and ^{14}C-labelled metabolite 500M02 (batch no. 933-2025; purity 98.8%) were used as reference compounds. Blood was sampled in the presence of ethylenediaminetetraacetic acid (EDTA) or lithium heparin (LiHep), and plasma was obtained.

Fig. 2. Chemical structure of pyraclostrobin labelled with ^{13}C at the pyrazole ring



Source: Birk, Lutz & Doebbe (2014)

For the EDTA plasma at time points 0.25 and 0.5 hour, the concentrations of radiolabelled pyraclostrobin were 1.429 mg/kg and 1.343 mg/kg, respectively. From time point 1 hour to 16 hours, the concentration almost reached a steady state and was generally higher than at time points 0.25 and 0.5 hour (3 hours: 2.615 mg/kg; 16 hours: 3.030 mg/kg). Only at time point 4 hours did the concentration decrease to 1.288 mg/kg. The results for the LiHep plasma were comparable with those of the EDTA plasma. The plasma concentrations are in accordance with those noted in the previously evaluated rat metabolism study (Annex 1, reference *100*). Analysis of the EDTA plasma supernatants at the different time intervals using high-performance liquid chromatography (HPLC) led to comparable metabolite patterns. The unchanged parent compound pyraclostrobin was identified at all time points. The concentration of radiolabelled pyraclostrobin ranged from 0.068 mg/kg (0.5 hour) to 0.290 mg/kg (16 hours). HPLC analysis of the LiHep plasma supernatants led to results similar to those with the EDTA plasma samples.

The masses corresponding to the parent pyraclostrobin and the metabolites 500M108, 500M29, 500M15, 500M46, 500M06, 500M104, 500M107, 500M30 and 500M106 were detected using HPLC with mass spectrometric (HPLC-MS) analysis of plasma supernatant samples. All metabolites that were identified in plasma in the previously evaluated rat metabolism study (Annex 1, reference *100*) were confirmed (pyraclostrobin, 500M06, 500M15 and 500M46). Moreover, metabolites that were identified in the previously described in vitro comparative study (Funk, Glaessgen & Kalyon, 2014) were also detected in rat plasma (500M108, 500M104, 500M107 and 500M106).

The identified metabolites were quantified in four samples (EDTA plasma supernatant at 3 hours; concentrated plasma supernatant of the EDTA blood pool sample at 0.25–16 hours; and LiHep plasma supernatants at 0.25 and 0.5 hour). HPLC peaks that contained more than one component were classified as to region (regions 1–4). Region 1 included 500M108 and 500M29, region 2, 500M15 and 500M108, region 3, 500M46 and 500M06, and region 4, 500M104, 500M107 and 500M30. In all four samples, the relative amounts of region 1, region 3 and region 4 were similar, ranging from 0.337 to 0.560 mg/kg for the EDTA plasma supernatant at 3 hours, from 0.172 to 0.268 mg/kg for the concentrated plasma supernatant of the EDTA blood pool sample at 0.25–16 hours, from 0.158 to 0.227 mg/kg for the LiHep plasma supernatant at 0.25 hour and from 0.227 to 0.331 mg/kg for the LiHep plasma supernatant at 0.5 hour. In all four samples, region 2 was the least abundant region and accounted for between 0.044 mg/kg (LiHep at 0.5 hour) and 0.111 mg/kg (EDTA at 3 hours). Pyraclostrobin was identified in all four samples, and the quantities corresponded well to the analyses of the EDTA plasma supernatant samples of the different time intervals (0.25–16 hours). Metabolite 500M106 was detected only in the LiHep plasma supernatant at 0.25 hour, where it was the least abundant component (0.030 mg/kg). The HPLC analyses are summarized in Table 2.

Table 2. Quantification of radioactive residues of pyraclostrobin in EDTA plasma supernatant at 3 hours, concentrated plasma supernatant of the EDTA blood pool sample at 0.25–16 hours and LiHep plasma supernatants at 0.25 and 0.5 hour

		Concentration of radioactivity (mg/kg)[a]			
Region	**Metabolite identified**	**EDTA 3 hours**	**EDTA 0.25–16 hours**	**LiHep 0.25 hour**	**LiHep 0.5 hour**
Identified					
	Pyraclostrobin	0.313	0.086	0.163	0.035
1	500M108	0.560	0.268	0.227	0.331
	500M29				
2	500M15	0.111	0.078	0.089	0.044
	500M108				
3	500M46	0.412	0.226	0.158	0.228
	500M06				
4	500M104				
	500M107	0.337	0.172	0.195	0.316
	500M30				
	500M106	nd	nd	0.030	nd
Characterized by HPLC					
Up to 3–5 further peaks (each below or equal to 0.218 mg/kg)		0.465	0.472	0.303	0.223
Total identified and characterized		2.197	1.303	1.164	1.177

EDTA: ethylenediaminetetraacetic acid; HPLC: high-performance liquid chromatography; LiHep: lithium heparin; nd: not detected
[a] Data taken from HPLC analysis.
Source: Birk, Lutz & Doebbe (2014)

The results indicated that pyraclostrobin was metabolized by typical phase I and II reactions. Its proposed metabolic pathway was:

- desmethoxylation of the side-chain;
- hydroxylation of the chlorophenyl pyrazole ring system;
- hydroxylation of the tolyl ring system;
- desmethylation of the side-chain; and
- cleavage of the amide bond in the side-chain.

The combination of these reactions followed by conjugation results in a large number of metabolites. Hydroxylation and glucuronic acid conjugation of the chlorophenyl and pyrazole rings of pyraclostrobin yield metabolite 500M29. Hydroxylation and glucuronic acid conjugation of only the pyrazole ring result in metabolites 500M46 and 500M104. Metabolite 500M30 is a cysteine conjugation product of pyraclostrobin, wherein cysteine is bound via its sulfur to the pyrazole ring. 500M30 is probably generated via conjugation of pyraclostrobin with glutathione, followed by enzymatic cleavage of the glutamic acid and the glycine of glutathione.

Desmethoxylation of pyraclostrobin yields the intermediate 500M07. Hydroxylation of the pyrazole ring of 500M07 results in the intermediate 500M08. Both 500M07 and 500M08 were identified in a previously evaluated rat metabolism study (Annex 1, reference *100*). Glucuronic acid conjugation of 500M08 results in metabolite 500M06. 500M06 might also be generated via *N*-desmethoxylation of 500M46.

Desmethylation of the *N*-methoxy side-chain of pyraclostrobin, followed by hydroxylation of the pyrazole ring and glucuronic acid conjugation, results in metabolite 500M15. Cleavage of the methylcarboxy group of pyraclostrobin yields metabolite 500M106. Conjugation of the secondary amine group of 500M106 with glucuronic acid results in metabolite 500M107. Cleavage of the methoxy moiety of 500M106, followed by hydroxylation and sulfate conjugation of the tolyl moiety, results in metabolite 500M108.

This proposed metabolic pathway of pyraclostrobin in rat plasma is illustrated in Fig. 3 (Birk, Lutz & Doebbe, 2014).

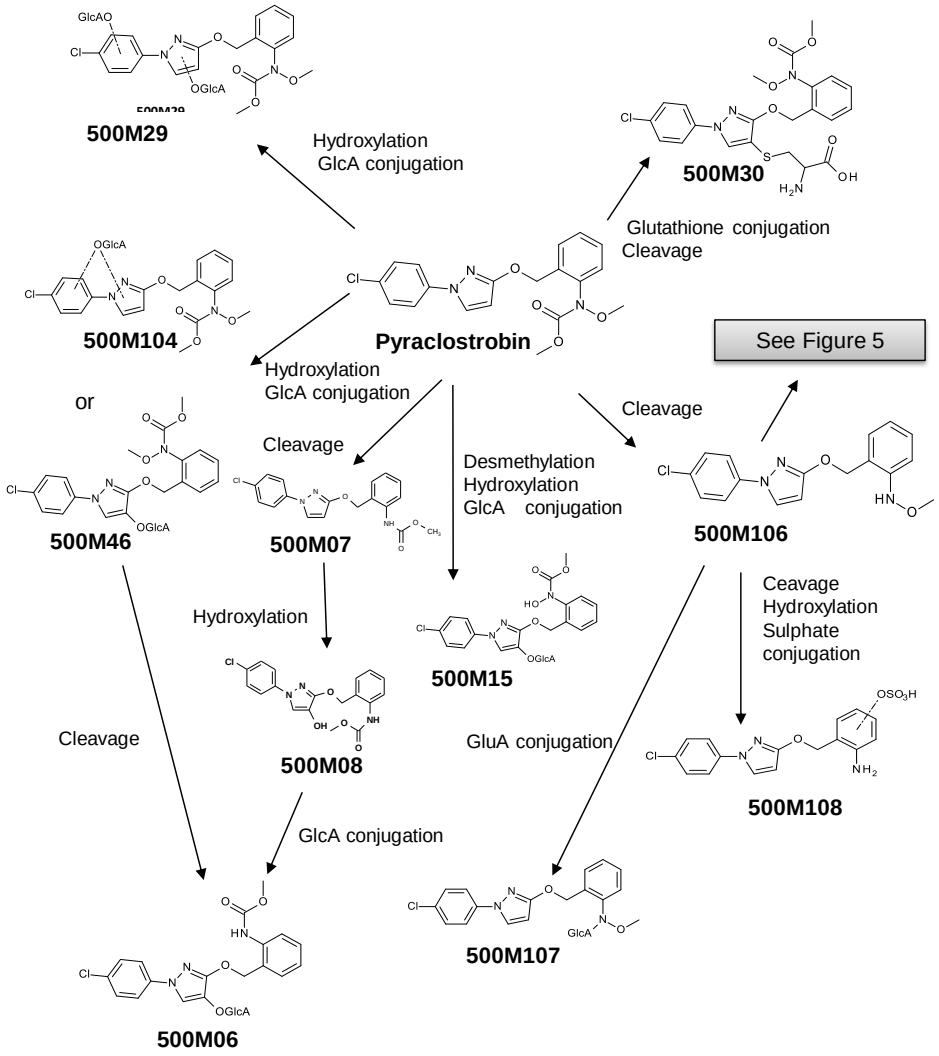
(d) Metabolism of pyraclostrobin in rat serum

A study was conducted to investigate the potential biotransformation of pyraclostrobin by enzymes in rat serum. [tol-^{14}C]Pyraclostrobin (lot/batch no. 566-5501; radiochemical purity 99.5%) at a concentration of 6 µmol/L was incubated with sex-mixed serum of Crl:WI(Han) rats (males aged 27–28 weeks and females aged 14 or 16 weeks). All experiments were performed in triplicate. After incubation for 0, 1, 3, 5 or 20 hours, the reaction was terminated by mixing with ice-cold acetonitrile. The radioactive residues in the supernatant resulting after centrifuged evaporation were determined by liquid scintillation counting, and an aliquot was analysed by HPLC with radiodetection. Selected replicates of the supernatants were analysed by HPLC-MS to investigate the presence of metabolites identified at concentrations above 5% of the applied radioactivity in the previously described comparative in vitro metabolism study (Funk, Glaessgen & Kalyon, 2014) performed on human hepatocytes and human liver microsomes.

Radio-HPLC analyses of the samples after incubation of radiolabelled pyraclostrobin at 6 µmol/L with rat serum allowed the identification of the parent compound and four relevant metabolites known from the previously described comparative in vitro metabolism study (Funk, Glaessgen & Kalyon, 2014). Within the first hour of incubation, no significant differences were observed in comparison with the zero incubation controls. After 3 hours, the concentration of pyraclostrobin started to decrease continuously to 66.29% of the applied radioactivity after 20 hours. The formation of metabolites 500M73, 500M106 and 500M02 and an isomer of 500M02 in trace amounts was observed after 1 hour. The concentration of all metabolites increased time-dependently,

with metabolite 500M106 being the main biotransformation product for all incubation periods (48.81% of the applied radioactivity after 20 hours).

Fig. 3. Proposed metabolic pathway of pyraclostrobin in rat plasma



Source: Birk, Lutz & Doebbe (2014)

A summary of the relevant metabolites of pyraclostrobin following incubation in rat serum is shown Table 3.

Table 3. Summary of relevant metabolites of pyraclostrobin (6 µmol/L) after incubation with rat serum

Incubation time (hours)	Sample description	Component (% of the applied radioactivity)				
		Pyraclostrobin	500M02	Isomer of 500M02	500M73	500M106
0	Supernatant	89.14	–	–	–	–
	Extract pellet 1	52.90	–	–	–	–
	Sum	**142.04**	–	–	–	–

Incubation time (hours)	Sample description	Component (% of the applied radioactivity)				
		Pyraclostrobin	500M02	Isomer of 500M02	500M73	500M106
1	Supernatant	79.93	0.29[a]	–	0.32[a]	0.78[a]
	Extract pellet 1	62.59	0.68[a]	0.27[a]	0.30[a]	0.57[a]
	Sum	**142.52**	**0.98[a]**	**0.27[a]**	**0.62[a]**	**1.34[a]**
3	Supernatant	84.90	0.60	–	0.58[a]	5.03[a]
	Extract pellet 1	53.48	1.78	0.69[a]	0.59[a]	1.60[a]
	Sum	**138.38**	**2.38**	**0.69[a]**	**1.17[a]**	**6.63[a]**
5	Supernatant	93.01	2.03	0.37[a]	0.95[a]	8.29[a]
	Extract pellet 1	ne	ne	ne	ne	ne
	Sum	**93.01**	**2.03**	**0.37[a]**	**0.95[a]**	**8.29[a]**
20	Supernatant	52.38	5.98	1.94	0.70	25.55
	Extract pellet 1	13.91	5.05	2.32	0.84	23.26
	Sum	**66.29**	**11.03**	**4.27**	**1.55**	**48.81**

ne: not extracted

[a] Peak assignment was based on comparison of the retention time and the metabolite pattern with the chromatogram obtained from analysis of the supernatants collected from assays incubated for 20 hours.

Source: Funk & Bellwon (2016b)

These results indicate that a significant biotransformation of pyraclostrobin occurred in rat serum within 20 hours, leading to metabolites. These metabolites were also formed in human hepatocytes or human liver microsomes (Funk & Bellwon, 2016b).

(e) Metabolism of metabolite 500M106 in male rats

The metabolism of 500M106, a major pyraclostrobin metabolite in humans, was investigated in four male Crl:WI(Han) rats (10 weeks of age) following the administration of a single oral dose of a mixture of ^{14}C-labelled 500M106 (lot/batch no. 1166-1100; chemical purity 95.2%; radiochemical purity 99.7%) and unlabelled 500M106 (lot/batch no. l83-166; purity 97.1%). The test item preparation was applied at a nominal dose of 10 mg/kg bw. Urine was sampled at time intervals of 0–6, 6–12 and 12–24 hours and afterwards at 24-hour intervals for up to 168 hours after treatment. Faeces were sampled at 24-hour intervals for up to 168 hours after treatment. After 168 hours, the rats were euthanized. In addition to the excreta samples, the total amount of radioactive residue was measured in the carcass of each animal. For balance estimates, the cage wash was also checked for radioactivity. Metabolites were identified by HPLC with tandem mass spectrometry (HPLC-MS/MS) and quantified by radio-HPLC (Thiaener & Bellwon, 2016).

The excretion of 500M106 was complete within the observation period. The main excretion route was via faeces, and detected portions of radioactive residues in faeces ranged from 84.61% to 89.89% of the dose within 168 hours. The excretion via urine ranged from 15.93% to 17.84% of the dose.

The combined faecal samples were extracted 3 times with acetonitrile and twice with water. The residue after acetonitrile and water extraction was further extracted 3 times with a mixture of acetonitrile and acetone.

The extractability of radioactive residues from faeces was moderate, and the main portion of the radioactive residues, ranging from 42.0% to 69.2% of the total radioactive residues (TRR), was extracted with acetonitrile. Low amounts were subsequently extracted with water and with the mixture

of acetonitrile and acetone, leading to radioactive residues after solvent extraction above or equal to 21.7% of the TRR. The residual radioactive residues after solvent extraction were subjected to a sequential solubilization procedure. Amounts up to 4.2% of the TRR were released by enzyme incubation (up to 8% of the TRR in total), and high portions were solubilized by applying hot alkaline treatment, accounting for up to 26.5% of the TRR.

All metabolites were present at 1% or more of the administered dose. Metabolites 500M109, 500M03, 500M05, 500M04 and 500M21 were identified in urine sampled within 48 hours. Metabolites 500M109 and 500M04 were quantified as the main components, accounting for up to 3.82% and 4.63% of the dose, respectively (average of four animals). Additional detected metabolites were present at levels ranging from 0.89% to 1.91% of the dose (average of four animals). In pooled and combined urine sampled within 48–168 hours, metabolites 500M109/500M03, 500M05/500M109, 500M04 and 500M21 were present at similar low levels, ranging from 0.12% to 0.49% of the dose. The administered compound 500M106 was not detected in urine sampled within 168 hours. Metabolites 500M04, 500M117, 500M105, 500M73/500M112, 500M01 and 500M02 as well as isomers of 500M01 and 500M02 were identified in faeces; metabolites 500M73/500M112 accounted for the main fraction (in total, 31.90% of the dose). Metabolites 500M01 and 500M02 were the next most abundant metabolites, accounting for 8.02% and 11.47% of the dose. Metabolite 500M04 was present at 6.18% of the dose, and the remaining metabolites 500M117 and 500M105 accounted for up to 0.39% of the dose. The applied compound 500M106 was not detected in faeces sampled within 168 hours.

The radioactive residues in urine are summarized in Table 4.

The major biotransformation steps in the metabolic pathway of 500M106 in male rats were considered to be as follows:

- cleavage of the N–O bond of the phenylamine moiety, followed by (a) hydroxylation, (b) dimerization with subsequent *N*-oxidation or (c) formation of formaldehyde adducts; and
- cleavage of the ether bridge between the pyrazole and phenylamine moiety, followed by conjugation (sulfation/glucuronidation) or hydroxylation and sulfation.

The proposed metabolic pathway of the metabolite 500M106 in male rats is illustrated in Fig. 4 (Fabian & Landsiedel, 2016).

Table 4. Summary of radioactive residues in urine obtained from rats during 0–168 hours

	Radioactive residues (% of dose)				
	Rat 1	**Rat 2**	**Rat 3**	**Rat 4**	**Rats 1–4**
Metabolite identity	**0–48 hours**	**0–48 hours**	**0–48 hours**	**0–48 hours**	**48–168 hours**
Identified					
500M109[a]	5.16	3.49	3.66	2.95	–
500M109 (RT 16.0–16.2)	0.90	0.90	1.00	1.69	–
500M109 (RT 17.6–18.4)	4.26	2.59	2.66	1.26	–
500M109/500M03	–	–	–	–	0.32
500M03	0.38	2.20	2.44	2.63	–
500M05/500M109	0.92	1.65	0.71	2.60	0.23
500M04	6.10	3.26	3.60	5.55	0.49
500M21	1.08	0.92	0.36	1.19	0.12
Total identified peaks	13.65	11.52	10.77	14.93	1.16

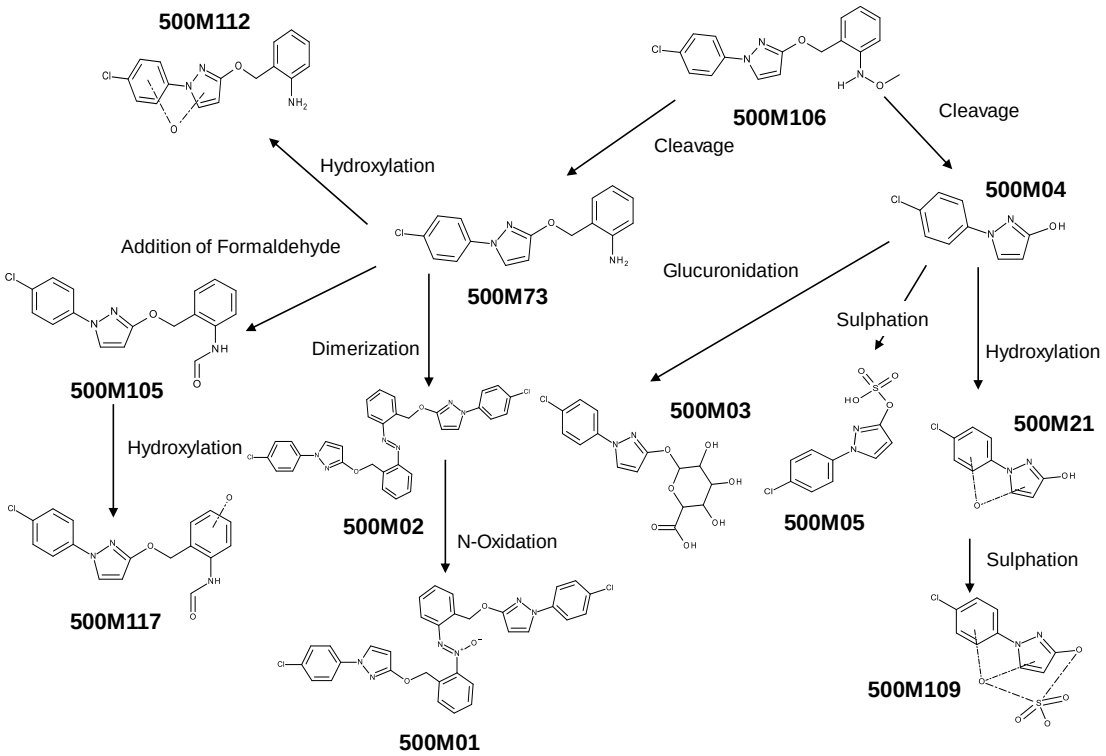
	Radioactive residues (% of dose)				
	Rat 1	**Rat 2**	**Rat 3**	**Rat 4**	**Rats 1–4**
Metabolite identity	**0–48 hours**	**0–48 hours**	**0–48 hours**	**0–48 hours**	**48–168 hours**
Characterized by HPLC					
No. of additional HPLC peaks	6	10	7	4	–
% of dose of maximum peak	0.78	0.83	0.78	0.75	–
Total characterized	2.36	4.46	3.51	1.95	–
Total identified and characterized	16.01	15.98	14.28	16.88	1.16

HPLC: high-performance liquid chromatography; RT: retention time

[a] For metabolite 500M109, no unambiguous structure is assigned, but it is given as a generic structure. Several isomers of 500M109 elute in three different peaks (RT 16.0–16.2 minutes, RT 17.6–18.4 minutes and RT 20.2–20.5 minutes) and even co-elute with metabolite 500M05 and two matrix adducts of metabolite 500M05 (RT 20.2–20.5 minutes), where the metabolite ratio could not be determined. Hence, the sum of peaks RT 16.0–16.2 minutes and RT 17.6–18.4 minutes is given additionally.

Source: Fabian & Landsiedel (2016)

Fig. 4. Proposed metabolic pathway of 500M106 in male rats



Source: Fabian & Landsiedel (2016)

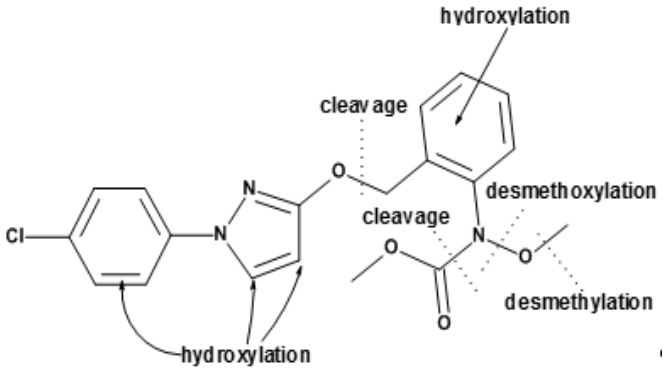
(f) Summary of biotransformation of pyraclostrobin

On the basis of the studies that were newly submitted to the present Meeting, including the comparative in vitro metabolism study and the investigations on metabolite 500M106, six steps were proposed for the biotransformation of pyraclostrobin in rats (Fig. 5):

1. desmethoxylation of the side-chain;
2. hydroxylation of the chlorophenyl pyrazole ring system;
3. hydroxylation of the tolyl ring system;
4. cleavage of the ether bond, resulting in chlorophenyl pyrazole or anthranilic acid derivatives;
5. desmethylation of the side-chain; and
6. cleavage of the amide bond in the side-chain.

The combination of these reactions with subsequent conjugation results in a large number of metabolites.

Fig. 5. Proposed biotransformation of pyraclostrobin in rats



Source: Annex 1, reference *100*; Birk, Lutz & Doebbe, 2014; Funk, Glaessgen & Kalyon, 2014; Fabian & Landsiedel, 2016; Funk & Bellwon, 2016a,b

An in vitro comparison of metabolic profiles among humans, rats, rabbits and dogs shows the same key degradation steps as were observed under in vivo conditions, and metabolites such as 500M04, 500M73, 500M108, 500M103, 500M104 and 500M88 were common to all test species. In humans and rabbits, cleavage of the amide bond, resulting in the formation of metabolite 500M106, was the major degradation pathway; 500M106 was subsequently metabolized by conjugation with glucuronic acid to form 500M107. Metabolite 500M02, formed by dimerization, was also identified in these two species. However, no unique human metabolite was detected, based on the study procedures for comparison at a level of 5% of the TRR.

In a rat dosed with 500M106, two major biotransformation steps were observed: 1) cleavage of the N–O bond of the phenylamine moiety, followed by (a) hydroxylation, (b) dimerization with subsequent *N*-oxidation or (c) formation of formaldehyde adducts; and 2) cleavage of the ether bridge between the pyrazole and phenylamine moiety, followed by conjugation (sulfation/glucuronidation) or hydroxylation and sulfation. Metabolite 500M02 was also considered to be covered by the investigations on 500M106, as it has been found in considerable amounts. An enzyme, human carboxylesterase, involved in the formation of 500M106 was confirmed to be present in rat serum.

The results indicate a significant biotransformation of pyraclostrobin, leading to metabolites that were also formed with human hepatocytes or human liver microsomes. In conclusion, 500M02,

500M106 and 500M107, identified as the metabolites in humans, were also identified in other species used for toxicological testing (Annex 1, reference *100*; Birk, Lutz & Doebbe, 2014; Funk, Glaessgen & Kalyon, 2014; Fabian & Landsiedel, 2016; Funk & Bellwon, 2016a,b).

2. Toxicological studies

2.1 Short-term studies of toxicity

(a) Exposure by inhalation

Two 4-week inhalation studies in rats were conducted.

In the first study, three groups of Wistar Crl:Wi(Han) rats (10 of each sex) were exposed, through the head and nose inhalation route, to liquid aerosols (1.6–1.9 μm) of pyraclostrobin (batch no. LJ 27822/199/b; purity 98.7%) dissolved in acetone at a nominal concentration of 0, 1, 30 or 300 mg/m^3 (equal to 0, 0.001, 0.03 and 0.3 mg/L) for 6 hours per day, 5 days per week, for 28 days.

Four male and three female rats died at the high concentration of 300 mg/m^3 (0.3 mg/L) between study days 7 and 24. This concentration is close to the acute 4-hour median lethal concentration (LC_{50}) of 0.58 mg/L observed for an aerosol of pyraclostrobin dissolved in acetone (Ma-Hock & Leibold, 2002). Body weight development in high-concentration (300 mg/m^3) males was impaired. This was evident from significantly lower body weights at day 21 (−6.6%) and from significantly lower body weight gains throughout the study when compared with the acetone control group males. Overall body weight gain of high-concentration males was decreased by 43%. The overall average daily feed intake of high-concentration males tended to be slightly lower, which is in line with the observed body weight effects in this group of animals. Haematology revealed increased white blood cell counts and an increase in absolute and relative neutrophil numbers in both sexes at 300 mg/m^3. This was probably the result of inflammatory processes in the respiratory tract (see below). No treatment-related clinical chemistry findings were noted.

Histopathology identified the respiratory tract (i.e. the nasal cavity, larynx and lungs) as well as the duodenum as target organs at 30 and 300 mg/m^3. A mild to moderate destruction of the olfactory epithelium in the nasal cavity was observed. These changes were characterized by (multi)focal atrophy and/or necrosis, (multi)focal reactive inflammation and signs of repair and regeneration as irregular architecture of the epithelium or gland-like structures. These findings are histomorphological correlates of different stages and intensities of the compound-related irritant effect. The changes in the respiratory epithelium were not as severe as those in the olfactory epithelium. There was minimal to moderate (multi)focal hyperplasia. The minimal to slight hyperplasia of the respiratory epithelium of the larynx observed in three high-concentration males was considered to be treatment related. In the lungs of all groups, a minimal to slight perivascular infiltration of inflammatory cells was observed. As the severity was slightly higher in the high-concentration females, this was considered to be treatment related. In addition, the number of histiocytes (severity) in the alveoli was higher in the high-concentration males, whereas the incidence was elevated in females at the intermediate and high concentrations. A treatment-related increase in the incidence and severity of diffuse mucosal hyperplasia in the duodenum was observed.

In conclusion, the low concentration of 1 mg/m^3 was identified as the no-observed-adverse effect concentration (NOAEC) for the first 4-week inhalation study in rats on the basis of histopathological changes in the nasal cavity, lungs and duodenum at 30 mg/m^3 (Gamer et al., 2005).

In the second study, four groups of Wistar rats (10 of each sex per group) were exposed, through the head and nose inhalation route, to liquid aerosols (1.4–2.2 μm) of pyraclostrobin (batch no. COD-001236; purity 99.02%) dissolved in acetone at a nominal concentration of 3, 10 or 30 mg/m^3 for 6 hours per day, 5 days per week, for 28 days. The study included recovery groups of 10 animals of each sex treated either with acetone (vehicle control) or with 30 mg/m^3 for 28 days followed by a 4-week treatment-free recovery period. The animals were examined for evident signs of toxicity and mortality.

Body weight and feed consumption were measured, an ophthalmoscopic examination was conducted, and haematology and blood chemistry parameters were measured. The exsanguinated animals were necropsied and assessed by gross pathology. The main organs were weighed, and all of the organs were examined histopathologically.

No treatment-related mortality or clinical signs of systemic toxicity were observed in any group. Fluctuations of body weight (body weight loss during inhalation exposure, body weight gain during the treatment-free weekends) were observed in all groups, including controls. No treatment-related changes in haematology or blood chemistry parameters were observed in treated groups.

A weight increase of the duodenum at 10 and 30 mg/m^3 was considered to be treatment related. Histopathology did not reveal any indication of systemic toxicity. However, a minimal to slight atrophy/necrosis of the olfactory epithelium in high-concentration males and females indicated local irritant effects, which were reversible within the 4-week recovery period. In some treated animals and one control animal, a small focal area at the base of the epiglottis was covered by flattened epithelium, which differed from the normal cuboidal to columnar laryngeal epithelium. This finding was also observed in two control animals and two treated animals in the recovery group. This minimal and focal change was regarded to be an adaptive, non-adverse response to the inhalation procedure and also occurred in controls.

The NOAEC for systemic toxicity in the second 4-week inhalation study in rats was 3 mg/m^3, based on the increased weight of the duodenum at 10 mg/m^3, whereas the NOAEC for local effects in the nasal cavity was 10 mg/m^3, based on the effects in the upper respiratory tract at 30 mg/m^3 (Ma-Hock et al., 2014).

Both inhalation studies indicated that pyraclostrobin induced direct irritating damage, and consequently inflammatory reaction, to the respiratory tract. Pyraclostrobin absorbed following inhalation exposure induced toxicity in the duodenum similar to that induced by oral treatment.

2.2 Long-term studies of toxicity and carcinogenicity

Additional data were made available on the histopathology of the 2-year toxicity and carcinogenicity studies in rats that had been evaluated by the 2003 Meeting (Mellert et al., 1999a,b), and two 2-year toxicity and carcinogenicity studies not available to the 2003 Meeting (Mellert, 2002b,c) were submitted.

In the previously evaluated carcinogenicity study (Annex 1, reference *100*), pyraclostrobin (purity 97.1%) was administered to groups of 50 male and 50 female Wistar rats at a dietary concentration of 0, 25, 75 or 200 parts per million (ppm) (equal to 0, 1.1, 3.4 and 9.0 mg/kg bw per day for males and 0, 1.5, 4.6 and 12.3 mg/kg bw per day for females, respectively) for 24 months. Impaired body weight development was observed in male and female rats at 200 ppm. Decreases in cumulative body weight gain approached 10% in males and 22% in females, but feed consumption was only slightly reduced, by about 4%, in females during the first 3 months of treatment. Histopathologically, the incidences of liver necrosis and liver adenomas were increased in males, but the incidence of liver carcinomas was unaffected. In the long-term study in rats that was conducted concurrently with this study of carcinogenicity (Mellert et al., 1999a), the incidence of liver adenomas was lower in treated groups than in the controls, liver carcinomas did not occur in a dose-related manner and the incidence of tumours overall was similar in all groups. On this basis, undue weight was not attached to the apparent increase in liver adenomas in males at 200 ppm. Erosion and ulcers in the glandular stomach were increased in males.

The NOAEL for the 24-month carcinogenicity study in rats was 75 ppm (equal to 3.4 mg/kg bw per day), on the basis of reduced body weight gain in both sexes and histopathological lesions in

liver and stomach in males at 200 ppm (equal to 9.2 mg/kg bw per day). There was no indication of a carcinogenic potential in rats (Mellert et al., 1999b).

Additional histopathological analysis of tissues and organs from the above carcinogenicity study was submitted to the present Meeting. The additional analysis focused on all male animals of the low- and mid-dose groups. The pathological investigations to determine histiocytic sarcomas revealed no malignant systemic tumours related to pyraclostrobin treatment in any group. The detailed histopathological evaluation of mid-dose males identified a low incidence of additional neoplastic findings that did not affect the overall tumour incidence (Mellert, 2002a).

The first carcinogenicity study that had not previously been evaluated by JMPR was conducted as an additional study to the carcinogenicity study by Mellert et al. (1999b). Pyraclostrobin (batch no. J.-Nr. 27882/191/c; purity 97.1%) was administered to groups of 50 male and 50 female Wistar rats at a dietary concentration of 0 or 400 ppm (equivalent to 20 mg/kg bw per day). As the maximum tolerated dose (MTD) was obtained at 200 ppm in the main study, this study was terminated after 399 study days without any further examinations.

The low incidence of mortality (one 400 ppm male and two and one females at 0 and 400 ppm, respectively) was not indicative of a treatment-related effect. No relevant clinical signs were observed. Body weight development was impaired in dosed males, as indicated by statistically significantly lower body weights from study day 7 onwards (11% decrease compared with controls). Body weight development of females was also impaired; however, body weights were statistically significantly lower from study day 70 onwards only (7% decrease compared with controls). Cumulative body weight gain was statistically significantly lower throughout the major part of the study for males and females (15% decrease in males and 12% decrease in females compared with controls). Consistently lower feed consumption was observed for males throughout the study and for females during major parts of the study.

Dietary administration of pyraclostrobin to rats at a concentration of 400 ppm for 399 days resulted in an impairment of body weight development in males and females. Treatment did not affect the survival of rats, and no relevant clinical signs were observed. The study was not designed to identify a NOAEL (Mellert, 2002b).

In the second carcinogenicity study that had not previously been evaluated by JMPR, pyraclostrobin (batch no. J.-Nr. 27882/191/c; purity 97.1%) was administered to groups of 50 female Wistar rats at a dietary concentration of 0 or 600 ppm (equivalent to 30 mg/kg bw per day). As the MTD was obtained at 200 ppm in the main study, the study was terminated after 426 study days without any further examinations.

No mortality or relevant clinical signs were observed. Body weight development was impaired in dosed females, as indicated by statistically significantly lower body weights from study day 35 onwards (12% decrease compared with controls). Cumulative body weight gain was statistically significantly lower throughout the major part of the study for dosed females (25% decrease compared with controls). Feed consumption by treated females was statistically significantly lower almost throughout the entire study (10% decrease compared with controls).

Dietary administration of pyraclostrobin to female rats at a concentration of 600 ppm for 426 days resulted in lower feed consumption and an impairment in body weight development. Treatment did not affect survival or elicit relevant clinical signs. The study was not designed to identify a NOAEL (Mellert, 2002c).

2.3 Reproductive and developmental toxicity

(a) Developmental toxicity

Rabbits

In the previously evaluated developmental toxicity study in rabbits (Schilling, Hellwig & Hildebrand, 1999; Annex 1, reference *100*), a NOAEL for maternal toxicity was not identified owing to a marked decrease in body weight gain and feed consumption at all doses. The pattern of the observations indicated that the decreased body weight gain and feed consumption were likely to have been caused by local gastrointestinal tract effects related to high concentrations of pyraclostrobin or to taste disturbance resulting from regurgitation or leakage of the gavaging solution. Consequently, the Meeting concluded that these observations did not reflect systemic toxicity caused by pyraclostrobin and were not used to establish the ARfD. The Meeting also concluded that the nutritional status of the does was likely to have been compromised by the marked transient reduction in feed intake.

It is for these reasons that the 2003 JMPR concluded that further information on the relationship between local irritation of the gastrointestinal tract and reduced body weight gains in pregnant rabbits and the effect of maternal nutritional deficit on fetal resorptions might allow the ARfD to be refined.

2.4 Special studies

(a) Immunotoxicity

Three immunotoxicity studies in mice were submitted.

In the first study, the ability of pyraclostrobin (batch no. COD-001236; purity 99.02%) to affect the natural killer cell–mediated immune response was investigated in 10 female B6C3F1 mice fed pyraclostrobin at a dietary concentration of 0, 50, 200 or 750 ppm (equal to 0, 13, 50 and 165 mg/kg bw per day, respectively) for 28 days. Additionally, 10 female mice were administered anti-Asialo-GM1 (positive control substance) via a single intravenous injection (0.2 mL/animal) on study day 27, the day prior to scheduled termination and necropsy. The spleen, thymus, lymph nodes (mandibular, mesenteric) and Peyer's patches were collected and examined histopathologically. Individual spleens were placed into individual tubes containing Earle's Balanced Salt Solution (EBSS) with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at 15 mmol/L and supplemented with gentamicin as a bacteriostat. After being weighed, the spleen samples were placed on crushed ice and shipped to ImmunoTox®, Inc. for natural killer cell analysis. The ability of natural killer cells (effector cells) to detect and destroy tumour cells was investigated ex vivo.

Impaired body weight development, decreased body weight gain and lower feed consumption were noted at the high dose. On day 28, body weight decreased by 12.7% and body weight gain decreased by 78.4%, compared with controls. Lower feed consumption was detected from day 0 to day 21. Treatment-related decreases in absolute and relative spleen and thymus weights were noted at the high dose. No histopathological evaluation of the thymus was performed. However, based on the substantial effect on body weight, a stress-related reduction in thymus weights can be assumed. The only treatment-related necropsy finding was a smaller thymus in two 750 ppm females. There was no test substance–related effect on natural killer cell activity at any dose. However, in line with the lower spleen weights, a lower number of spleen cells was noted at the high dose. The significantly higher (25%) spleen cell numbers at the low dose were not test substance related due to lack of an effect in the 200 ppm group. For the positive control (anti-Asialo-GM1), a statistically significant decrease in the natural killer cell activity was observed at effector target ratios of 50:1 and greater (Table 5).

The NOAEL for systemic toxicity was 200 ppm (equal to 50 mg/kg bw per day), based on reduced body weight gain and feed consumption and reduced thymus and spleen weights at 750 ppm (equal to 165 mg/kg bw per day). The NOAEL for immunotoxicity was 750 ppm (equal to 165 mg/kg bw per day), the highest dose tested (Smiraldo, 2012a).

Table 5. Summary of a 28-day natural killer cell immunotoxicity study in female mice

Finding	Pyraclostrobin				Anti-Asialo-GM1
	0 ppm	50 ppm	200 ppm	750 ppm	
Organ weight					
Absolute spleen weight (mg)	60.6	70.4	57.4	33.1**	–
Relative spleen weight (%)	0.27	0.32	0.26	0.17**	–
Absolute thymus weight (mg)	49.1	50.9	46.7	25.0**	–
Relative thymus weight (%)	0.22	0.23	0.21	0.13**	–
Gross pathology					
Thymus small	0/10	0/10	0/10	2/10	0/10
Natural killer activity					
Effector target ratio 200:1	6.2 ± 0.5	5.3 ± 0.4	6.0 ± 0.5	6.3 ± 1.1	1.1± 0.6**
Effector target ratio 100:1	2.0 ± 0.2	1.9 ± 0.3	1.8 ± 0.3	1.4 ± 0.5	0.1 ± 0.4**
Effector target ratio 50:1	1.2 ± 0.3	0.9 ± 0.3	1.2 ± 0.3	0.6 ± 0.3	0.1 ± 0.3*
Effector target ratio 25:1	1.1 ± 0.4	0.8 ± 0.4	0.9 ± 0.3	0.6 ± 0.3	0.4 ± 0.3
Effector target ratio 12.5:1	1.1 ± 0.3	1.1 ± 0.3	0.9 ± 0.3	0.8 ± 0.3	0.5 ± 0.3
Effector target ratio 6.25:1	0.8 ± 0.3	0.8 ± 0.1	0.3 ± 0.3	0.7 ± 0.3	0.5 ± 0.2
Number of spleen cells ($\times 10^7$)	9.5 ± 0.6	11.9 ± 0.9*	9.1 ± 0.5	5.3 ± 0.4**	10.0 ± 0.4

ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$ (Dunnett's test for homogenous data, Wilcoxon rank test for non-homogenous data)

[a] Values may not calculate exactly due to rounding of figures.

Source: Smiraldo (2012a)

In the second study, pyraclostrobin (batch no. COD-001236; purity 99.02%) was administered to groups of 10 female mice at a dietary concentration of 0, 50, 200 or 750 ppm (equal to 0, 13, 50 and 202 mg/kg bw per day, respectively) for 28 days. Additionally, 10 female mice were administered cyclophosphamide monohydrate (CPS) (batch no. 079K1569; purity 100.5%), the positive control substance, via intraperitoneal injection once daily during study days 24–27 at a dose of 50 mg/kg bw per day and a dosing volume of 10 mL/kg bw. On study day 24, all animals were immunized via an intravenous tail vein injection with 0.2 mL of 7.5×10^7 sheep red blood cells (sRBCs) in EBSS with HEPES (except for a single animal in the 750 ppm group that was immunized via intraperitoneal injection with 0.4 mL of 7.5×10^7 sRBCs in EBSS with HEPES). Mortality, body weight and feed consumption were determined. The exsanguinated animals were necropsied. Spleen, thymus, lymph nodes (mandibular, mesenteric) and Peyer's patches were collected and – except for spleen – placed in 10% neutral-buffered formalin for potential further histopathology. Individual spleens were placed into individual tubes containing EBSS with HEPES at 15 mmol/L and supplemented with gentamicin as a bacteriostat. After being weighed, the spleen samples were placed on crushed ice and shipped to ImmunoTox®, Inc. for the conduct of the splenic antibody-forming cell (AFC) assay.

Treatment-related clinical signs were restricted to the high dose (750 ppm) and consisted of decreased defecation, faeces smaller than normal and yellow material around the urogenital area. No mortality was observed in this study. Statistically significant and treatment-related effects on body weight development were observed only at the high dose. A body weight loss was observed (−5% compared with day 0) during the first week of treatment, and body weight was lower by 11.0% compared with controls on day 28. Statistically significantly lower feed consumption was observed at 200 and 750 ppm for interval days 0–7 (−16% and −25%, respectively, compared with controls). There were no macroscopic findings noted at the scheduled necropsy. Treatment-related and statistically significant decreases in terminal body weight and absolute and relative thymus and spleen weights were

observed in the high-dose animals (Table 6). The lower spleen weights correlated with the lower number of spleen cells. No histopathological evaluation of the thymus was performed. However, based on the substantial effect on body weight, a stress-related reduction of thymus weights can be assumed.

Table 6. Summary of spleen AFC responses to the T cell–dependent antigen, sRBCs, in female mice treated for 28 days with pyraclostrobin

	Pyraclostrobin				CPS
Parameter	**0 ppm**	**50 ppm**	**200 ppm**	**750 ppm**	**50 mg/kg bw**
Organ weights					
Absolute spleen weight (g)	92.4	85.2	82.6	55.4*	46.3*
Relative spleen weight (%)	0.41	0.39	0.37	0.27**	0.21*
Absolute thymus weight (g)	41.3	42.1	39.9	25.5*	13.1*
Relative thymus weight (%)	0.18	0.19	0.18	0.12*	0.06*
AFC responses					
Spleen cells ($\times 10^7$)	14.03	12.32	12.12	7.71**	5.07**
IgM AFC/10^6 spleen cells	2 939	1 559**	1 523*	1 210*	0**
IgM AFC/spleen ($\times 10^3$)	419	189**	184**	93**	0**

AFC: antibody-forming cell; bw: body weight; CPS: cyclophosphamide monohydrate; IgM: immunoglobulin M; ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$ (Dunnett's test)
Source: Smiraldo (2012b)

Test substance–related statistically significantly lower spleen cell numbers (−45%) were noted in the 750 ppm dose group compared with the vehicle controls. As expected, a statistically significant decrease in the spleen cell number (−64%) was observed in the positive control group (CPS). An apparent, statistically significant suppression of the humoral immune response was observed in all treated groups when evaluated as specific activity (AFC/10^6 spleen cells) or total spleen cell activity (AFC/spleen). However, the overall functional responses of the vehicle control and the pyraclostrobin-exposed groups were enhanced compared with historical control data from vehicle control animals of the same sex and strain. In the vehicle control animals from the pyraclostrobin study, the specific activity was more than twice the mean of 1206 AFC/10^6 spleen cells observed in the historical control data. Similarly, the mean responses of all pyraclostrobin-exposed animals ranged from 1210 to 1559 AFC/10^6 spleen cells, whereas historical control values averaged 1206 AFC/10^6 spleen cells (historical control minimum/maximum of study means: 1116/1499 AFC/10^6 spleen cells) – that is, the response of the pyraclostrobin-treated animals was within or slightly above the historical control range (only values below the historical control range would indicate an immunotoxic effect). The reason for the increased AFC activity in controls could not be determined. Thus, the results of this assay cannot be conclusively interpreted. The positive control group displayed statistically significant decreases in specific activity (−100%) and total spleen activity (−100%), compared with the vehicle control group (Table 6).

In conclusion, test substance–related lower spleen cell numbers were noted in the 750 ppm group (−45%). An apparent suppression of the humoral component of the immune system was noted in all test substance–treated groups when evaluated using the concurrent control of this AFC assay. However, the functional response of the vehicle control was substantially above the historical control mean, and the pyraclostrobin-exposed groups were within or close to the historical control range of the same sex and strain of mice. Therefore, the results of the humoral immune response of this study are not conclusively interpretable (Smiraldo, 2012b).

In the third study, the immunotoxic potential of pyraclostrobin (batch no. COD-001236; purity 99.02%) in 10 female B6C3F1 mice was analysed using dietary concentrations of 0, 50, 200 and 750

ppm (equal to 0, 14, 55 and 191 mg/kg bw per day, respectively) for 28 days. This was the second assay to assess the immunoglobulin M (IgM)–mediated immune response, as the first assay was not interpretable regarding immunotoxicity due to a higher activity of the vehicle control, which exceeded the historical control range by a factor of 2. Additionally, 10 female mice were administered CPS, the positive control substance, via intraperitoneal injection once daily during study days 24–27 at a dose of 50 mg/kg bw per day and a dosing volume of 10 mL/kg bw.

No clinical signs indicative of toxicity were observed throughout the study. Statistically significant and treatment-related effects on body weight development were observed only at the high dose. On day 28, body weight was decreased by 9.8%, and body weight gain was decreased by 90.9%, compared with controls. Test substance–related lower feed consumption was observed in the 750 ppm group throughout the study (−10.5% to −18.5%, compared with the vehicle controls). There were no test substance–related macroscopic findings noted at the scheduled necropsy. Treatment-related and statistically significant decreases in terminal body weight and absolute and relative thymus and spleen weights were observed in the 750 ppm group animals (Table 7). The lower spleen weights correlated with the lower number of spleen cells. No histopathological evaluation of the thymus was performed. However, based on the substantial effect on body weight, a stress-related reduction of thymus weights can be assumed.

Table 7. Summary of a second AFC immunotoxicity study in female mice treated for 28 days with pyraclostrobin

	Pyraclostrobin				CPS
Parameter	**0 ppm**	**50 ppm**	**200 ppm**	**750 ppm**	**50 mg/kg bw**
Organ weights					
Absolute spleen weight (g)	94.1	89.4	87.5	67.5*	44.7**
Relative spleen weight (%)	0.42	0.40	0.41	0.33**	0.21**
Absolute thymus weight (g)	45.0	46.0	43.6	29.1**	15.6**
Relative thymus weight (%)	0.20	0.21	0.20	0.14*	0.07**
AFC responses					
Spleen cells ($\times 10^7$)	15.01	12.52*	12.54	9.01**	3.85**
IgM AFC/10^6 spleen cells	990	1 003	1 001	1 459**	0**
IgM AFC/spleen ($\times 10^3$)	148	125	122	129	0**

AFC: antibody-forming cell; bw: body weight; CPS: cyclophosphamide monohydrate; IgM: immunoglobulin M; ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$ (Dunnett's test); the positive control was compared with the vehicle control using Student's *t*-test

Source: Smiraldo (2012c)

A treatment-related lower number of spleen cells (−40%) was noted at the high dose (750 ppm). The statistically significantly lower spleen cell number at the low dose (50 ppm; −17%) was not considered treatment related, because the values at 50 and 200 ppm were the same and statistical significance was most likely due to the decreased variability within the group. In contrast to the lower spleen cell number, the number of AFCs per spleen was not affected by treatment. Accordingly, at the high dose, a statistically significantly higher (47%) specific activity (AFC/10^6 spleen cells) was noted, compared with the vehicle control group. There were no effects on the absolute and specific activities at the intermediate and low doses. In the positive control group, the total absence of AFCs resulted in a 100% decrease in total and specific AFC activities (Table 7).

The study director reported the no-observed-effect level (NOEL) for immune effects to be 200 ppm (equal to 55 mg/kg bw per day), based on decreased spleen and thymus weights and a lower number of spleen cells at 750 ppm (equal to 191 mg/kg bw per day) (Smiraldo, 2012c). However, the lack of

effects on immunoglobulin A (IgA) and the substantial effect on body weight suggest that the effects on organ weights and on the number of spleen cells are related to the lower body weight, rather than a direct effect on the immune system. In addition, no effect indicating immunosuppression was observed in any study with this compound.

The NOAEL for systemic toxicity was 200 ppm (equal to 55 mg/kg bw per day), based on reduced body weight gain and feed consumption, decreased thymus and spleen weights, and low numbers of spleen cells at 750 ppm (equal to 191 mg/kg bw per day). The NOAEL for immunotoxicity was 750 ppm (equal to 191 mg/kg bw per day), the highest dose tested (Smiraldo, 2012c).

(b) Phototoxicity

Pyraclostrobin (batch no. COD-001236; purity 99.02%) was tested for its ability to induce phototoxic effects in Balb/c 3T3 cells in vitro. The photo-cytotoxicity was estimated by means of the neutral red uptake (NRU) method. Three experiments were carried out with and without irradiation with an ultraviolet A (UVA) source. Vehicle and positive controls were included in each experiment. The first experiment failed to fulfil the acceptance criteria, and the results of the second experiment were confirmed in a third experiment. The latter two experiments fulfilled the acceptance criteria.

Based on an initial range-finding phototoxicity test for the determination of the experimental concentrations, the following concentrations were tested with and without UVA irradiation: 0, 0.5, 1.0, 2.2, 4.6, 10.0, 21.5, 46.4 and 100.0 µg/mL. Precipitation was seen at the top concentration of 100 µg/mL with and without irradiation. In the absence and the presence of UVA irradiation, cytotoxicity was noted, and median effective concentration (EC_{50}) values were calculated.

Based on the results of this study, the test substance was predicted to have no phototoxic potential, as indicated by photo-irritancy factor (PIF) values of 1.6 and 0.9 for the second and third experiments, respectively. The threshold for a negative response is less than or equal to 2. The positive control chlorpromazine led to the expected increased cytotoxicity with UVA irradiation, as indicated by PIF values of 29.8 and 40.8.

Under the experimental conditions of this study, pyraclostrobin is not considered to be a phototoxic substance in the in vitro 3T3 NRU phototoxicity test using Balb/c 3T3 cells (Cetto & Landsiedel, 2012, 2014).

(c) Effects on iron levels in blood serum and urine of rats

A study was conducted to determine the level of iron in serum and urine after oral administration of pyraclostrobin (batch no. CP029053; purity 99.0%) to groups of 10 male and 10 female Wistar rats at a dietary concentration of 0, 50, 500 or 1500 ppm (equal to 0, 3.8, 33.9 and 73.9 mg/kg bw per day for males and 0, 4.1, 37.4 and 78.3 mg/kg bw per day for females, respectively) over a period of 14 days. Clinical signs, body weight and feed consumption were determined. Iron and transferrin levels in blood (day 14) were determined for all animals. For determination of iron in urine, individual animals were transferred to metabolism cages (withdrawal of feed and water), and urine was collected overnight.

No abnormalities were detected in any animal. No animals died during the study. Feed consumption and body weight development were impaired in both sexes at and above 500 ppm. At the high dose, an overall body weight loss was observed.

Serum iron concentrations were dose- and time-dependently decreased in mid- and high-dose animals by up to 50% when compared with the controls. Serum transferrin levels and urinary iron excretion were not affected by treatment. The slight decrease in transferrin concentration in the serum of the high-dose males on day 7 was not consistent over time and was regarded to be incidental and not toxicologically relevant. No treatment-related effects were noted at 50 ppm (Table 8).

Table 8. Summary of effects on iron levels in blood serum and urine of rats administered pyraclostrobin in the diet for 14 days

	0 ppm	50 ppm	500 ppm	1 500 ppm
Males				
Blood chemistry				
Serum iron (µmol/L)				
Day 7	47.01	44.84	36.61	34.85*
Day 14	54.54	46.71	37.72**	27.41**
Serum transferrin (g/L)				
Day 7	5.50	5.34	5.45	4.74**
Day 14	6.38	5.80	6.07	5.85
Urine analysis				
Iron (nmol/L[a])				
Day 15	3.97	3.27	2.69	3.60
Females				
Blood chemistry				
Serum iron (µmol/L)				
Day 7	59.07	60.09	46.48**	57.49
Day 14	53.61	58.31	45.55*	41.97**
Serum transferrin (g/L)				
Day 7	5.40	5.23	5.62	4.92
Day 14	5.86	5.68	6.13	5.63
Urine analysis				
Iron (nmol/L[a])				
Day 15	2.61	2.44	2.78	2.89

ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$ (Kruskal Wallis plus Wilcoxon test)
[a] The units for iron concentrations in urine are not clear from the original report.
Source: Mellert et al. (2003a)

This mechanistic study indicated that pyraclostrobin-induced anaemia was related to lower absorption of iron from the gut, resulting in lower serum iron levels (Mellert et al., 2003a).

(d) Effects on oxidative stress in liver

Pyraclostrobin (batch no. CP029053; purity 99.0%) was administered to groups of 10 male Wistar rats at a dietary concentration of 0, 75 or 200 ppm (equal to 0, 5.1 and 13.4 mg/kg bw per day for 14 days and 0, 5.3 and 13.6 mg/kg bw per day for 28 days, respectively) for 14 or 28 days. Feed consumption and body weights were determined weekly. The animals were examined for signs of toxicity or mortality at least once a day. Potential induction of oxidative stress by pyraclostrobin in liver was determined by measuring lipid peroxidation.

No animals died during the study. No abnormal clinical signs were detected. Lipid peroxidation in liver was decreased markedly after 14 days in animals receiving 200 ppm (416, 346.7 and 236.2 nmol malondialdehyde [MDA] equivalents per gram liver at 0, 75 and 200 ppm, respectively). After 28 days, lipid peroxidation was still decreased in both treated groups, although to a smaller extent (539.5, 467.3

and 460.1 nmol MDA equivalents per gram liver at 0, 75 and 200 ppm, respectively). The reduction of thiobarbituric acid–reactive material is not indicative of an induction of oxidative stress in the liver and is not considered to represent an adverse effect (Mellert, Beimborn & Van Ravenzwaay, 2003).

(e) In vitro haemolytic potential

In order to clarify the possible haemolytic potential of pyraclostrobin, the effect on haemolysis was investigated in vitro using 0.001%, 0.01% and 0.1% weight per volume (w/v) pyraclostrobin (batch no. CP029053; purity 99.0%) solutions. The assay is based on the integrity of the erythrocyte membrane after incubation with the test compound. Haemoglobin crosses the cell membrane into the test solution after damage of the membrane. The concentration of free haemoglobin can then be determined and correlated directly with the damage caused to the erythrocyte membrane by the test compound. To demonstrate any haemolytic effect, photographs of the erythrocyte suspension and the supernatant were taken.

Pyraclostrobin did not cause haemolysis in the in vitro haemolysis test even at relatively high concentrations (0.1% w/v) and after stirring the erythrocyte suspension for 2 hours (Deckardt & Van Ravenzwaay, 2003).

(f) Combination study of pyraclostrobin with vitamin B_{12}

Pyraclostrobin (batch no. LJ-Nr.27882/199/b; purity 98.7%) was administered to groups of 12 male Wistar rats at a dietary concentration of 0 or 1500 ppm (equivalent to 150 mg/kg bw per day) over a period of 4 weeks. Animals received additionally 0.1 mL vitamin B_{12} subcutaneously each day. Feed consumption and body weights were determined weekly. The animals were examined for clinical signs of toxicity or mortality at least once a day. Haematological examinations and serum iron determinations were carried out on days 7, 14, 21 and 27. At necropsy, pH was determined in forestomach and glandular stomach.

The administration of pyraclostrobin resulted in decreased feed consumption and body weight. The decreases were similar in the groups with and without vitamin B_{12} administration. Haematology examinations revealed an iron deficiency anaemia after administration of pyraclostrobin. The administration of vitamin B_{12} did not affect pyraclostrobin-induced changes in red blood cell parameters or serum iron level. The pH measurement in forestomach and glandular stomach did not reveal any substance-related changes. The absolute and relative weights of the duodenum were increased in the groups receiving pyraclostrobin either with or without vitamin B_{12} administration. There was basically no difference between the two groups. No effect was seen in the group receiving vitamin B_{12} only.

In conclusion, the simultaneous administration of excessive vitamin B_{12} and pyraclostrobin did not inhibit pyraclostrobin-induced anaemia, serum iron deficiency or duodenal weight increase. The pH values in the stomach did not show any treatment-related effects. This study showed evidence that serum iron deficiency is the causative agent for anaemia and duodenal thickening (Mellert, 2003a; Mellert et al., 2003b).

(g) Combination study of dimoxystrobin with iron

Dimoxystrobin, which is a strobilurin fungicide similar to pyraclostrobin, was administered to groups of 10 male Wistar rats at a dietary concentration of 0 or 4500 ppm (equal to 0, 206.6 and 171.2 mg/kg bw per day for controls, 4500 ppm or 4500 ppm plus iron complex group, respectively) over a period of 14 days and to groups of 10 female Wistar rats at a dietary concentration of 0, 500 or 4500 ppm (equal to 0, 37.7, 17.7, 191.3 and 84.7 mg/kg bw per day for controls, 500 ppm, 500 ppm plus iron complex, 4500 ppm and 4500 ppm plus iron complex, respectively) over a period of 7 days. Simultaneously, additional groups received an iron complex (Myofer® 100) intramuscularly (males once daily at a dose of 100 mg/kg bw on study days 0, 7, 11 and 13, and females twice daily at a dose of 50 mg/kg bw from day 2 to day 6). Feed consumption and body weights were determined weekly.

The animals were examined for signs of toxicity or mortality at least once a day. On several days, the following serum parameters were determined: iron, transferrin (males only) and unsaturated iron binding capacity (males only). At necropsy, duodenal weights were determined.

Iron levels in the serum were increased in all groups receiving the iron complex (with and without dimoxystrobin) and reduced in the groups receiving dimoxystrobin only. Feed consumption was statistically significantly decreased in all treated males and in females treated with 4500 ppm dimoxystrobin and/or iron complex. Both the administration of 4500 ppm dimoxystrobin and the administration of the iron complex caused a significant impairment of body weight. In the groups receiving 4500 ppm dimoxystrobin and iron complex, the effect was additive. Duodenal weights were statistically significantly increased in the 500 ppm (females) and 4500 ppm (both sexes) groups. Treatment with 500 ppm dimoxystrobin and iron complex as well as 4500 ppm dimoxystrobin and iron complex led to a lower increase in duodenal weights in all treatment groups. The increase was not statistically significant in females of the 4500 ppm plus iron complex treatment group when compared with controls. Moreover, the increase was not dose dependent. Although the duodenal weights did not completely reach the control values, these data indicate that iron administered to animals treated with dimoxystrobin had an inhibitory effect on the increase in duodenal weights, in the sense of preventing effects on the duodenum that are caused by a reduction in serum iron levels following treatment with dimoxystrobin.

In conclusion, this study indicates that the administration of iron complex can reduce the dimoxystrobin-induced increase in duodenal weights. Dimoxystrobin-induced iron deficiency might therefore be a causative factor for increased duodenal weights (Mellert, 2003b; Mellert et al., 2002; Mellert & Kaufmann, 2004).

(h) Effect of dimoxystrobin on iron absorption and transport in the duodenum

In a non-GLP-compliant study investigating the effect of dimoxystrobin on the mucosal uptake and transfer of iron into the carcass after oral administration, groups of five female Wistar rats were treated with control diet or diet containing 4500 ppm dimoxystrobin (equivalent to 450 mg/kg bw per day) for 24, 96 or 168 hours. The everted duodenums of these rats, removed under anaesthesia, were incubated for 5 minutes in buffer containing $^{59}Fe^{2+}$-ascorbate, and Fe^{2+} uptake was measured by counting radioactivity in the duodenal segments and by autoradiography. Iron transfer across the serosal membrane (i.e. iron transfer into the body) was determined with tied-off duodenal segments in anaesthetized rats that were exposed to 4500 ppm dimoxystrobin in the feed for 24 or 96 hours. $^{59}Fe(nitrilotriacetate)_2$ was injected into duodenal segments. After varying time intervals (10, 20 and 40 minutes), the animals were terminated, and the duodenal segments were removed, washed and weighed. Radioactivity in the duodenal segments and the remaining carcass, representing mucosal retention and mucosal transfer, respectively, was determined by gamma counting. The sum of the mucosal retention and mucosal transfer represents the total mucosal uptake.

Mucosal iron uptake showed a statistically significant reduction after treatment with dimoxystrobin for 96 and 168 hours. This was confirmed by autoradiography performed on animals that had been treated for 168 hours compared with those on control diet. Mucosal iron uptake was slightly and statistically non-significantly reduced after treatment of rats with dimoxystrobin for 24 hours. Iron transfer across the serosal membrane (i.e. iron transfer into the body) was significantly reduced after a 96-hour treatment with dimoxystrobin, with the reduction of iron transfer being proportional to the reduction in uptake. After 24 hours of treatment, no significant effect was found.

In summary, the results of this study clearly demonstrate that repeated treatment of rats with dimoxystrobin considerably reduces both uptake of iron by the duodenum mucosa and transfer of iron into the body (Srai, 2003).

2.5 Toxicity of metabolites

New acute, short-term toxicity and genotoxicity studies for seven metabolites of pyraclostrobin were submitted.

(a) 500M04 (pyrazolon)

Toxicity data submitted on metabolite 500M04 included studies on acute oral toxicity in rats, irritation to eye and skin in rabbits, hypersensitization in guinea-pigs and short-term oral toxicity in rats (summarized in Table 9). In vitro and in vivo genotoxicity studies with this metabolite are described in section (c) on genotoxicity below.

Table 9. Summary of acute toxicity, irritation, sensitization and short-term toxicity of 500M04

Route (method)	Species/strain	Purity (%); batch no.	Result	Reference
Oral (gavage)	Wistar rats	100.5; 27967/95	LD_{50} > 2 000 mg/kg bw in both sexes	Kuehlem (1997a)
Skin irritation	New Zealand white rabbits	100.5; 27967/95	Negative	Kuehlem (1997b)
Eye irritation	New Zealand white rabbits	100.5; 27967/95	Weakly positive	Kuehlem (1997c)
Dermal sensitization (maximization test)	Dunkin-Hartley guinea-pigs	100.5; 27967/95	Not sensitizing	Kuehlem & Hellwig (1997)
Ninety-day oral toxicity	Wistar rats	99.6; L84-174	NOAEL = 100 mg/kg bw for males, 300 mg/kg bw for females	Buesen et al. (2013)

bw: body weight; LD_{50}: median lethal dose; NOAEL: no-observed-adverse-effect level

Acute oral toxicity

500M04 was investigated in a study in which a group of three male and three female Wistar rats were administered the metabolite by gavage at a dose of 200 or 2000 mg/kg bw. The test material was administered in 0.5% tylose CB 30000 at a dosing volume of 10 mL/kg bw. The animals were starved overnight prior to dosing. Mortality and signs of reaction to treatment were recorded during a subsequent 14-day observation period. The surviving animals were killed on the following day. All animals were subjected to necropsy.

No deaths occurred after oral administration of 200 or 2000 mg/kg bw. At a dose of 2000 mg/kg bw, male rats showed an impaired or poor general state, dyspnoea, apathy and staggering up to 1 day after administration, whereas female rats did not show any symptoms. Likewise, no symptoms were noted for males or females at the dose of 200 mg/kg bw. Normal body weight gain was observed during the 14 days of the observation period with the exception of one 200 mg/kg bw female, which displayed a body weight loss during the second week of observation. No remarkable findings were observed at necropsy. The LD_{50} was greater than 2000 mg/kg bw (Kuehlem, 1997a).

Skin irritation in rabbits

The potential of 500M04 to irritate the skin of rabbits was assessed by semi-occluded application of 0.5 g of the test material to the closely clipped dorsa of three New Zealand white rabbits for 4 hours. Dermal reactions were assessed 1, 24, 48 and 72 hours after removal of the dressings.

No erythema or oedema was observed in any rabbit after 24–72 hours. 500M04 did not show a skin irritation potential under the test conditions chosen (Kuehlem, 1997b).

Eye irritation in rabbits

The potential of 500M04 to cause damage to the conjunctiva, iris or cornea was assessed in three New Zealand white rabbits that were subjected to a single ocular instillation of 0.1 mL of 500M04. Ocular reactions were examined 1, 24, 48 and 72 hours after treatment.

No ocular reactions on the cornea or iris were observed. Slight to moderate conjunctival redness and chemosis were observed in all animals at the 1-hour reading point. Discharge was observed only in one animal. At the 72-hour reading point, all effects were fully reversed.

Based on the findings of this study, 500M04 is slightly irritating to the eye under the test conditions chosen (Kuehlem, 1997c).

Sensitization in guinea-pigs

For the determination of the potential sensitizing properties of 500M04, a maximization test based on the method of Magnusson and Kligman was conducted using a control group and a treated group of five and 10 female Pirbright white Dunkin Hartley guinea-pigs, respectively. The test substance concentrations for the main test were selected based on the results of the pretests and the results of the first challenge. The intradermal induction was performed with a 5% test substance preparation in 1% aqueous tylose CB 30000 solution or in Freund's complete adjuvant/0.9% aqueous sodium chloride solution (1:1). The epicutaneous induction was conducted with a 25% test substance preparation in 1% aqueous tylose CB 30000 solution. Two challenges were performed 14 and 21 days after percutaneous induction.

After the first challenge with a 10% test substance preparation, very slight to well-defined skin reactions were observed in three test group animals. The second challenge with a 10% substance preparation did not cause any skin reactions.

Based on the results of this study and applying the evaluation criteria, it was concluded that 500M04 does not have a sensitizing effect on the skin of the guinea-pig in the maximization test under the test conditions chosen (Kuehlem & Hellwig, 1997).

Short-term oral toxicity in rats

Three groups of 10 male and 10 female Wistar rats received 500M04 (batch no. L84-174; purity 99.6%) at a target dose of 100, 300 or 1000 mg/kg bw per day (mean intakes 103, 302 and 1017 mg/kg bw per day for males and 106, 316 and 1066 mg/kg bw per day for females, respectively) for 3 months by the dietary route. A further group of 10 male and 10 female rats received the basal control diet as a contemporaneous control. Mortality, clinical signs, body weight, feed consumption and ophthalmological parameters were measured. Water consumption was monitored from day 49 onwards. A functional observational battery (FOB) was performed on all animals. Haematology and blood chemistry parameters were measured. All animals were terminated, necropsied and assessed by gross pathology. The organs were sampled, weighed and examined histopathologically.

No effects on clinical signs, mortality, body weight, feed consumption, ophthalmology or FOB test parameters were detected. Daily drinking-water consumption was consistently increased in both sexes at the high dose (1000 mg/kg bw per day) throughout the observation period, with males more severely affected than females (Table 10). The increased water consumption is also evident if the average water consumption between days 49 and 91 is calculated. The effect on water consumption in mid- and low-dose animals was neither consistent over time nor dose dependent, and the difference in the overall mean water consumption relative to the controls was marginal. Thus, only the effect at 1000 mg/kg bw per day was considered to be treatment related.

Table 10. Summary of findings in short-term toxicity study in rats administered 500M04

	Males				Females			
Finding	**0 mg/kg bw per day**	**100 mg/kg bw per day**	**300 mg/kg bw per day**	**1 000 mg/kg bw per day**	**0 mg/kg bw per day**	**100 mg/kg bw per day**	**300 mg/kg bw per day**	**1 000 mg/kg bw per day**
Water consumption								
g/animal[a]	22.5	23.0	23.4	31.5	20.5	21.3	20.5	24.5
% relative to control[a]	–	2.2	4.1	39.7	–	4.2	−0.1	19.5
Haematology								
RBCs (10^{12}/L)	8.82	8.80	8.66	8.02**	7.53	7.41	7.58	7.54
HGB (mmol/L)	9.1	9.1	9.1	8.8*	8.4	8.3	8.5	8.4
MCV (fL)	49.7	50.1	50.4	53.5**	52.4	52.6	53.2	53.1
MCH (fmol)	1.04	1.04	1.05	1.10*	1.12	1.12	1.12	1.12
Ret (%)	2.0	1.9	2.2	2.9*	2.6	2.9	2.9	2.6
Clinical chemistry								
Total protein (g/L)	62.99	62.03	61.77	59.01**	64.11	64.98	65.03	66.65
Triglycerides (mmol/L)	1.16	1.18	1.44	1.86**	0.64	0.49	0.58	0.97
Urine analysis								
Urine volume (mL)	3.2	3.9	3.5	6.5**	2.4	2.7	3.0	3.9**
Specific gravity (g/L)	1 060	1 055	1 062	1 040**	1 071	1 062	1 051	1 045**
Crystal[b]	2	2	3*	3**	2	2	2	2
Histopathology: Kidney[c]								
Eosinophic material, renal pelvis	0/10	0/10	1/10 (2.0)	6/10 (2.8)	0/10	0/10	0/10	1/10 (2.0)
Hyperplasia, urothelial	0/10	0/10	1/10 (1.0)	7/10 (1.4)	0/10	0/10	0/10	3/10 (2.0)
Mineralization, medulla	2/10 (1.0)	4/10 (1.0)	0/10	7/10 (1.0)	8/10 (1.3)	9/10 (1.3)	10/10 (1.6)	10/10 (2.3)
Nephropathy chronic	4/10 (1.0)	7/10 (1.0)	5/10 (1.0)	9/10 (1.3)	6/10 (1.0)	1/10 (1.0)	1/10 (1.0)	9/10 (1.3)
Ulceration, papillary	0/10	0/10	0/10	1/10 (2.0)	0/10	0/10	0/10	0/10
Histopathology: Spleen[c]								
Haematopoiesis, extramedullary	6/10 (1.0)	10/10 (1.4)	10/10 (1.5)	10/10 (2.0)	8/10 (1.2)	–	–	9/10 (1.2)

bw: body weight; HGB: haemoglobin; MCH: mean cell haemoglobin; MCV: mean corpuscular volume; RBC: red blood cells; Ret: reticulocytes; *: $P \leq 0.05$; **: $P \leq 0.01$ (Kruskal-Wallis and Wilcoxon-tests, two-sided)

[a] Values were calculated based on mean individual daily consumption. Values may not calculate exactly due to rounding of mean values; no statistics were performed because $n = 2$.

[b] Semiquantitative parameter: Grade 0 = none, Grade 1 = few, Grade 2 = many, Grade 3 = masses.

[c] Mean severity grading given in parentheses. Histopathological findings were graded minimal (Grade 1), slight (Grade 2), moderate (Grade 3), marked (Grade 4) and massive/severe (Grade 5). The mean severity is the sum of the gradings divided by the incidence.

Source: Buesen et al. (2013)

Treatment-related haematological changes were restricted to high-dose males and consisted of a slight regenerative normochromic-macrocytic anaemia, indicated by decreased erythrocyte counts and haemoglobin values and increased mean corpuscular volume, mean cell haemoglobin and relative reticulocyte counts. Treatment-related clinical chemistry findings were restricted to high-dose males and consisted of decreased total protein and globulin values as well as increased triglyceride levels. Urine analysis revealed increased mean urine volumes, which were accompanied by lower specific gravity of the urine in both sexes at 1000 mg/kg bw per day. This is consistent with the higher water consumption noted at this dose. In addition, more crystals of unknown origin were found in the urine sediment of males at and above 300 mg/kg bw per day. The aforementioned findings were considered to be treatment related (Table 10).

No statistically significant absolute or relative organ weight differences were observed. There were no treatment-related gross necropsy findings. Treatment-related histopathological findings were observed in the kidneys of mid-dose males and high-dose males and females as well as in the spleen of high-dose males.

In the kidney, the eosinophilic material was localized in the lumen of the renal pelvis in males at 300 and 1000 mg/kg bw per day and in females at 1000 mg/kg bw per day. The urothelial hyperplasia most probably represented a reactive response to the presence of the eosinophilic material in the renal pelvis in males at 300 and 1000 mg/kg bw per day and in females at 1000 mg/kg bw per day. The increased mineralization was seen in the medulla at 1000 mg/kg bw per day in both sexes. Chronic progressive nephropathy showed a slightly increased incidence and severity in both sexes at the high dose. Papillary ulceration was detected at 1000 mg/kg bw per day in males. Increased severity of extramedullary haematopoiesis in the spleen was detected in high-dose males (Table 10).

The NOAEL was 100 mg/kg bw per day, based on renal lesions and urine analysis findings in males at 300 mg/kg bw per day (Buesen et al., 2013).

(b) 500M106

Short-term oral toxicity in rats

500M106 (batch no. L87-218; purity 97.7%) was administered to groups of 10 male and 10 female Wistar rats by gavage at a target dose of 0, 100, 300 or 1000 mg/kg bw per day for 28 days. The test item was administered as a suspension in 0.5% aqueous carboxymethylcellulose. Mortality, body weight, feed consumption, water consumption, ophthalmological examination, a FOB and urine analysis were conducted. Haematology and clinical chemistry parameters were examined. All animals were terminated, necropsied and assessed by gross pathology. The organs were sampled, weighed and examined histopathologically.

No test substance–related effects on clinical signs, mortality, ophthalmoscopy, body weight, feed consumption, FOB test parameters, motor activity or clinical chemistry parameters were observed.

Treatment-related and adverse haematological changes were restricted to high-dose males and females and consisted of a slight, regenerative normochromic-normocytic anaemia, indicated by decreased erythrocyte counts, haemoglobin and haematocrit in males and increased absolute reticulocyte counts in both sexes. Platelet counts were significantly higher in all treated male groups. However, the values were within the range observed in historical control animals of this strain and age (683–973 $\times 10^9$/L). A statistically significant increase in the number of reticulocytes was noted in both sexes at the low dose. Again, the numbers were within the historical control range (males: 102.1–184.6 $\times 10^9$/L; females: 102.2–189.8 $\times 10^9$/L) and not dose related (Table 11).

Changes in absolute and relative organ weights were noted in high-dose males and consisted of increased liver and duodenum weights (Table 11). There were no treatment-related gross necropsy findings.

Table 11. Summary of 4-week oral toxicity study of 500M106 in rats

	Males				Females			
Finding	**0 mg/kg bw per day**	**100 mg/kg bw per day**	**300 mg/kg bw per day**	**1 000 mg/kg bw per day**	**0 mg/kg bw per day**	**100 mg/kg bw per day**	**300 mg/kg bw per day**	**1 000 mg/kg bw per day**
Haematology								
RBC (10^{12}/L)	7.86	7.67	7.88	7.35**	7.15	7.04	6.88	6.9
HGB (mmol/L)	8.8	8.8	8.8	8.4**	8.2	8.2	8.0	8.3
HCT (%)	41.7	41.5	41.4	39.4**	37.5	37.3	36.8	36.8
Platelets (10^9/L)[a]	712	772*	775*	817**	756	676	711	733
Reticulocytes (10^9/L)[b]	141.2	172.4**	151.7	248.1**	146.1	173.7*	191.5	211.7**
Organ weights								
Absolute duodenum weight (g)	0.477	0.508	0.485	0.532*	0.442	0.432	0.425	0.456
Relative duodenum weight (%)	0.176	0.185	0.184	0.200*	0.256	0.245	0.241	0.263
Absolute liver weight (g)	7.32	7.57	7.23	8.00*	4.70	4.68	4.83	4.95
Relative liver weight (%)	2.69	2.75	2.73	3.00**	2.72	2.65	2.74	2.84*
Absolute spleen weight (g)	0.583	0.531	0.549	0.578	0.462	0.373	0.407	0.419
Relative spleen weight (%)	0.214	0.193	0.207	0.217	0.266	0.211	0.231	0.240
Histopathology: Liver[c]								
Hypertrophy, centrilobular	0/10	0/10	0/10	4/10 (1.0)	0/10	0/10	0/10	0/10
Histopathology: Spleen[c]								
Haematopoiesis, extramedullary	2/10 (1.0)	2/10 (1.0)	3/10 (1.0)	8/10 (1.9)	1/10 (1.0)	0/10	3/10 (1.0)	6/10 (1.5)

bw: body weight; HCT: haematocrit; HGB: haemoglobin; RBC: red blood cells; *: $P \leq 0.05$; **: $P \leq 0.01$ (Kruskal-Wallis plus Wilcoxon test, two-sided)

[a] Historical control data for platelets: males: 683–973 × 10^9/L.

[b] Historical control data for reticulocytes: males: 102.1–184.6 × 10^9/L; females: 102.2–189.8 × 10^9/L.

[c] Mean severity grading given in parentheses. Histopathological findings were graded minimal (Grade 1), slight (Grade 2), moderate (Grade 3), marked (Grade 4) and massive/severe (Grade 5). The mean severity is the sum of the gradings divided by the incidence.

Source: Buesen et al. (2017)

Treatment-related histopathological findings were restricted to the high dose and noted in the liver of high-dose males and the spleen of high-dose males and females. In the liver of high-dose males, a minimal centrilobular hepatocellular hypertrophy was noted, which correlated with the increased liver weights. In the spleen, an increased incidence and severity of extramedullary haematopoiesis was noted,

which correlated with the slight anaemia observed at this dose. There was no histopathological correlate to the slightly increased absolute and relative duodenum weights (Table 11).

The NOAEL for 500M106 in rats was 300 mg/kg bw per day, on the basis of the treatment-related effects on red blood cell parameters (regenerative anaemia in both sexes), duodenum (increased weight in males), liver (increased weight and hepatocellular hypertrophy in males) and spleen (extramedullary haematopoiesis) at 1000 mg/kg bw per day (Buesen et al., 2017).

(c) Genotoxicity

Several metabolites of pyraclostrobin were evaluated for potential genotoxicity in in vitro tests for mutagenicity in bacterial and mammalian cells, for chromosome damage (clastogenicity) and for unscheduled DNA synthesis. The results of these studies are summarized in Table 12.

Table 12. Summary of in vitro and in vivo genotoxicity studies with metabolites of pyraclostrobin

Metabolite/study	Test object	Concentration	Purity (%), lot/batch no.	Results	Reference
500M04 (pyrazolon)					
In vitro					
Reverse mutation test	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2*uvr*A	10, 33, 100, 333, 1 000, 2 600 and 5 200 µg/plate ±S9	97.6, L83-44-2	Negative	Woitkowiak (2012)
Forward mutation test	Chinese hamster ovary cells (*Hprt* locus)	Experiment 1: 21.9, 43.8, 87.5, 175 and 350 µg/mL +S9; 21.9, 43.8, 87.5 and 175 µg/mL −S9 Experiment 2: 31.3, 62.5, 125 and 250 µg/mL +S9; 62.5, 125, 250, 500 and 1 000 µg/mL −S9	99.6, L84-174	Negative	Schulz & Landsiedel (2012a)
Chromosomal aberrations	Chinese hamster ovary cells (V79 cells)	62.5, 125 and 250 µg/mL ±S9; 100, 150, 200 and 250 µg/mL +S9	97.6, L83-44-2	Positive with S9	Schulz & Landsiedel (2012b)
In vivo					
Micronucleus test	Bone marrow cells from NMRI mice (five males)	500, 1 000 and 2 000 mg/kg bw (once by gavage, analysed 24 hours after treatment) 2 000 mg/kg bw (once by gavage, analysed 48 hours after treatment)	99.6, L84-174	Negative	Fabian & Landsiedel (2013); Schulz & Landsiedel (2013a)

Metabolite/study	Test object	Concentration	Purity (%), lot/batch no.	Results	Reference
500M24					
In vitro					
Reverse mutation test	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	33, 100, 333, 1 000, 2 500 and 5 000 µg/plate ±S9	98.8, L82-125	Negative	Woitkowiak (2014)
Forward mutation test	L5178Y mouse lymphoma cells ($Tk^{+/-}$ locus)	287.5, 575, 1 150 and 2 300 µg/mL ±S9; 431.3, 862.5, 1 725 and 2 300 µg/mL +S9; 143.8, 287.5, 575, 1 150 and 2 300 µg/mL −S9	98.8, L82-125	Negative	Schulz & Landsiedel (2014a)
Chromosomal aberrations	Chinese hamster ovary cells (V79 cells)	575, 1 150 and 2 300 µg/mL ±S9; 1 150, 1 725 and 2 300 µg/mL −S9	98.8, L82-125	Positive without S9	Schulz & Landsiedel (2014b)
In vivo					
Micronucleus test	Bone marrow cells from NMRI mice (five males)	500, 1 000 and 2 000 mg/kg bw (once by gavage, analysed 24 hours after treatment) 2 000 mg/kg bw (once by gavage, analysed 48 hours after treatment)	98.8, L82-125	Negative	Dony (2014); Schulz & Landsiedel (2016a)
500M49					
In vitro					
Reverse mutation test	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	33, 100, 333, 1 000, 2 500 and 5 000 µg/plate ±S9	100.0, L82-115	Negative	Woitkowiak (2013a)
Forward mutation test	L5178Y mouse lymphoma cells ($Tk^{+/-}$ locus)	125, 250, 500, 1 000 and 2 000 µg/mL ±S9; 375, 750 and 1 500 µg/mL +S9; 62.5 µg/mL −S9	100.0, L82-115	Negative	Schulz & Landsiedel (2014c)
Chromosomal aberrations	Chinese hamster ovary cells (V79 cells)	250, 500, 1 000 and 2 000 µg/mL ±S9	100.0, L82-115	Negative	Schulz & Landsiedel (2014d)

Metabolite/study	Test object	Concentration	Purity (%), lot/batch no.	Results	Reference
500M51					
In vitro					
Reverse mutation test	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	10, 33, 100, 333, 1 000, 2 600 and 5 200 µg/plate ±S9	97.9, L82-117	Negative	Woitkowiak (2013b)
Forward mutation test	L5178Y mouse lymphoma cells (*Tk*$^{+/-}$ locus)	250, 500, 1 000 and 2 000 µg/mL ±S9; 375, 750, 1 500 and 2 000 µg/mL +S9; 7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1 000 and 2 000 µg/mL −S9	97.9, L82-117	Negative	Schulz & Landsiedel (2014e)
Chromosomal aberrations	Chinese hamster ovary cells (V79 cells)	500, 1 000 and 2 000 µg/mL +S9; 125, 250, 500, 1 000 and 2 000 µg/mL −S9	97.9, L82-117	Negative	Schulz & Landsiedel (2014f)
500M76					
In vitro					
Reverse mutation test	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	22, 110, 550, 2 750 and 5 500 µg/plate ±S9	94.5, 01586-236	Negative	Engelhardt & Hoffmann (2000)
Forward mutation test	Chinese hamster ovary cell (*Hprt* locus)	62.5, 125, 250, 500, 750 and 1 000 µg/mL +S9; 12.5, 25, 50, 100, 200 and 400 µg/mL −S9 9.38, 18.75, 37.5, 75, 150 and 300 µg/mL −S9	94.5, 01586-236	Negative	Engelhardt & Leibold (2003)
Chromosomal aberrations	Chinese hamster ovary cells (V79 cells)	250, 500 and 750 µg/mL +S9; 125, 250 and 500 µg/mL −S9; 700, 750 and 800 µg/mL +S9; 500, 550 and 600 µg/mL −S9	94.5, 01586-236	Positive with and without S9	Schulz & Landsiedel (2013b, 2014g)
In vivo					
Micronucleus test	Bone marrow cells from NMRI mice (five males)	125, 250 and 500 mg/kg bw (once by gavage, analysed 24 hours after treatment) 500 mg/kg bw (once by gavage,	99.0, L83-122	Negative	Schulz & Landsiedel (2012c)

Metabolite/study	Test object	Concentration	Purity (%), lot/batch no.	Results	Reference
		analysed 48 hours after treatment)			
500M02					
In vitro					
Reverse mutation test	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	4, 20, 100, 500, 2 500 and 5 000 µg/plate ±S9	99.9, 01185-022	Negative	Engelhardt & Hoffmann (1999)
		33, 100, 333, 1 000, 2 500 and 5 000 µg/plate ±S9	98.3, L85-192	Negative	Woitkowiak (2016)
Forward mutation test	L5178Y mouse lymphoma cells ($Tk^{+/-}$ locus)	4.69, 9.38, 18.75, 37.5, 75 and 150 µg/mL ±S9; 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 µg/mL ±S9	98.3, L85-192	Negative	Schulz & Landsiedel (2016b)
Micronucleus test	Human lymphocytes	4.3, 6.6, 7.6, 9.9, 13.2 and 14.8 µg/mL +S9; 4.3, 7.6, 13.2, 22.2, 33.3 and 100 µg/mL −S9	98.3, L84-192	Negative	Chang (2016a)
500M106					
In vitro					
Reverse mutation test	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	3.3, 10, 33, 100, 333, 1 000, 2 600 and 5 200 µg/plate ±S9	97.6, L87-58	Negative	Woitkowiak & Landsiedel (2016)
Forward mutation test	L5178Y mouse lymphoma cells ($Tk^{+/-}$ locus)	1.56, 3.13, 6.25, 12.5, 25, 50 and 100 µg/mL ±S9; 4.69, 9.38, 18.75, 37.5, 50, 75 and 100 µg/mL +S9	97.6, L87-58	Positive with S9	Schulz & Landsiedel (2016c)
Micronucleus test	Human lymphocytes	1.9, 2.6, 3.3, 3.5, 4.6, 5.8, 6.1, 8.0, 10.7, 14.0, 18.7, 32.7 and 100 µg/mL ±S9	97.6, L87-58	Negative	Chang (2016b)
In vivo					
Micronucleus test	Bone marrow cells from NMRI mice (5 males)	500, 1 000 and 2 000 mg/kg bw (once by gavage, analysed 24 hours after treatment)	97.6, L87-58	Negative	Dony (2016)
		2 000 mg/kg bw (once by gavage, analysed 48 hours after treatment)			

Metabolite/study	Test object	Concentration	Purity (%), lot/batch no.	Results	Reference
Muta™Mouse gene mutation assay	Male CD2-LacZ80/HazfBR mice (Muta™Mouse)	100, 300 and 1 000 mg/kg bw (gavage for 28 consecutive days)	97.6, L87-58	Negative	Ueda (2017)

bw: body weight; *Hprt*: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction from rat liver homogenate; *Tk*: thymidine kinase

3. Observations in humans

Manufacturing plant personnel are monitored by regular medical examinations. There are no specific parameters available for monitoring the effects of pyraclostrobin. The surveillance programme includes a general physical examination, including neurological status, red and white blood cell counts and liver enzymes. Adverse health effects suspected to be related to pyraclostrobin exposure have not been observed (BASF, 2017).

Information on 33 persons exposed to pyraclostrobin was published in the Morbidity and Mortality Weekly Report series of the United States Centers for Disease Control and Prevention (Gergely & Calvert, 2008). In all but one incident, the exposure was to spray drift from aerial application. The most severe incident pertained to 27 workers who were exposed to off-target drift from a nearby field while removing tassels from corn plants (to prevent autopollination and enable hybridization). Some workers reported feeling wet droplets on their skin and seeing mist coming from the aircraft. All workers received skin decontamination on-site by a hazardous materials team before being transported to an emergency department for observation until their symptoms resolved.

The most common symptom was upper respiratory tract pain or irritation (26 patients), followed by chest pain (20 patients). Three patients had nausea, and one patient each had pruritis, skin redness, eye pain, weakness, headache, dizziness and chest pain. According to United States National Institute for Occupational Safety and Health classifications, low-severity illness or injury includes "illnesses manifested by skin, eye, or upper respiratory irritation". These illnesses might also include fever, headache, fatigue or dizziness. Typically, the illness or injury resolves without treatment, and time lost from work or normal activities is less than 3 days.

The other incidents pertained to single cases with off-target drift of pyraclostrobin from nearby aerial applications. The individuals were exposed by riding a motorcycle near a field or by spray drifting to their home yard. Symptoms reported were headache, eye pain partially associated with conjunctivitis and dizziness. The last case was from a crop-duster pilot who – when his plane crashed during take-off – was exposed to spilling of the liquid fungicide.

Comments

Biochemical aspects

Based on in vivo studies and in vitro incubations with rat serum or plasma, six biotransformation reactions were observed: 1) desmethoxylation of the side-chain, 2) hydroxylation of the chlorophenyl pyrazole ring system, 3) hydroxylation of the tolyl ring system, 4) cleavage of the ether bond, resulting in chlorophenyl pyrazole or anthranilic acid derivatives, 5) desmethylation of the side-chain and 6) cleavage of the amide bond in the side-chain. The combination of these reactions with subsequent conjugation resulted in a large number of metabolites (Birk, Lutz & Doebbe, 2014).

An in vitro comparison of metabolic profiles among rats, rabbits, dogs and humans shows the same key degradation steps in all species. The metabolites 500M04, 500M73, 500M108, 500M103, 500M104 and 500M88 were common to all test species. 500M02, 500M106 and 500M107, identified as the metabolites in humans, were also identified in other species used for toxicological testing (Funk, Glaessgen & Kalyon, 2014; Fabian & Landsiedel, 2016; Funk & Bellwon, 2016a,b).

Toxicological data

Additional data were made available on the histopathology of the 2-year toxicity and carcinogenicity studies in rats that had been evaluated by the 2003 Meeting (Mellert et al., 1999a,b), and two supplementary carcinogenicity studies not available to the 2003 Meeting (Mellert, 2002b,c) were submitted. The two new studies were conducted at concentrations (400 and 600 ppm, respectively) higher than those used in the first study. As these concentrations were above the MTD because of toxicity resulting in early termination, these studies provided no additional information relevant to the risk assessment.

Three new immunotoxicity studies were submitted, one of which was uninterpretable due to the inconsistent results in negative and positive controls.

In one immunotoxicity study, female mice were administered pyraclostrobin in the diet at 0, 50, 200 or 750 ppm (equal to 0, 13, 50 and 165 mg/kg bw per day, respectively) for 28 days. The NOAEL for immunotoxicity was 750 ppm (equal to 165 mg/kg bw per day), the highest dose tested. The NOAEL for systemic toxicity was 200 ppm (equal to 50 mg/kg bw per day), based on reduced body weight gain and feed consumption and reduced thymus and spleen weights at 750 ppm (equal to 165 mg/kg bw per day). The reduced spleen and thymus weights were considered to be secondary to the extreme reductions in body weight gain in mice receiving 750 ppm (Smiraldo, 2012a).

Another immunotoxicity study was conducted in female mice administered pyraclostrobin in the diet at 0, 50, 200 or 750 ppm (equal to 0, 14, 55 and 191 mg/kg bw per day, respectively) for 28 days. The NOAEL for immunotoxicity was 750 ppm (equal to 191 mg/kg bw per day), the highest dose tested. The NOAEL for systemic toxicity was 200 ppm (equal to 55 mg/kg bw per day), based on reduced body weight gain and feed consumption, decreased thymus and spleen weights, and low numbers of spleen cells at 750 ppm (equal to 191 mg/kg bw per day). The reduced spleen and thymus weights were considered secondary to the extreme reductions in body weight gain in mice receiving 750 ppm (Smiraldo, 2012c).

The Meeting concluded that pyraclostrobin is not immunotoxic.

A study of phototoxicity in vitro indicated that pyraclostrobin was not phototoxic (Cetto & Landsiedel, 2012, 2014).

Two repeated-dose inhalation studies in rats were submitted. In the first study, rats were exposed, through the head and nose inhalation route, to an aerosol of pyraclostrobin at 0, 1, 30 or 300 mg/m^3 for 6 hours per day, 5 days per week, for 4 weeks (Gamer et al., 2005). In the second study, rats were exposed, through the head and nose inhalation route, to an aerosol of pyraclostrobin at 0, 3, 10 or 30 mg/m^3 for 6 hours per day, 5 days per week, for 4 weeks (Ma-Hock et al., 2014). In both studies, there were local irritant effects leading to inflammation of the nasal tract at 30 mg/m^3. In addition, mucosal hyperplasia in the duodenum was observed at 30 mg/m^3 in the first study (NOAEC of 1 mg/m^3) and not seen in the second study at any tested concentration.

Additional studies indicated that the mucosal hyperplasia observed in mice, rats and dogs following repeated dietary exposure could be induced by reduced uptake of iron in the duodenum, resulting in lower serum iron levels, but not by local irritation (Mellert et al., 2003a,b; Srai, 2003). It is unlikely that this mode of action would be applicable to effects seen after a single dose (Appendix 3).

Toxicological data on metabolites and/or degradates

500M04

500M04 (pyrazolon) is a metabolite in rats, rabbits and humans. Its acute oral LD_{50} is greater than 2000 mg/kg bw (Kuehlem, 1997a). 500M04 was not irritating to the skin of rabbits (Kuehlem, 1997b), slightly irritating to the eye of rabbits (Kuehlem, 1997c) and not sensitizing in guinea-pigs (Kuehlem & Hellwig, 1997).

500M04 was tested for genotoxicity in an adequate range of in vitro and in vivo assays. It gave negative results for gene mutation (Schulz & Landsiedel, 2012a; Woitkowiak, 2012) and a positive response in an in vitro chromosomal aberration assay (Schulz & Landsiedel, 2012b), but it was negative in an in vivo micronucleus test (Schulz & Landsiedel, 2013a).

In a 3-month toxicity study in which rats were administered 500M04 by the dietary route 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 kidney effects at 300 mg/kg bw per day (Buesen et al., 2013). The Meeting noted that effects on the kidney were also identified with the parent compound in a short-term toxicity study in rats evaluated by the 2003 JMPR, but at higher doses (Annex 1, reference *100*). Other changes noted for the parent compound (e.g. reductions in body weight and feed consumption, effects on clinical chemistry parameters, liver hypertrophy and mucosal hypertrophy in the duodenum) were not observed for 500M04 in the 3-month toxicity study.

The Meeting concluded that the toxicity of 500M04 was lower than that of pyraclostrobin.

500M106

500M106 is a metabolite in rats, rabbits and humans. In a 28-day study in which rats were administered 500M106 by gavage at a dose of 0, 100, 300 or 1000 mg/kg bw per day, the NOAEL was 300 mg/kg bw per day, based on effects on the duodenum, liver, spleen and haematological system at 1000 mg/kg bw per day (Buesen et al., 2017). These effects were also observed for pyraclostrobin, but at much higher doses (reviewed by 2003 JMPR; Annex 1, reference *100*).

500M106 was tested for genotoxicity in an adequate range of in vitro and in vivo assays. Negative results were seen in an Ames test (Woitkowiak & Landsiedel, 2016) and an in vitro micronucleus assay (Chang, 2016b). In a forward mutation test, 500M106 was positive with S9 (Schulz & Landsiedel, 2016c). In two genotoxicity studies in vivo (micronucleus assay in mice and Muta™Mouse transgenic mouse model), 500M106 showed no genotoxicity (Dony, 2016; Ueda, 2017).

The Meeting concluded that the toxicity of 500M106 was similar to or lower than that of pyraclostrobin.

500M02

500M02 was tested for genotoxicity in an adequate range of in vitro assays. No evidence of genotoxicity was found (Engelhardt & Hoffmann, 1999; Chang, 2016a; Schulz & Landsiedel, 2016b; Woitkowiak, 2016).

The subchronic toxicity of 500M02 was considered to be tested in the 28-day rat study with 500M106 (Buesen et al., 2017), as 500M02 is metabolically formed from 500M106 to a substantial extent (~11.5% of the dose).

The Meeting concluded that, on the basis of its formation from 500M106 and the absence of genotoxicity, the toxicity of 500M02 was similar to or lower than that of pyraclostrobin.

500M24

500M24 was tested for genotoxicity in an adequate range of in vitro and in vivo assays. It gave negative results for gene mutation (Schulz & Landsiedel, 2014a; Woitkowiak, 2014) and a positive response in an in vitro chromosomal aberration assay (Schulz & Landsiedel, 2014b), but it was negative in an in vivo micronucleus test (Dony, 2014; Schulz et al., 2016a).

500M49

500M49 was tested for genotoxicity in an adequate range of in vitro assays. No evidence of genotoxicity was found (Woitkowiak, 2013a; Schulz & Landsiedel, 2014c,d).

500M51

500M51 was tested for genotoxicity in an adequate range of in vitro assays (Woitkowiak, 2013b; Schulz & Landsiedel, 2014e,f). No evidence of genotoxicity was found.

500M76

500M76 was tested for genotoxicity in an adequate range of in vitro and in vivo assays, producing mainly negative results (Engelhardt & Hoffmann, 2000; Engelhardt & Leibold, 2003; Schulz & Landsiedel, 2012c). It gave a positive response in an in vitro chromosomal aberration assay (Schulz & Landsiedel, 2013b, 2014b), but it was negative in an in vivo micronucleus test (Schulz & Landsiedel, 2012c).

500M07 (plant metabolite)

No toxicological information on 500M07 was submitted to the present Meeting; however, 500M07 is a rat metabolite found in serum and is formed early in the metabolic pathway.

Human data

Information on 33 cases of accidental exposure to pyraclostrobin was submitted (Gergely & Calvert, 2008). In almost all incidents, the exposure was to spray drift from aerial application. The most severe incident involved 27 subjects. Skin, eye and upper respiratory irritation were frequently reported.

Toxicological evaluation

The Meeting concluded that no revision of the ADI established by the 2003 Meeting was necessary.

The Meeting established a new ARfD of 0.7 mg/kg bw, based on the overall NOAEL of 5.8 mg/kg bw per day in 90-day and 1-year dog feeding studies (evaluated by the 2003 Meeting). A safety factor of 8 (2.5 for interspecies toxicodynamic differences, 3.2 for interindividual toxicodynamic differences) was applied. The previous ARfD was withdrawn.

Vomiting and diarrhoea seen during the first week of dosing of dogs with feed at 11 mg/kg bw per day (Annex 1, reference *100*) were identified as the critical effects. These critical effects are considered to be secondary to a direct, local effect of pyraclostrobin on the gastrointestinal tract, which is local concentration related and independent of absorption and metabolism (Appendix 2). Therefore, the default 100-fold safety factor was modified based on the scheme outlined by the International Programme on Chemical Safety (IPCS) on chemical-specific adjustment factors (IPCS, 2005) by removing the interindividual and interspecies toxicokinetic factors of 3.2 and 4, respectively.

The Meeting concluded that the effects secondary to local irritation following gavage dosing with pyraclostrobin were not relevant to human dietary risk assessment, and therefore the basis for the previously established ARfD was no longer applicable, as this was a gavage study in rabbits.

Acute reference dose (ARfD)

0.7 mg/kg bw

Critical end-points for setting guidance values for exposure to pyraclostrobin

Absorption, distribution, excretion and metabolism in mammals	
Metabolism	Desmethoxylation of the side-chain Hydroxylation of the chlorophenyl pyrazole ring system Hydroxylation of the tolyl ring system Cleavage of the ether bond, resulting in chlorophenyl pyrazole or anthranilic acid derivatives Desmethylation of the side-chain Cleavage of the amide bond in the side-chain
Toxicologically significant compounds in animals and plants	Pyraclostrobin
Other toxicological studies	
Immunotoxicity	No immunotoxicity
Phototoxicity	No phototoxicity
Studies on toxicologically relevant metabolites	
500M04 (pyrazolon)	Oral LD_{50} > 2 000 mg/kg bw (rats) 3-month oral toxicity study NOAEL 100 mg/kg bw per day (rats) No evidence of genotoxicity in vivo
500M24	No evidence of genotoxicity in vivo
500M49	No evidence of genotoxicity in vitro
500M51	No evidence of genotoxicity in vitro
500M76	No evidence of genotoxicity in vivo
500M02	No evidence of genotoxicity in vitro
500M106	Four-week oral toxicity study NOAEL 300 mg/kg bw per day (rats) No evidence of genotoxicity in vivo
Human data	
	Skin, eye or upper respiratory irritation

Summary

	Value	Study	Safety factor
ARfD	0.7 mg/kg bw	Ninety-day and 1-year feeding studies in dogs	8

References

BASF (2017). Pyraclostrobin (BAS 500 F). "Joint Meeting on Pesticide Residues" (JMPR). Toxicology: monograph. Unpublished report. BASF SE, Ludwigshafen/Rhein and Limburgerhof, Germany. BASF document ID 2017/1211. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Birk B, Lutz T, Doebbe A (2014). Further investigations of metabolites in rat plasma samples after dosing with ^{14}C-pyraclostrobin. Unpublished report. BASF SE, Limburgerhof, Germany. BASF document ID 2014/1315930. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Buesen R, Strauss V, Moreno MCR, Becker M, Van Ravenzwaay B (2013). Reg. No. 298327 (metabolite of BAS 500 F, pyraclostrobin) – repeated dose 90-day oral toxicity study in Wistar rats – administration via the diet. Unpublished report. BASF SE, Limburgerhof, Germany. BASF document ID 2013/1042164. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Buesen R, Strauss V, Moreno MCR, Becker M, Van Ravenzwaay B (2017). Reg. No. 399379 (metabolite of BAS 500 F, pyraclostrobin) – repeated-dose 28-day oral toxicity study in Wistar rats – administration by gavage. Unpublished report. BASF SE, Limburgerhof, Germany. BASF document ID 2017/1288407. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Cetto V, Landsiedel R (2012). BAS 500 F (pyraclostrobin) – in vitro 3T3 NRU phototoxicity test. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2012/1189936. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Cetto V, Landsiedel R (2014). Amendment no. 1 – BAS 500 F (pyraclostrobin) – in vitro 3T3 NRU phototoxicity test. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2014/1092412. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Chang S (2016a). Reg. No. 369315 (metabolite of BAS 500 F, pyraclostrobin): micronucleus test in human lymphocytes in vitro. Unpublished report. Envigo CRS GmbH, Rossdorf, Germany. BASF document ID 2016/1135652. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Chang S (2016b). Reg. No. 399379 (metabolite of BAS 500 F, pyraclostrobin) – micronucleus test in human lymphocytes in vitro. Unpublished report. Envigo CRS GmbH, Rossdorf, Germany. BASF document ID 2016/1109533. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Deckardt K, Van Ravenzwaay B (2003). In vitro hemolysis test with BAS 500 F (screening). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2003/1013685. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Dony E (2014). Reg. No. 5916421 (metabolite of BAS 500 F, pyraclostrobin): micronucleus assay in bone marrow cells of the mouse. Unpublished report. Harlan Cytotest Cell Research GmbH, Rossdorf, Germany. BASF document ID 2013/1389659. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Dony E (2016). Reg. No. 399379 (metabolite of BAS 500 F, pyraclostrobin), micronucleus assay in bone marrow cells of the mouse. Unpublished report. Envigo CRS GmbH, Rossdorf, Germany. BASF document ID 2016/1295116. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Engelhardt G, Hoffmann HD (1999). *Salmonella typhimurium*/*Escherichia coli* reverse mutation assay (Ames standard plate test and Prival preincubation test) with Reg. No. 369315. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 1999/10736. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Engelhardt G, Hoffmann HD (2000). *Salmonella typhimurium*/*Escherichia coli* reverse mutation assay (standard plate test and preincubation test) with Reg. No. 413038. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2000/1000005. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Engelhardt G, Leibold E (2003). In vitro gene mutation test with Reg. No. 413038 in CHO cells (*HPRT* locus assay). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2003/1004384. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Fabian E, Landsiedel R (2013). ^{14}C-Reg. No. 298327 (metabolite of BAS 500 F, pyraclostrobin) – study on the kinetics in mice. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2012/1278425. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Fabian E, Landsiedel R (2016). ^{14}C-Reg. No. 399379 (metabolite of BAS 500 F, pyraclostrobin) – study on kinetics in Wistar rats after oral administration. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2015/1241735. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Funk D, Bellwon P (2016a). Influence of WWL229 on the in-vitro metabolism of ^{14}C-BAS 500 F with human hepatocytes and human liver cytosol. Unpublished report. BASF SE, Limburgerhof, Germany. BASF document ID 2016/1225031. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Funk D, Bellwon P (2016b). Metabolisation of ^{14}C-BAS 500 F in rat serum. Unpublished report. BASF SE, Limburgerhof, Germany. BASF document ID 2016/1225032. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Funk D, Glaessgen WE, Kalyon B (2014). Comparative in-vitro metabolism with ^{14}C-BAS 500 F. Unpublished report. BASF SE, Limburgerhof, Germany. BASF document ID 2014/1001562. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Gamer AO, Deckardt K, Burkhardt S, Van Ravenzwaay B (2005). BAS 500 F – subacute inhalation study in Wistar rats – 20 aerosol exposures during 4 weeks. Unpublished report. BASF SE, Limburgerhof, Germany. BASF document ID 2005/1013950. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Gergely RM, Calvert GM (2008). Acute pesticide poisoning associated with pyraclostrobin fungicide – Iowa, 2007. MMWR Morb Mortal Wkly Rep. 56(51):1343–5 (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5651a3.htm, accessed 26 September 2018).

IPCS (2005). Chemical-specific adjustment factors for interspecies differences and human variability: guidance document for use of data in dose/concentration–response assessment. Genera: World Health Organization, International Programme on Chemical Safety (Harmonization Project Document No. 2; http://apps.who.int/iris/handle/10665/43294, accessed 26 September 2018).

Kuehlem C (1997a). Study on the acute oral toxicity of pyrazolon in rats. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 1997/10963. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Kuehlem C (1997b). Study on the acute dermal irritation/corrosion of pyrazolon in the rabbit. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 1997/10964. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Kuehlem C (1997c). Study on the acute eye irritation of pyrazolon in the rabbit. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 1997/10965. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Kuehlem C, Hellwig J (1997). Pyrazolon – maximization test in guinea pigs. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 1997/10968. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Ma-Hock L, Leibold E (2002). BAS 500 F – acute inhalation toxicity study in Wistar rats – 4-hour liquid aerosol exposure. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2002/1012053. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Ma-Hock L, Strauss V, Treumann S, Becker M, Van Ravenzwaay B (2014). BAS 500 F (pyraclostrobin) – repeated dose 28-day inhalation toxicity study with recovery period in Wistar rats, aerosol exposure. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2014/1003946. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W (2002a). Amendment no. 1: BAS 500 F – carcinogenicity study in Wistar rats. Administration in the diet for 24 months. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2002/1005114. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W (2002b). Summary of results – BAS 500 F – first supplementary carcinogenicity study in Wistar rats – administration in the diet for 24 months. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2002/1004123. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W (2002c). BAS 500 F – second supplementary carcinogenicity study in female Wistar rats – administration in the diet for 24 months. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2002/1004124. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W (2003a). Amendment no. 1 to the report: BAS 500 F – administration in the diet over 4 weeks with simultaneous subcutaneous administration of vitamin B_{12}. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2003/1014738. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W (2003b). Amendment no. 1 to the report: Screening study BAS 505 F – administration in the diet with simultaneous parenteral administration of iron to Wistar rats. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2003/1009197. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Kaufmann W (2004). Amendment no. 2 to the report: Screening study BAS 505 F – administration in the diet with simultaneous parenteral administration of iron to Wistar rats. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2004/1010601. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Beimborn DB, Van Ravenzwaay B (2003). BAS 500 F – determination of lipid peroxidation in the liver of male Wistar rats. Administration in the diet for 14 and 28 days. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2003/1009622. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Deckardt K, Gembardt C, Pappritz G, Hildebrand B (1999a). BAS 500F – chronic toxicity study in Wistar rats, administration in the diet for 24 months. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 99/11672. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany [cited in Annex 1, reference *100*].

Mellert W, Deckardt K, Gembardt C, Pappritz G, Hildebrand B (1999b). BAS 500F – carcinogenicity study in Wistar rats, administration in the diet for 24 months. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 99/11868. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany [cited in Annex 1, reference *100*].

Mellert W, Deckardt K, Kaufmann W, Leibold E (2002). Screening study BAS 505 F – administration in the diet with simultaneous parenteral administration of iron to Wistar rats. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2002/1013984. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Deckardt K, Gembart C, Van Ravenzwaay B (2003a). BAS 500 F – determination of iron in urine and serum of Wistar rats – administration in the diet over 14 days. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2003/1009534. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Deckardt K, Kuttler K, Hellwig J (2003b). BAS 500 F – administration in the diet over 4 weeks with simultaneous subcutaneous administration of vitamin B_{12}. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2003/1013463. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schilling K, Hellwig J, Hildebrand B (1999). BAS 500F – prenatal developmental toxicity study in Himalayan rabbits, oral administration (gavage). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 1999/11512. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany [cited in Annex 1, reference *100*].

Schulz M, Landsiedel R (2012a). Reg. No. 298327 (metabolite of BAS 500 F, pyraclostrobin) – in vitro gene mutation test in CHO cells (*HPRT* locus assay). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2012/1272482. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2012b). Reg. No. 298327 (metabolite of BAS 500 F, pyraclostrobin) – in vitro chromosome aberration assay in V79 cells. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2012/1185707. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2012c). Reg. No. 413038 (metabolite of BAS 500 F, pyraclostrobin) – micronucleus test in bone marrow cells of the mouse. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2012/1220183. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2013a). Reg. No. 298327 (metabolite of BAS 500 F, pyraclostrobin) – micronucleus test in bone marrow cells of the mouse. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2013/1026779. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2013b). Reg. No. 413038 (metabolite of BAS 500 F, pyraclostrobin) – in vitro chromosome aberration assay in V79 cells. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2012/1044766. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2014a). Reg. No. 5916421 (metabolite of BAS 500 F, pyraclostrobin) – in vitro gene mutation test in L5178Y mouse lymphoma cells ($TK^{+/-}$ locus assay, microwell version). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2013/1298449. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2014b). Reg. No. 5916421 (metabolite of BAS 500 F, pyraclostrobin) – in vitro micronucleus assay in V79 cells (cytokinesis block method). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2013/1363549. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2014c). Reg. No. 5916420 (metabolite of BAS 500 F, pyraclostrobin) – in vitro gene mutation test in L5178Y mouse lymphoma cells ($TK^{+/-}$ locus assay, microwell version). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2013/1298447. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2014d). Reg. No. 5916420 (metabolite of BAS 500 F, pyraclostrobin) – in vitro micronucleus assay in V79 cells (cytokinesis block method). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2013/1361921. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2014e). Reg. No. 78810 (metabolite of BAS 500 F, pyraclostrobin) – in vitro gene mutation test in L5178Y mouse lymphoma cells ($TK^{+/-}$ locus assay, microwell version). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2013/1298448. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2014f). Reg. No. 78810 (metabolite of BAS 500 F, pyraclostrobin) – in vitro micronucleus assay in V79 cells (cytokinesis block method). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2013/1361922. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2014g). Amendment no. 1 – Reg. No. 413038 (metabolite of BAS 500 F, pyraclostrobin) – in vitro chromosome aberration assay in V79 cells. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2014/1145892. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2016a). Amendment no. 1: Cytogenetic study in vivo with BAS 500 F in the mouse micronucleus test. Single oral administration. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2016/1309356. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2016b). Reg. No. 369315 (metabolite of BAS 500 F, pyraclostrobin) – in vitro gene mutation test in L5178Y mouse lymphoma cells (*TK* locus assay, microwell version). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2016/1225056. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2016c). Reg. No. 399379 (metabolite of BAS 500 F, pyraclostrobin) – in vitro gene mutation test in L5178Y lymphoma cells ($TK^{+/-}$ locus assay, microwell version). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2016/1171390. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Smiraldo PG (2012a). A 28-day oral (dietary) natural killer cell immunotoxicity study of BAS 500 F in female B6C3F1 mice. Unpublished report. WIL Research Laboratories Inc., Ashland, OH, USA. BASF document ID 2011/1035857. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Smiraldo PG (2012b). A 28-day oral (dietary) antibody forming cell immunotoxicity study of BAS 500 F in female B6C3F1 mice. Unpublished report. WIL Research Laboratories Inc., Ashland, OH, USA. BASF document ID 2012/1020986. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Smiraldo PG (2012c). A 28-day oral (dietary) antibody forming cell immunotoxicity study of BAS 500 F in female B6C3F1 mice. Unpublished report. WIL Research Laboratories Inc., Ashland, OH, USA. BASF document ID 2012/1084176. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Srai K (2003). BAS 505 F – study on the effect on the mucosal iron transport in female Wistar rats – administration in the diet. Unpublished report. Royal Free and University College Medical School, London, United Kingdom. BASF document ID 2003/1014047. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Thiaener J, Bellwon P (2016). Metabolism investigation of ^{14}C-Reg. No. 399379 (metabolite of BAS 500F, pyraclostrobin) in faeces and urine of male Wistar rats. Unpublished report. BASF SE, Limburgerhof, Germany. BASF document ID 2015/1198492. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Ueda M (2017). Reg. No. 399379 (metabolite of BAS 500 F, pyraclostrobin): transgenic mice (Muta™Mouse) gene mutation assay. Unpublished report. BSRC – Biosafety Research Center, Iwata Shizuoka, Japan. BASF document ID 2017/1002442. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Woitkowiak C (2012). Reg. No. 298327 (metabolite of BAS 500 F, pyraclostrobin) – *Salmonella typhimurium* / *Escherichia coli* reverse mutation assay. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2012/1220416. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Woitkowiak C (2013a). Reg. No. 5916420 (metabolite of BAS 500 F, pyraclostrobin) – *Salmonella typhimurium* / *Escherichia coli* reverse mutation assay. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2013/1281928. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Woitkowiak C (2013b). Reg. No. 78810 (metabolite of BAS 500 F, pyraclostrobin) – *Salmonella typhimurium* / *Escherichia coli* reverse mutation assay. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2013/1255749. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Woitkowiak C (2014). Reg. No. 5916421 (metabolite of BAS 500 F, pyraclostrobin) – *Salmonella typhimurium*/*Escherichia coli*, reverse mutation test. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2013/1323364. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

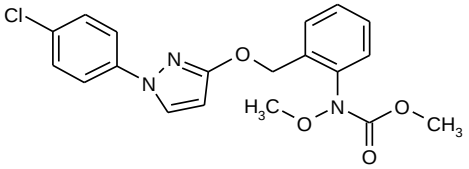
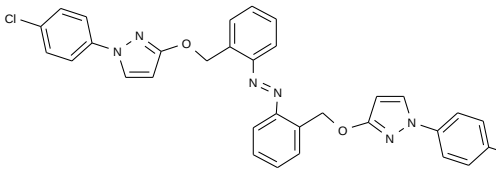
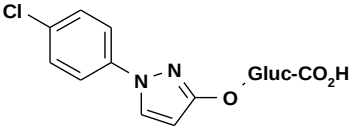
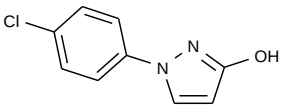
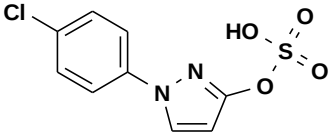
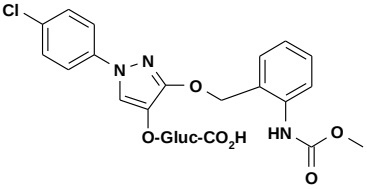
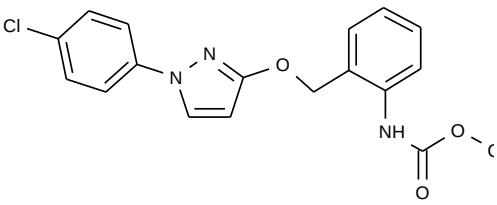
Woitkowiak C (2016). Reg. No. 369315 (metabolite of BAS 500 F, pyraclostrobin) – *Salmonella typhimurium*/*Escherichia coli* – reverse mutation assay (Prival modification). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2016/1200488. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

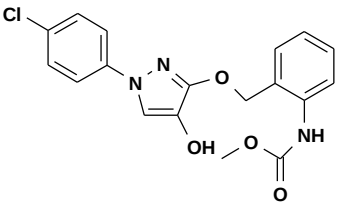
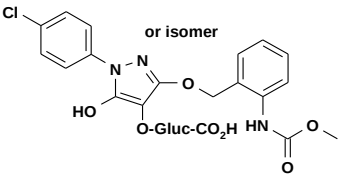
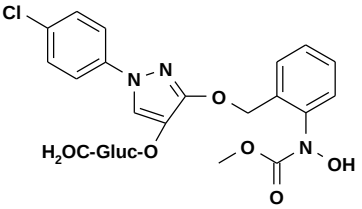
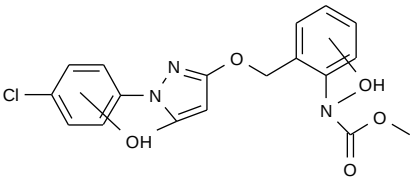
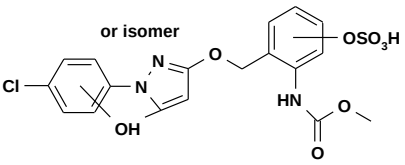
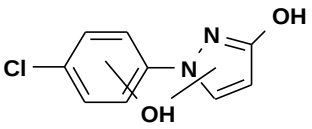
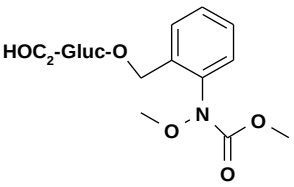
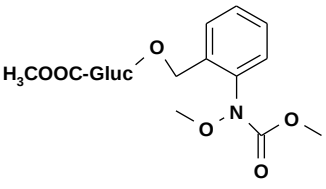
Woitkowiak C, Landsiedel R (2016). Reg. No. 399379 (metabolite of BAS 500 F, pyraclostrobin) – *Salmonella typhimurium* / *Escherichia coli* – reverse mutation assay. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2016/1119837. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

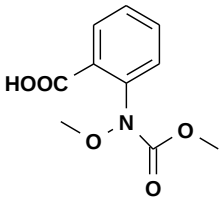
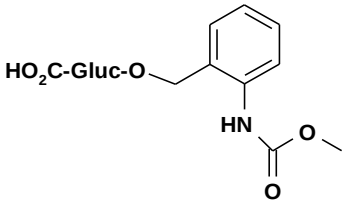
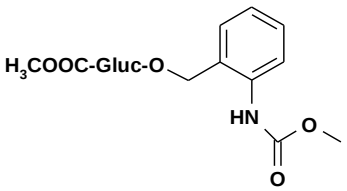
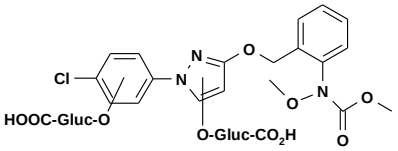
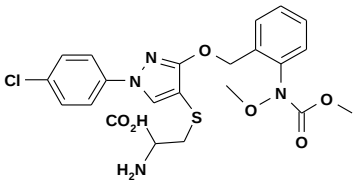
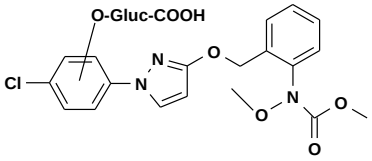
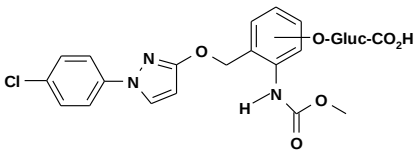
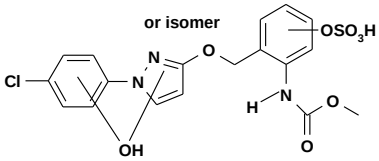
Appendix 1: List of metabolites of pyraclostrobin

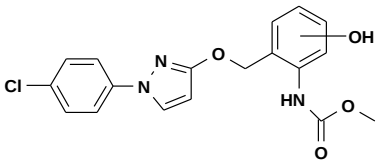
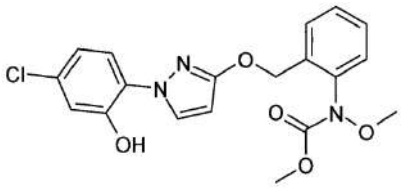
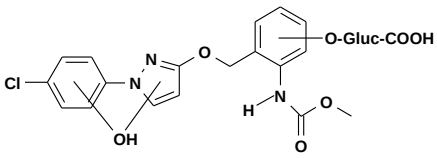
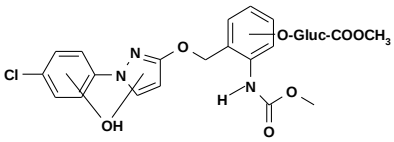
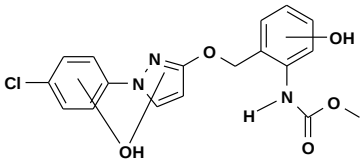
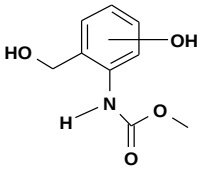
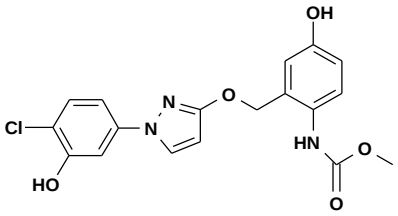
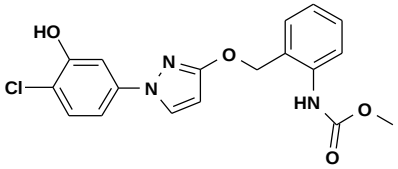
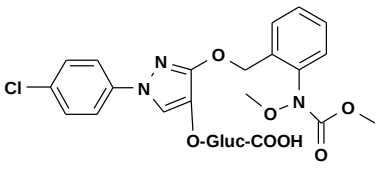
The metabolites of pyraclostrobin are identified in Table A1-1.

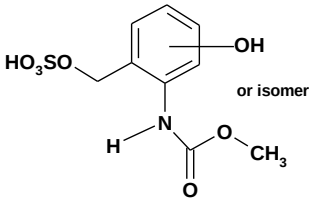
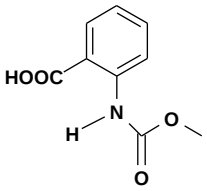
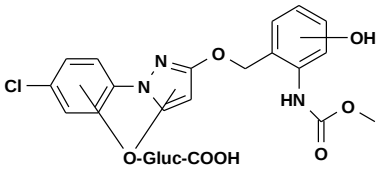
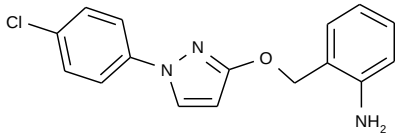
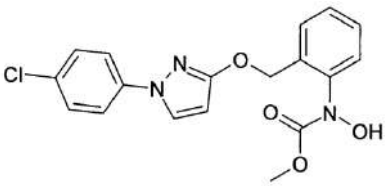
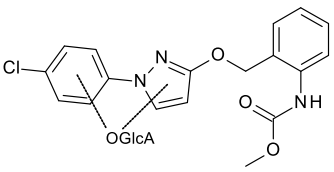
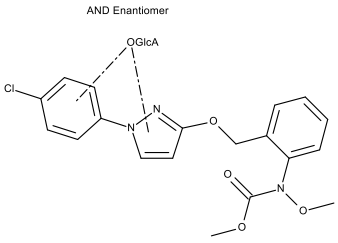
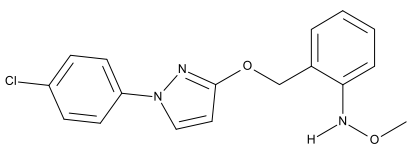
Table A1-1. Identification of metabolites of pyraclostrobin in animals and humans

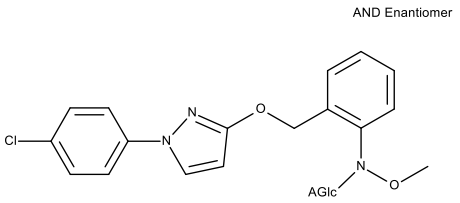
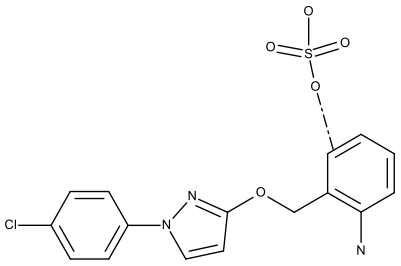
Metabolite code	Reg. no.	Synonyms	CAS no.	Compound found in:	Structure
BAS 500 F (pyraclostrobin)	304428	500M00	175013-18-0		
500M02	369315	BF 500-7	Not assigned	Human, dog, rabbit (in vitro)	
500M03	Not assigned	na	Not assigned	Rat Human, rabbit (in vitro)	
500M04	298327	BF 500-5	76205-19-1	Rat Human, rat, rabbit, dog (in vitro)	
500M05	Not assigned	na	Not assigned	Rat	
500M06	Not assigned	na	Not assigned	Rat (in vivo, plasma) Human, rat, rabbit (in vitro)	
500M07	340266	BF 500-3	512165-96-7	Rat	

Metabolite code	Reg. no.	Synonyms	CAS no.	Compound found in:	Structure
500M08	Not assigned	na	Not assigned	Rat (in vivo, plasma)	
500M13	Not assigned	na	Not assigned	Rat (in vivo, plasma)	
500M15	Not assigned	na	Not assigned	Rat (in vivo, plasma)	
500M18	Not assigned	na	Not assigned	Rat	
500M19	Not assigned	na	Not assigned	Rat	
500M21	Not assigned	na	Not assigned	Rat	
500M22	Not assigned	na	Not assigned	Rat	
500M23	Not assigned	na	Not assigned	Rat	

Metabolite code	Reg. no.	Synonyms	CAS no.	Compound found in:	Structure
500M24	5916421	na	Not assigned	Rat	
500M25	Not assigned	na	Not assigned	Rat	
500M26	Not assigned	na	Not assigned	Rat	
500M29	Not assigned	na	Not assigned	Rat (in vivo, plasma)	
500M30	Not assigned	na	Not assigned	Rat (in vivo, plasma)	
500M31	Not assigned	na	Not assigned	Rat (in vivo, plasma)	
500M32	Not assigned	na	Not assigned	Rat (in vivo, plasma)	
500M33	Not assigned	na	Not assigned	Rat	

Metabolite code	Reg. no.	Synonyms	CAS no.	Compound found in:	Structure
500M34	Not assigned	na	Not assigned	Rat (in vivo, plasma)	
500M35	412040	na	Not assigned	Rat (in vivo, plasma)	
500M37	Not assigned	na	Not assigned	Rat (in vivo, plasma)	
500M38	Not assigned	na	Not assigned	Rat (in vivo, plasma)	
500M39	Not assigned	na	Not assigned	Rat	
500M40	Not assigned	na	Not assigned	Rat	
500M44	Not assigned	na	Not assigned	Rat	
500M45	Not assigned	na	Not assigned	Rat (in vivo, plasma)	
500M46	Not assigned	na	Not assigned	Rat (in vivo, plasma)	

Metabolite code	Reg. no.	Synonyms	CAS no.	Compound found in:	Structure
500M48	Not assigned	na	Not assigned	Rat	
500M51	78810	na	6268-38-8	Rat	
500M52	Not assigned	na	Not assigned	Rat	
500M73	358672	BF 500-4	Not assigned	Human, rat, rabbit, dog (in vitro)	
500M88	322410	BF 500-1	220897-76-7	Human, rat, rabbit, dog (in vitro)	
500M103	Not assigned	na	Not assigned	Human, rat, rabbit (in vitro)	
500M104	Not assigned	na	Not assigned	Rat (in vivo, plasma) Human, rat, rabbit (in vitro)	
500M106	399379	na	Not assigned	Rat (in vivo, plasma) Human, rabbit (in vitro)	

Metabolite code	Reg. no.	Synonyms	CAS no.	Compound found in:	Structure
500M107	Not assigned	na	Not assigned	Rat (in vivo, plasma) Human, rabbit (in vitro)	
500M108	Not assigned	na	Not assigned	Rat (in vivo, plasma) Human, rat, rabbit (in vitro)	

CAS: Chemical Abstracts Service; na: not applicable; Reg. no.: registration number
Source: Funk, Glaessgen & Kalyon (2014)

Appendix 2: Summary of potential direct/secondary local effects induced by pyraclostrobin in toxicity studies

The potential direct or secondary local effects induced by pyraclostrobin in toxicity studies in mice, rats, rabbits and dogs are summarized in Table A2-1.

Table A2-1. Effects induced by pyraclostrobin in toxicity studies

Study (route)	Species	NOAEL for local effect	Local effect	Secondary effect to local effect	Reference
Skin irritation	Rabbits	–	Slightly irritating	–	Annex 1, reference *100*
Eye irritation	Rabbits	–	Slightly irritating	–	Annex 1, reference *100*
Three-month oral toxicity (feeding)	Mice	9.2 mg/kg bw per day	Erosion/ulcer of glandular stomach	–	Annex 1, reference *100*
Three-month oral toxicity (feeding)	Dogs	5.8/6.2 mg/kg bw per day	–	Vomiting and diarrhoea during first 2 weeks	Annex 1, reference *100*
Twelve-month oral toxicity (feeding)	Dogs	5.4 mg/kg bw per day	–	Vomiting and diarrhoea during first week	Annex 1, reference *100*
Four-week inhalation (inhalation, nasal)	Rats	10 mg/m^3 (overall)	Inflammation, necrosis, atrophy, regeneration in of nasal cavity, larynx and/or lung	–	Gamer et al. (2005); Ma-Hock et al. (2014)
Two-year carcinogenicity (feeding)	Rats	3.4 mg/kg bw per day	Erosion/ulcer of stomach	–	Annex 1, reference *100*
Developmental toxicity (gavage)	Rats	10 mg/kg bw per day (maternal)	–	Decreased feed consumption on GDs 6–8 (maternal)	Annex 1, reference *100*
Developmental toxicity (gavage)	Rabbits	3 mg/kg bw per day (maternal) 5 mg/kg bw per day (embryo/fetal)[a]	–	Lower body weight gain immediately after dosing (maternal) Implantation loss/reduced fetal weight (embryo/fetal)	Annex 1, reference *100*

ARfD: acute reference dose; bw: body weight; GD: gestation day; NOAEL: no-observed-adverse-effect level

[a] Point of departure for setting ARfD in 2003.

Appendix 3: Proposed mode of action for the induction of mucosal hyperplasia in the duodenum by strobilurin fungicides, including pyraclostrobin

The hypothesized mode of action (MOA) and relevance to humans for pyraclostrobin-induced mucosal hyperplasia in the duodenum is assessed using the International Programme on Chemical Safety (IPCS) framework for analysing the relevance of a non-cancer MOA for humans (IPCS, 2007).

Data on toxicity of pyraclostrobin, orysastrobin and dimoxystrobin

1. Occurrence of duodenal mucosal hyperplasia in studies on toxicity of pyraclostrobin

In subchronic oral (dietary admixture) studies on the toxicity of pyraclostrobin in mice, rats and dogs, duodenal mucosal thickening and hyperplasia were detected. The results of these studies are summarized in Table A3-1. The duodenal changes observed in the short-term studies were not accompanied by inflammatory or degenerative changes in the small intestines. The dose ranges of the long-term studies in mice and rats were lower than the NOAELs for this effect in the short-term studies, and no such changes or related neoplastic lesions were observed in these long-term studies (Annex 1, reference *100*).

Table A3-1. Histopathology of duodenal lesions in oral toxicity studies with pyraclostrobin[a]

Study/dose/finding	Males						Females					
91-day study in mice												
Dose (mg/kg bw per day)	0	9.2	30.4	119	274	476	0	12.9	40.4	162	374	635
Thickening of mucosa	0/10	0/10	0/10	10/10	10/10	10/10	0/10	0/10	0/10	6/10	10/10	9/10
18-month carcinogenicity study in mice												
Dose (mg/kg bw per day)	0	1.4	4.1	17.2			0	1.6	4.8	20.5	32.8	
Thickening of mucosa	0/10	0/10	0/10	0/10			0/10	0/10	0/10	0/10	0/10	
4-week study in rats												
Dose (mg/kg bw per day)	0	1.8	9.0	42	120		0	2.0	9.6	47	126	
Mucosal hyperplasia	–	–	–	P	P		–	–	–	P	P	
91-day study in rats												
Dose (mg/kg bw per day)	0	4	11	35	69	106	0	4	13	41	80	119
Mucosal hyperplasia	2/10	1/10	1/10	4/10	5/10	10/10	2/10	1/10	2/10	1/10	1/10	10/10
24-month carcinogenicity study in rats												
Dose (mg/kg bw per day)	0	1.2	3.4	9.2			0	1.5	4.6	12.6		
Mucosal hyperplasia	–	–	–	–			–	–	–	–		

Study/dose/finding	Males					Females				
3-month study in dogs										
Dose (mg/kg bw per day)	0	2.8	5.8	12.9		0	3.0	6.2	13.6	
Mucosal hypertrophy	0/5	0/5	0/5	2/5		0/5	0/5	0/5	1/5	
12-month study in dogs										
Dose (mg/kg bw per day)	0	2.7	5.4	10.8		0	2.7	5.4	11.2	
Mucosal hypertrophy	0/5	0/5	0/5	0/5		0/5	0/5	0/5	0/5	

–: no lesions; bw: body weight; P: present; the incidence was increased, but detailed information was not submitted for the 2018 JMPR

[a] Yellow cells show the increased incidence of duodenal lesions induced by pyraclostrobin.

Source: Annex 1, reference *100*

2. Relationship of iron deficiency anaemia with duodenal mucosal changes induced by pyraclostrobin treatment

Duodenal hyperplastic changes in rats and mice were accompanied by the typical iron deficiency hypochromic microcytic anaemia (decreased levels of haemoglobin, mean corpuscular volume, mean cell haemoglobin and/or mean cell haemoglobin concentration), although less clearly so at the lowest-observed-adverse-effect level (LOAEL), where anaemia was barely detectable (Annex 1, reference *100*) (Table A3-2). In fact, in the 90-day toxicity study in dogs, no finding indicating anaemia was observed at the doses at which mild duodenal changes were observed in 1–2/5 animals.

Serum iron concentrations and transferrin levels and urinary iron excretion were not examined in any standard toxicity studies in mice, rats or dogs.

Table A3-2. Anaemia and duodenal changes in oral toxicity studies with pyraclostrobin[a]

	Males						Females					
91-day study in mice												
Dose (mg/kg bw per day)	0	9.2	30.4	119	274	476	0	12.9	40.4	162	374	635
Haemoglobin (mmol/L)	11.8	11.6	11.6	11.4	11.4	10.6***	11.4	11.5	11.2	11.0	10.9**	10.4**
Haematocrit (L/L)	0.57	0.56	0.55	0.54*	0.54*	0.52*	0.52	0.53	0.52	0.52	0.51	0.50
MCV (10^{-15} L)	48.3	48.0	47.3	47.0*	46.7*	42.6*	46.4	46.7	46.3	46.8	46.0	42.6
MCH (10^{-15} mol/L)	0.99	0.99	1.00	1.00	0.98	0.87**	1.02	1.01	1.00	1.00	0.98*	0.90**
MCHC (mmol/L)	20.6	20.7	21.1	21.1	21.0	20.5	22.0	21.6	21.7	21.3***	21.2***	21.0***
Duodenal hyperplastic change	–	–	–	+	+	+	–	–	–	+	+	+

	Males						Females					
4-week study in rats												
Dose (mg/kg bw per day)	0	1.8	9.0	42	120		0	2.0	9.6	47	126	
RBC	–	–	–	–	–		–	–	–	↓	↓	
Haemoglobin	–	–	–	–	↓		–	–	–	↓	↓	
Duodenal hyperplastic change	–	–	–	+	+		–	–	–	+	+	
91-day study in rats												
Dose (mg/kg bw per day)	0	4	11	35	69	106	0	4	13	41	80	119
Erythrocytes (10^{12}/L)	8.5	8.5	8.8	8.6	8.4	8.2	8.0	7.9	8.0	7.7	7.4**	7.1**
Haemoglobin (mmol/L)	9.7	9.5	9.8	9.7	9.5	9.4	9.2	9.3	9.3	9.3	8.7*	8.6**
Haematocrit (L/L)	0.43	0.43	0.44	0.44	0.44	0.43	0.41	0.41	0.42	0.42	0.40	0.39
MCV (10^{-15} L)	50.6	50.1	50.2	51.3	52.3*	52.9*	51.5	52.0	52.1	53.9***	53.8***	54.8***
MCH (10^{-15} mol/L)	1.14	1.12	1.12	1.13	1.14	1.15	1.16	1.18	1.17	1.20**	1.19*	1.21**
MCHC (mmol/L)	22.5	22.3	22.3	21.9**	21.8**	21.7***	22.6	22.6	22.4	22.4	22.2***	22.2***
Duodenal hyperplastic change	–	–	–	+	+	+	–	–	–	–	–	+
3-month study in dogs												
Dose (mg/kg bw per day)	0	2.8	5.8	12.9			0	3.0	6.2	13.6		
Haematology	–	–	–	–			–	–	–	–		
Duodenal hyperplastic change	–	–	–	+			–	–	–	+		
12-month study in dogs												
Dose (mg/kg bw per day)	0	2.7	5.4	10.8			0	2.7	5.4	11.2		
Haematology	–	–	–	–			–	–	–	–		

–: no lesions; +: present; ↓: decrease (detailed information was not submitted for the 2018 JMPR); bw: body weight; MCH: mean cell haemoglobin; MCHC: mean cell haemoglobin concentration; MCV: mean corpuscular volume; RBC: red blood cells; *: $P \leq 0.05$; **: $P \leq 0.02$; ***: $P \leq 0.002$ (Kruskal-Wallis plus Mann-Whitney *U*-test)

[a] Yellow cells show the effects induced by pyraclostrobin.

Source: Annex 1, reference *100*

Although pyraclostrobin is a weak irritant, no mucosal damage to the duodenum, such as inflammation, erosion or ulcer, was observed in any of the toxicity studies in mice, rats and dogs. Therefore, the duodenal hyperplasia observed in toxicity studies is not considered a regenerative change

in response to mucosal damage, as is seen with a variety of cytotoxic and highly irritating chemical substances (Meek et al., 2003).

3. Effects on iron levels in serum and urine in rats

In a study conducted to determine the levels of iron in serum and urine after oral administration of pyraclostrobin to Wistar rats for 14 days in the diet at 0, 50, 500 or 1500 ppm (equal to 0, 3.8, 33.9 and 73.9 mg/kg bw per day for males and 0, 4.1, 37.4 and 78.3 mg/kg bw per day for females, respectively), serum iron concentrations were dose- and time-dependently decreased in both sexes at 500 and 1500 ppm, with up to a 50% decrease in males, and serum transferrin levels were decreased in males and females at 1500 ppm (Table A3-3). Iron levels in the urine were not affected by the treatment. This study suggested that pyraclostrobin-induced anaemia was related to lower absorption of iron from the gut, resulting in lower iron levels in serum (Mellert et al., 2003).

Table A3-3. Summary of changes in iron or transferrin in the serum by pyraclostrobin in rats

	Males				**Females**			
	0 ppm	**50 ppm**	**500 ppm**	**1 500 ppm**	**0 ppm**	**50 ppm**	**500 ppm**	**1 500 ppm**
Serum iron (μmol/L)								
Day 7	47.01	44.84	36.61	34.85*	59.07	60.09	46.48**	57.49
Day 14	54.54	46.71	37.72**	27.41**	53.61	58.31	45.55*	41.97**
Serum transferrin (g/L)								
Day 7	5.50	5.34	5.45	4.74**	5.40	5.23	5.62	4.92
Day 14	6.38	5.80	6.07	5.85	5.86	5.68	6.13	5.63

bw: body weight; *: $P \leq 0.05$; **: $P \leq 0.01$
Source: Mellert et al. (2003)

In a study in which orysastrobin, a strobilurin fungicide similar to pyraclostrobin (Fig. A3-1), was administered to male Wistar rats in feed at a concentration of 0, 10, 100 or 2500 ppm (equal to 0, 0.7, 7.4 and 143 mg/kg bw per day, respectively) for 14 days, decreases in serum iron and transferrin levels and an increase in the weight of the duodenum were observed in the rats at 2500 ppm. No further details were provided (FSCJ, 2005).

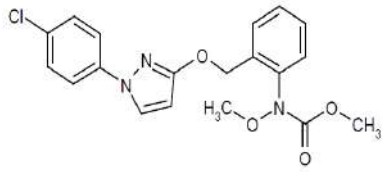
4. Mucosal proliferative lesions in duodenum in rats treated with orysastrobin

Data on other strobilurin fungicides were added to the present analysis to provide support for the findings on pyraclostrobin.

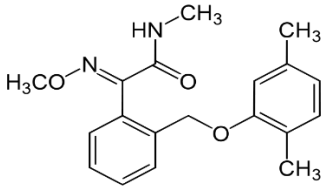
It has been reported that orysastrobin, another strobilurin fungicide, induces duodenal changes similar to those induced by pyraclostrobin (FSCJ, 2005; Van Ravenzwaay et al., 2007). The mucosal hyperplasia was observed in both sexes of mice, rats and dogs (Table A3-4). In rats exposed to the carcinogenic dose of 2500 ppm orysastrobin, increased epithelial cell proliferation was detected in the duodenal mucosa after 4 weeks, and decreased serum iron levels, increased serum transferrin levels and unsaturated iron capacity were seen after 1 week. Most of these changes are reversible; however, some hyperplastic lesions progressed to tumours in the duodenum of rats and mice. The carcinogenic effect in the duodenum was presumably caused by an attempt to increase iron absorption from the gastrointestinal tract in order to compensate for the markedly decreased serum iron levels. The reversible mechanism of action with a clear threshold dose and the absence of mutagenic potential in vivo were reported (Van Ravenzwaay et al., 2007).

Fig. A3-1. Chemical structures of pyraclostrobin, orysastrobin and dimoxystrobin

Pyraclostrobin



Dimoxystrobin



Orysastrobin

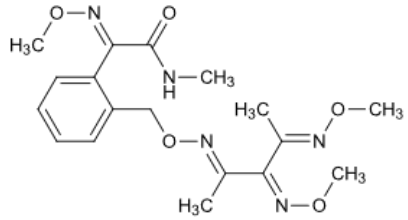


Table A3-4. Histopathology of duodenal lesions in oral toxicity studies of orysastrobin

Species	Duration	Dietary concentrations (ppm)	NOAEL for duodenal lesions (ppm)	Findings
Rats	90 days	0, 300, 1 000, 3 000, 5 000	<300	Thickening of duodenal mucosa
	24 months	0, 100, 500, 2 500	100	Thickening of duodenal mucosa
			500	Carcinoma and/or adenoma in duodenum
Mice	18 months	0, 100, 500, 2 000	500	Thickening of duodenal mucosa
			100	Focal hyperplasia in pylorus (at the transition of glandular stomach to duodenum)
			500	Adenocarcinoma in duodenum
Rat (mechanistic study)	4 weeks	0, 10, 100, 2 500	100	Increased epithelial cell proliferation activity (BrdU immunohistochemistry)
	2 weeks	0, 10, 100, 2 500	100	Decreased serum iron and increased serum transferrin and unsaturated iron capacity

BrdU: 5-bromo-2′-deoxyuridine; NOAEL: no-observed-adverse-effect level; ppm: parts per million
Source: Modified Tables 2 and 3 in Van Ravenzwaay et al. (2007)

Orysastrobin increased the incidences of tumours in the duodenum in rats and mice at the highest dose, suggesting a carcinogenic potential by long-term and high-dose exposure to strobilurin fungicides that induce duodenal hyperplasia. Duodenal hyperplasias following treatment with pyraclostrobin were observed in short-term studies, but no increases in hyperplasia or tumours were observed in long-term studies in rats and mice. The highest doses (200 ppm in rats; 180 ppm in mice) in the carcinogenicity studies were lower than those in the short-term studies in rats and mice. The doses, however, were considered to be acceptable for the evaluation of carcinogenicity, because long-term treatment with pyraclostrobin at 360 ppm in mice seriously depressed their body weights, indicating that the dose exceeded the MTD. Although strobilurin fungicides have the potential to promote the formation of duodenal tumours from mucosal hyperplasia due to continuous stimulation by negative feedback in response to the low iron level in the serum in rodents (FSCJ, 2005), long-term treatment with pyraclostrobin is not concluded to be carcinogenic in the duodenum.

5. Cell proliferation activity

Cell proliferation activity in the duodenum in mice and rats was studied after administration of orysastrobin (see Fig. A3-1 above), a strobilurin fungicide similar to pyraclostrobin.

In a 4-week study in which male C57BL/6J Rj mice were fed orysastrobin at a concentration of 0, 10, 100 or 2000 ppm (equal to 0, 1.9, 20.9 and 437 mg/kg bw per day, respectively), cell proliferation activity in the duodenum was increased at 2000 ppm. The increase showed a recovery after the diet was withdrawn for 2 weeks. No further details were provided (FSCJ, 2005).

In a 4-week study in which male Wistar rats were fed orysastrobin at a concentration of 0, 10, 100 or 2500 ppm (equal to 0, 0.6, 6.1 and 148 mg/kg bw per day, respectively), cell proliferation activity in the duodenum was increased at 2500 ppm. The increase showed a recovery after the diet was withdrawn for 2 weeks. No further details were provided (FSCJ, 2005).

No such studies have been conducted with pyraclostrobin.

6. Effect of dimoxystrobin on iron absorption and transport in the duodenum

In a study investigating the effect of dimoxystrobin, a compound similar to pyraclostrobin, on the mucosal uptake and transfer of iron into the carcass after oral administration in rats treated with dimoxystrobin at 0 or 4500 ppm (equivalent to 450 mg/kg bw per day) in the diet for 24, 96 or 168 hours, the everted duodenums were incubated to measure Fe^{2+} uptake in the duodenal segments. Iron transfer across the serosal membrane (i.e. iron transfer into the body) was measured using tied-off duodenal segments in rats exposed to 4500 ppm dimoxystrobin in the feed for 24 or 96 hours (Srai, 2003).

Mucosal iron uptake showed a statistically significant reduction after treatment with dimoxystrobin for 96 and 168 hours. The autoradiographic data confirmed the reduction of iron uptake. The data show the decrease in density of silver grains along the villus length, suggesting the reduction in uptake by the treatment.

Iron transfer across the serosal membrane (i.e. iron transfer into the body) was significantly reduced after a 96-hour treatment with dimoxystrobin, with the reduction of iron transfer being proportional to the reduction in uptake. After 24 hours of treatment, no significant effect was found. The results are summarized in Table A3-5.

In summary, the results clearly demonstrated that repeated treatment of rats with dimoxystrobin considerably reduced both uptake of iron by the duodenal mucosa and transfer of iron into the body (Srai, 2003).

Table A3-5. Iron transfer across the serosal membranes in the duodenum of dimoxystrobin-treated rats

Incubation times with iron	Treatment period with dimoxystrobin	Group	Mucosal retention[a]	Mucosal transfer[a]	Total mucosal uptake[a]
10 minutes	24 hours	Control	16.6 ± 3.7[b]	29 ± 10.2	45.6 ± 12.3
		Treated	17.8 ± 5.1	15.3 ± 5*	33.1 ± 8.9
	96 hours	Control	41.1 ± 18.5	10.6 ± 3.8	51.7 ± 21
		Treated	13.4 ± 9.5*	4.6 ± 2.1*	18 ± 9.4*
20 minutes	24 hours	Control	8.7 ± 3.5	12.6 ± 7.9	21.3 ± 10.4
		Treated	10.3 ± 2.2	13.3 ± 3.6	23.6 ± 5.6
	96 hours	Control	34.5 ± 9.7	37.7 ± 5.3	72.2 ± 11.7
		Treated	12.4 ± 2.4*	5.7 ± 2**	18.1 ± 2.6**
40 minutes	24 hours	Control	42.8 ± 10.5	33.6 ± 11.1	76.3 ± 13.8
		Treated	38.4 ± 4.9	20.9 ± 10.4	59.2 ± 13.3
	96 hours	Control	27.1 ± 10.8	38.9 ± 13.6	66.1 ± 15.8
		Treated	36.2 ± 17.7	17.5 ± 8.7*	53.7 ± 24.9

*: $P < 0.05$; **: $P < 0.01$

[a] Radioactivity of ^{59}Fe as measured by gamma counting in the intestinal tissue or in the carcass was referred to as mucosal retention or mucosal transfer, respectively. The sum of mucosal retention and mucosal transfer represents total mucosal uptake.

[b] Mean ± standard deviation.

Source: Srai (2003)

7. Combination study of dimoxystrobin with trivalent iron

Dimoxystrobin was administered to groups of 10 male Wistar rats at a dietary concentration of 0 or 4500 ppm (equal to 0, 206.6 and 171.2 mg/kg bw per day for controls, 4500 ppm or 4500 ppm plus iron complex group, respectively) over a period of 14 days and to groups of 10 female Wistar rats at a dietary concentration of 0, 500 or 4500 ppm (equal to 0, 37.7, 17.7, 191.3 and 84.7 mg/kg bw per day for controls, 500 ppm, 500 ppm plus iron complex, 4500 ppm and 4500 ppm plus iron complex, respectively) over a period of 7 days. Simultaneously, additional groups received an iron complex (Myofer® 100) intramuscularly (males once daily at a dose of 100 mg/kg bw on study days 0, 7, 11 and 13, and females twice daily at a dose of 50 mg/kg bw from day 2 to day 6).

Iron levels in the serum were increased in all groups receiving the iron complex (with and without dimoxystrobin) and reduced in the groups receiving dimoxystrobin only. Duodenal weights were statistically significantly increased in the 500 ppm (females) and 4500 ppm (both sexes) groups. In the iron complex groups at 500 and 4500 ppm (both sexes), the increase in duodenal weights was lower than in the corresponding dimoxystrobin groups. The increases in duodenal weights in the iron complex groups were not statistically significant in females compared with controls. Although the duodenal weights did not completely reach the control values, these data indicate that iron administered to animals treated with dimoxystrobin had an inhibitory effect on the increase in duodenal weights, in the sense of preventing effects on the duodenum that are caused by a reduction in serum iron levels following treatment with dimoxystrobin.

In immunohistochemical staining with proliferative cell nuclear antigen (PCNA), the number of positive cells was increased by approximately 200% at 4500 ppm with or without the iron complex in males. In females, the number of positive cells was increased by approximately 150% at 500 ppm with or without the iron complex and at 4500 ppm without the iron complex. The number of positive

cells at 4500 ppm with the iron complex was reduced and similar to that in the control group with the iron complex.

In conclusion, the administration of an iron complex can reduce the dimoxystrobin-induced increase in duodenal weights. Dimoxystrobin-induced iron deficiency might therefore be a causative factor for increased duodenal weights (Mellert et al., 2002; Mellert, 2003; Mellert & Kaufmann, 2004).

Postulated MOA for pyraclostrobin-induced mucosal hyperplasia of the duodenum in rodents and dogs

The data for pyraclostrobin support an MOA involving the following key events:

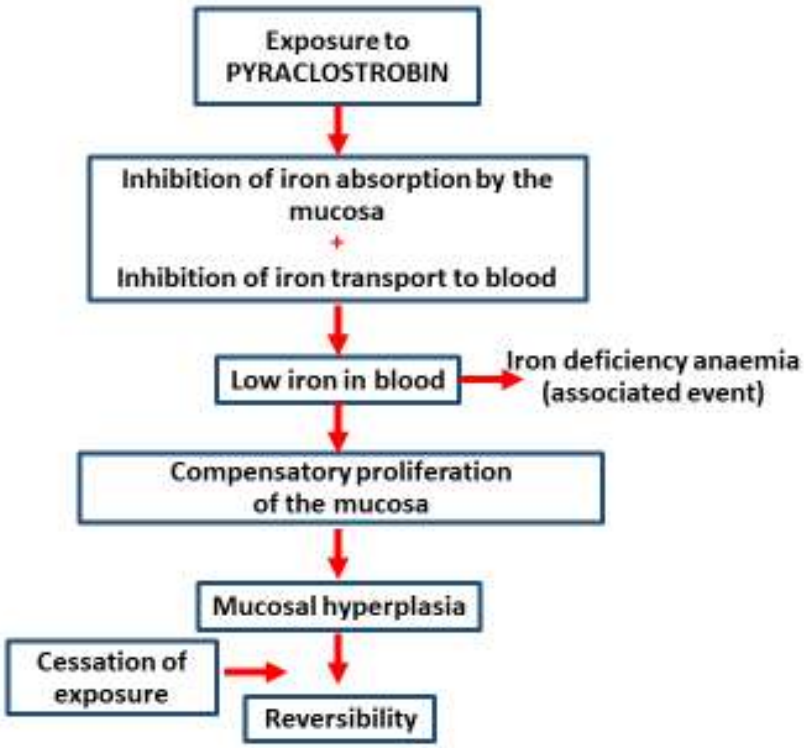
- inhibition of iron absorption from mucosa and iron transport to the circulatory system in the duodenum;
- iron deficiency in blood;
- increased cell proliferation activity in the mucosa of the duodenum as a compensatory response to the iron-deficient status in blood;
- reversible mucosal hyperplasia in the duodenum upon cessation of exposure;

and the following associated event:

- iron deficiency anaemia.

A diagrammatic representation of the postulated MOA is given in Fig. A3-2.

Fig. A3-2. Proposed MOA for the induction of mucosal hyperplasia in the duodenum by pyraclostrobin



Dose–response relationship and concordance

Key event: inhibition of iron absorption and transfer

In the report on inhibition of iron absorption and transfer, there was no description of a dose–response relationship because the study was conducted using a single-dose treatment with dimoxystrobin, a compound similar to pyraclostrobin (Srai, 2003).

Key event: Serum iron levels (see Table A3-3)

In rats treated with pyraclostrobin at 0, 50, 500 or 1500 ppm for 14 days in the diet, the NOAEL for the decrease in iron level in the serum was 50 ppm (equal to 3.8 mg/kg bw per day), with a LOAEL of 500 ppm (equal to 33.9 mg/kg bw per day) (Mellert et al., 2003).

Key event: Mucosal hyperplasia (see Table A3-1)

Mucosal hyperplasia following exposure to pyraclostrobin showed clear dose dependency in mice, rats and dogs. The NOAEL for mucosal hyperplasia in mice was 30.4 mg/kg bw per day, with a LOAEL of 119 mg/kg bw per day, in a 91-day oral toxicity study. The NOAEL for mucosal hyperplasia in rats was 11 mg/kg bw per day, with a LOAEL of 35 mg/kg bw per day, in a 90-day oral toxicity study. The NOAEL for mucosal hyperplasia in dogs was 5.8 mg/kg bw per day, with a LOAEL of 12.9 mg/kg bw per day, in a 90-day study. Long-term studies conducted in mice and rats were conducted at lower doses than the NOAELs for the 90-day studies, and no mucosal hyperplasia was observed in either study (Annex 1, reference *100*).

Key event: Cell proliferation activity

No data on cell proliferation activity in animals treated with pyraclostrobin were obtained.

In male mice treated with orysastrobin, a strobilurin fungicide similar to pyraclostrobin, at a dietary concentration of 0, 10, 100 or 2000 ppm (equal to 0, 1.9, 20.9 and 437 mg/kg bw per day, respectively) for 4 weeks, the NOAEL for cell proliferation activity in the duodenum was 100 ppm, with a LOAEL of 2000 ppm. The increase showed a recovery after the diet was withdrawn for 2 weeks (FSCJ, 2005).

In male rats treated with orysastrobin at a dietary concentration of 0, 10, 100 or 2500 ppm (equal to 0, 0.6, 6.1 and 148 mg/kg bw per day, respectively) for 4 weeks, the NOAEL for cell proliferation activity in the duodenum was 100 ppm, with a LOAEL of 2500 ppm. The increase showed a recovery after the diet was withdrawn for 2 weeks (FSCJ, 2005).

Associated event: Anaemia (see Table A3-2)

In a 3-month study in mice, the NOAEL for haematological effects was 30.4 mg/kg bw per day, with a LOAEL of 119 mg/kg bw per day. In a 4-week study in rats, the NOAEL for haematological effects was 9.6 mg/kg bw per day, with a LOAEL of 47 mg/kg bw per day. In a 3-month study in rats, the NOAEL for haematological effects was 11 mg/kg bw per day, with a LOAEL of 35 mg/kg bw per day. No haematological effects were observed in dogs (Annex 1, reference *100*).

The dose concordance of key and associated events from studies with pyraclostrobin or other strobilurin fungicides in mice, rats and dogs is summarized in Table A3-6.

Table A3-6. Dose concordance of key and associated events in the MOA for the induction of mucosal hyperplasia in the duodenum in mice, rats and dogs by pyraclostrobin

	NOAEL (mg/kg bw per day)	LOAEL (mg/kg bw per day)
Key events		
Decreased serum iron level		
Rat 14-day	3.8	33.9
Mucosal hyperplasia		
Mouse 3-month	30.4	119
Rat 4-week	9.0	42
Rat 3-month	11	35
Dog 3-month	5.8	12.9
Associated event		
Anaemia		
Mouse 3-month	30.4	119
Rat 4-week	9.6	47
Rat 3-month	11	35
Dog 3-month	12.9	–[a]

bw: body weight; LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level
[a] No findings indicating anaemia.

Temporal relationship

The temporal relationships among each key or associated event and pyraclostrobin treatment are summarized in Table A3-7.

Table A3-7. Temporal relationships of key and associated events for the induction of mucosal hyperplasia of the duodenum by pyraclostrobin or other strobilurin fungicides in mice, rats and dogs

	Within 96 hours	7 days	14 days	4 weeks	3 months
Key event: inhibition of iron absorption or transport					
Dimoxystrobin[a]					
Inhibition of absorption/transfer of iron in the duodenum	YES				
Key event: low iron in blood					
Pyraclostrobin					
Decreased iron and transferrin levels in serum in rats		YES	YES		
Orysastrobin					
Decreased serum iron level in rats			YES		
Key events: mucosal hyperplasia and cell proliferation					
Pyraclostrobin					
Mucosal hyperplasia					
Mouse					YES

	Within 96 hours	7 days	14 days	4 weeks	3 months
Rat				YES	YES
Dog					YES
Orysastrobin					
Cell proliferation activity in the duodenum					
Mice				YES	
Rats				YES	
Associated event					
Pyraclostrobin					
Anaemia					
Mouse					YES
Rat				YES	YES
Dog					

[a] See structure in Fig. A3-1 above.

Strength, consistency and specificity of association of the duodenum response with key events

The key and associated events occurred (Table A3-6) at increasing doses, in the order shown in the proposed MOA (Fig. A3-2). In fact, the dose–response relationships of the key events assessed for pyraclostrobin were consistent with the proposed sequence in the MOA.

The key events (Table A3-7) also indicated a clear, well-matched temporal relationship with the duodenum response in each step of the proposed MOA (Fig. A3-2), because the inhibitions of the absorption and transfer of iron in the duodenum, the initial event, occurred very quickly (within 96 hours), followed by the low iron levels in the serum after 7 days, the increased cell proliferation activity in the duodenum after 4 weeks and the mucosal hyperplasia in the duodenum after 4 weeks or 3 months. Most MOA studies were conducted using rats. However, from the available data, it can be concluded that there was no remarkable temporal difference in the events leading to mucosal hyperplasia in mice, rats or dogs.

Although the data on inhibition of the absorption and transfer of iron were obtained from studies using dimoxystrobin (Srai, 2003) only, given the similar toxicological profiles of the strobilurins, including the decrease in serum iron (Mellert et al., 2002) and mucosal cell proliferation following exposure to orysastrobin (Mellert & Kaufmann, 2004), they can be applied, at least qualitatively, to pyraclostrobin.

Biological plausibility

1. Occurrence of duodenal mucosal lesions in iron deficiency in rats

A study in which rats were treated with an iron-deficient diet has shown that this treatment results in duodenal changes similar to those observed with pyraclostrobin. Administration of an iron-deficient diet to Wistar rats resulted within 14 days in reduced serum iron concentrations, hypochromic microcytic anaemia, and an increase in duodenal epithelial cell proliferation (Cunha et al., 2008). After 5 weeks of iron deficiency, hypochromic microcytic anaemia and a clear increase in duodenal weight but no pronounced effects on cell proliferation were observed. Increased duodenal weights corresponded to significant increases in mucosal area, indicating a diffuse, simple mucosal hyperplasia. The sequence of events following iron depletion thus appears to be: 1) reduced serum iron levels, 2) induction of hypochromic microcytic anaemia, 3) increased duodenal epithelial cell proliferation and 4) increased duodenal weight (increased mucosal area). Iron deficiency anaemia was reversible after a

2-week recovery period, while duodenal weights were still increased. Intramuscular iron supplementation in animals fed with iron-deficient diet resulted in the maintenance of body iron levels at normal values, and neither anaemia nor increased duodenal cell proliferation was detected after 14 days. Thus, increased duodenal mucosal hyperplasia was shown to be secondary to depletion of body iron and consequent anaemia and possibly reflects an attempt to increase iron absorption to counteract iron deficiency.

2. Effect of dimoxystrobin on iron absorption and transport in the duodenum

In a study investigating the effect of dimoxystrobin, a compound similar to pyraclostrobin, on the mucosal uptake and transfer of iron into the carcass after oral administration in rats treated with dimoxystrobin at 0 or 4500 ppm (equivalent to 450 mg/kg bw per day) in the diet for 24, 96 or 168 hours, the everted duodenums were incubated to measure Fe^{2+} uptake in the duodenal segments. Iron transfer across the serosal membrane (i.e. iron transfer into the body) was measured using tied-off duodenal segments in rats exposed to 4500 ppm dimoxystrobin in the feed for 24 or 96 hours (Srai, 2003).

Mucosal iron uptake showed a statistically significant reduction after treatment with dimoxystrobin for 96 and 168 hours. The autoradiographic data confirmed the reduction of iron uptake. The data show the decrease in density of silver grains along the villus length, suggesting the reduction in uptake by the treatment.

Iron transfer across the serosal membrane (i.e. iron transfer into the body) was significantly reduced after a 96-hour treatment with dimoxystrobin, with the reduction of iron transfer being proportional to the reduction in uptake.

In summary, the results clearly demonstrated that repeated treatment of rats with dimoxystrobin considerably reduced both uptake of iron by the duodenal mucosa and transfer of iron into the body (Srai, 2003).

On the basis of the chemical similarity of dimoxystrobin to pyraclostrobin, inhibition of both the absorption and the transfer of iron in the duodenum likely occurred as a result of pyraclostrobin treatment.

Alternative MOA hypotheses

1. Mucosal hyperplasia as a consequence of cytotoxicity and regenerative proliferation

High concentrations of hexavalent chromium (Cr(VI)), captan and folpet induced duodenal tumours in mice (Thompson et al., 2017) as a consequence of cytotoxicity-induced regenerative proliferation.

When B6C3F1 mice were exposed to 180 ppm Cr(VI) in their drinking-water, 12 000 ppm captan in their feed or 16 000 ppm folpet in their feed for 28 days, villous enterocyte hypertrophy and mild crypt epithelial hyperplasia were observed in all exposed mice.

Folpet is not genotoxic in vivo and causes duodenal glandular tumours in mice, but not in rats. Folpet reacts with thiol groups and is rapidly hydrolysed at pH 7. Both reactions produce thiophosgene, which reacts with thiols and other functional groups. At sufficiently high, prolonged dietary doses, folpet irritates the mouse duodenum, resulting in cytotoxicity, with consequent regenerative proliferation and ultimately tumour development (Gordon, Cohen & Singh, 2012). Based on MOA analysis and assessment of human relevance, folpet, like captan, another trichloromethylthio-related fungicide with similar toxic and carcinogenic effects, is not likely to be a human carcinogen at doses that do not cause cytotoxicity and regenerative proliferation (Cohen et al., 2010).

The sequence of events involving cytotoxicity and regenerative proliferation is a common MOA for tumorigenesis by non–DNA-reactive chemicals (Meek et al., 2003).

This MOA should be excluded for pyroclostrobin because of the following:

- This MOA appears applicable to mice only, whereas duodenal hyperplasia induced by strobilurin fungicides was found in several species.
- Common histopathological findings in this MOA are inflammation or degenerative change, which have not been observed with strobilurin fungicides.

2. Brunner's gland hyperplasia induced by vascular endothelial growth factor

Vascular endothelial growth factor receptor tyrosine kinase inhibitors are reported to cause reversible mucosal hyperplasia (adenosis) in the duodenum of rats (Inomata et al., 2014). At 4 weeks, there was degeneration and necrosis of Brunner's gland epithelium accompanied by neutrophil infiltration around the affected glands. The main function of Brunner's gland is to protect the duodenal mucosa from the acidic gastric contents and abrasion of the intestinal mucosa by the passage of rough ingesta. At 13 weeks, the inflammation was more extensive and followed by reactive hyperplasia of the duodenal epithelium. Similar changes were not present in similar time-course studies in dogs and monkeys, suggesting that this is a rodent- or species-specific change (Inomata et al., 2014).

This MOA should be excluded because the target is Brunner's gland and because of the morphological findings (adenosis, which is observed in the muscular layer to the serosa).

3. Direct damage to DNA in the duodenal epithelium

Pyraclostrobin is not genotoxic in vitro or in vivo. Although no data were provided on DNA damage to the duodenum by the treatment, there is no reason to conclude that pyraclostrobin causes direct damage to DNA in the duodenal epithelium. Some genotoxic carcinogens, such as methylnitronitrosoguanidine, are known to induce tumours in various areas of the gastrointestinal tract in experimental animals, but there are no reports on induction of tumours in the duodenum only.

This MOA should be excluded because pyraclostrobin does not exhibit genotoxicity. There is no evidence of tumour induction in the duodenum only by genotoxic carcinogens.

Uncertainty, inconsistency or data gaps

The data for the present analysis were obtained not only from studies on pyraclostrobin, but also from studies on similar strobilurin fungicides – specifically, dimoxystrobin and orysastrobin. Several key events were evaluated based on the studies on these other strobilurin fungicides. Therefore, there are uncertainties or data gaps related to the lack of studies on pyraclostrobin on the basis of which to evaluate some of the key events. Although the level of inhibitory activity of iron absorption and transfer varied depending on the compound, the comparison of data on duodenal hyperplasia induced by several strobilurin fungicides contributes to the consistency of this MOA.

Human relevance of the proposed MOA

1. Is the weight of evidence sufficient to establish the MOA in animals?

Mucosal hyperplasia in the duodenum was consistently observed in mice, rats and dogs, with dose responsiveness (Table A3-8). Whereas serum iron levels or absorption and transfer of iron in the duodenum were not measured in mice or dogs, morphological and physiological features related to iron absorption in the duodenum are considered common among mice, rats, dogs and humans based on current knowledge. No evidence of inflammatory reaction or degeneration of the duodenal mucosa was obtained in any species. Overall, cell proliferation and consequently mucosal hyperplasia will occur as a compensatory response to lower iron levels in the serum due to inhibition of the uptake of iron in those species.

Table A3-8. Comparison of key and associated events for pyraclostrobin in animal species

Key/associated events	Mice	Rats	Dogs	Humans	Comments
Key events					
Inhibition of absorption and transfer of iron	NE	Yes	NE	Likely	Evidence from dimoxystrobin, a strobilurin fungicide similar to pyraclostrobin
Decrease in serum iron level	Likely	Yes	Likely	Likely	Suggestive based on haematological profiles of anaemia
Cell proliferation	Yes	Yes	NE	Likely	Evidence from orysastrobin, a strobilurin fungicide similar to pyraclostrobin
Mucosal hyperplasia in the duodenum	Yes	Yes	Yes	Likely	
Associated event					
Iron deficiency anaemia in response to low serum iron levels	Yes	Yes	Yes	Yes	Serum iron levels were not measured in mice or dogs

NE: not examined

2. Can the human relevance of the MOA be reasonably excluded on the basis of fundamental/ qualitative differences in key events between humans and animals?

Pyraclostrobin is a non-genotoxic substance, and no carcinogenicity was observed in rats or mice. Hyperplasia of the duodenal mucosa due to pyraclostrobin exposure was observed in all animal species examined (mice, rats and dogs). It was revealed from various studies in rats that iron deficiency occurs as a result of pyraclostrobin exposure, and mucosal hyperplasia occurs to enlarge the surface area for the absorption of iron in the feed from the duodenum. In general, hyperplasia is a change that shows recoverability when the cause is eliminated (cessation of exposure).

In humans, iron deficiency anaemia is one of the most common diseases worldwide. Iron deficiency anaemia attributable to nutritional deficiency or blood loss remains the most common, treatable anaemia in the world (Brugnara, 2003; Muñoz, Villar & Garcia-Erce, 2009). The most common cause of iron deficiency anaemia is blood loss from lesions in the gastrointestinal tract (Liu & Kaffes, 2012). Once the cause for the underlying blood loss or dietary deficiency is identified, the finding of anaemia with microcytic hypochromic erythrocytes in conjunction with abnormal serum biochemical indices (low iron, low transferrin saturation, low ferritin) usually leads to the administration of oral iron supplements, with improvement of anaemia in the vast majority of cases.

Intestinal structure and function in 11 children with iron deficiency anaemia were reported (Ercan et al., 1991). In six cases, there were histological abnormalities of small intestinal mucosa in varying degrees, consisting of villous damage, increased activity in the crypts, increased lymphoplasmocytic infiltration and changes in the surface epithelium. Ultrastructurally, microvilli lesions, mitochondrial changes and an increase in lysosomes were observed. These changes were due to impairment of cell metabolism of small intestinal epithelia. However, mucosal hyperplasia is not detected in human patients with iron deficiency anaemia.

The structure and function of the human duodenum are similar to those of mice, rats and dogs (Treuting, Valasek & Dintzis, 2012). After chronic exposure to strobin compounds, a similar duodenal mucosal hyperplastic lesion may be observed if iron deficiency anaemia occurs in humans.

3. Can the human relevance of the MOA be reasonably excluded on the basis of quantitative differences in either kinetics or dynamics factors in experimental animals and humans?

There are no quantitative data comparing the kinetics or dynamics of the inhibition of iron uptake in the duodenum among humans and experimental animals. More information is necessary to enable a quantitative comparison.

Conclusion

The MOA is considered to be an interspecies MOA, because there are no qualitative differences between mucosal hyperplasias and their effective doses among rodents and dogs. Major MOA studies on key events were conducted in rats; however, anatomical and functional similarities in the duodenum among these species result in similar effects on iron absorption and transport in the duodenum by the treatment. Clear evidence of a relationship between mucosal hyperplasia and iron deficiency anaemia, which is a common type of anemia in humans, has not been provided, and there was no related report on occupational workers. In contrast, the effects on iron absorption and transport in the duodenum observed in rodents and dogs are considered to be common to humans due to the anatomical and functional similarities in the duodenum.

In conclusion, a similar MOA is plausible in humans following long-term and high-dose exposure to strobilurin fungicides.

References for Appendix 3

Brugnara C (2003). Iron deficiency and erythropoiesis: new diagnostic approaches. Clin Chem. 49:1573–8.

Cohen SM, Gordon EB, Singh P, Arce GT, Nyska A (2010). Carcinogenic mode of action of folpet in mice and evaluation of its relevance to humans. Crit Rev Toxicol. 40:531–45.

Cunha GC, Van Ravenzwaay B, Mellert W, Kaufmann W (2008). Iron deficiency causes duodenum mucosal hyperplasia in male Wistar rats. Toxicol Lett. 177:156–67.

Ercan O, Ulukutlu L, Ozbay G, Arda O (1991). Intestinal effects of iron deficiency anemia in children. Turk J Pediatr. 33:85–98.

FSCJ (2005). Orysastrobin. Food Safety Commission of Japan (https://www.fsc.go.jp/fsciis/evaluationDocument/show/kya20081030045, accessed 26 September 2018) (in Japanese).

Gordon E, Cohen SM, Singh P (2012). Folpet-induced short term cytotoxic and proliferative changes in the mouse duodenum. Toxicol Mech Methods. 22:54–9.

Inomata A, Nakano-Ito K, Fujikawa Y, Sonoda J, Hayakawa K, Ohta E et al. (2014). Brunner's gland lesions in rats induced by a vascular endothelial growth factor receptor inhibitor. Toxicol Pathol. 42:1267–74.

IPCS (2007). Part 1: IPCS framework for analysing the relevance of a cancer mode of action for humans and case-studies. Part 2: IPCS framework for analysing the relevance of a non-cancer mode of action for humans. Geneva, Switzerland: World Health Organization, International Programme on Chemical Safety (Harmonization Project Document No. 4; http://www.inchem.org/documents/harmproj/harmproj/harmproj4.pdf, accessed 26 September 2018).

Liu K, Kaffes AJ (2012). Iron deficiency anemia: a review of diagnosis, investigation and management. Eur J Gastroenterol Hepatol. 24:109–16.

Meek ME, Bucher JR, Cohen SM, Dellarco V, Hill RN, Lehman-McKeeman LD et al. (2003). A framework for human relevance analysis of information on carcinogenic modes of action. Crit Rev Toxicol. 33:591–653.

Mellert W (2003). Amendment no. 1 to the report: Screening study BAS 505 F – administration in the diet with simultaneous parenteral administration of iron to Wistar rats. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2003/1009197. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Kaufmann W (2004). Amendment no. 2 to the report: Screening study BAS 505 F – administration in the diet with simultaneous parenteral administration of iron to Wistar rats. Unpublished report. BASF AG,

Ludwigshafen/Rhein, Germany. BASF document ID 2004/1010601. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Deckardt K, Kaufmann W, Leibold E (2002). Screening study BAS 505 F – administration in the diet with simultaneous parenteral administration of iron to Wistar rats. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2002/1013984. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Deckardt K, Gembart C, Van Ravenzwaay B (2003). BAS 500 F – determination of iron in urine and serum of Wistar rats – administration in the diet over 14 days. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2003/1009534. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Muñoz M, Villar I, Garcia-Erce JA (2009). An update on iron physiology. World J Gastroenterol. 15:4617–26.

Srai K (2003). BAS 505 F – study on the effect on the mucosal iron transport in female Wistar rats – administration in the diet. Unpublished report. Royal Free and University College Medical School, London, United Kingdom. BASF document ID 2003/1014047. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Thompson CM, Wolf JC, McCoy A, Suh M, Proctor DM, Kirman CR et al. (2017). Comparison of toxicity and recovery in the duodenum of B6C3F1 mice following treatment with intestinal carcinogens captan, folpet, and hexavalent chromium. Toxicol Pathol. 45:1091–1101.

Treuting PM, Valasek MA, Dintzis SM (2012). Upper gastrointestinal tract. In: Treuting PM, Dintzis SM, editors. Comparative anatomy and histology. A mouse and human atlas. London, United Kingdom: Academic Press; 155–75.

Van Ravenzwaay B, Akiyama M, Landsiedel R, Kieczka H, Cunha G, Schneider S et al. (2007). Toxicological overview of a novel strobilurin fungicide, orysastrobin. J Pestic Sci. 32:270–7.