

MANDESTROBIN

First draft prepared by
Luca Tosti,¹ David Eastmond² and Carl Cerniglia³

¹ Department of Biomedical and Clinical Sciences, University of Milan, and International Centre for Pesticides and Health Risk Prevention, ASST Fatebenefratelli Sacco, Milan, Italy

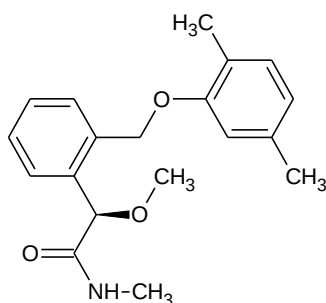
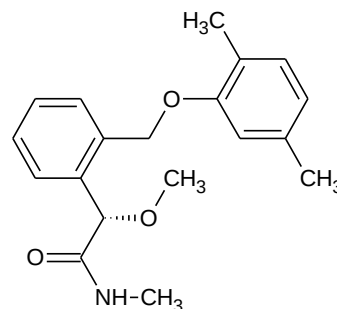
² Department of Molecular, Cell and Systems Biology, University of California, Riverside, California, United States of America (USA)

³ Division of Microbiology, National Center for Toxicological Research, Food and Drug Administration, Jefferson, Arkansas, USA

Explanation.....	295
Evaluation for acceptable intake.....	296
1. Biochemical aspects	296
1.1 Absorption, distribution and excretion	297
1.2 Biotransformation	301
2. Toxicological studies	311
2.1 Acute toxicity	311
(a) Lethal doses	311
(b) Dermal irritation	313
(c) Ocular irritation.....	313
(d) Dermal sensitization.....	313
2.2 Short-term studies of toxicity	313
(a) Oral administration	314
(b) Dermal application.....	337
2.3 Long-term studies of toxicity and carcinogenicity	337
2.4 Genotoxicity	351
(a) In vitro	352
(b) In vivo	353
2.5 Reproductive toxicity	353
(a) Multigeneration studies.....	353
(b) Developmental toxicity	368
2.6 Special studies.....	374
(a) Neurotoxicity	374
(b) Immunotoxicity	378
(c) Mechanistic studies.....	380
(d) Toxicity studies on metabolites and impurities.....	387
(e) Microbiological effects	394
3. Observations in humans	395
Comments.....	395
Toxicological evaluation	400
References	403

Explanation

Mandestrobin is the common name approved by the International Organization for Standardization for 2-[2-[(2,5-dimethylphenoxy)methyl]phenyl]-2-methoxy-*N*-methylacetamide (International Union of Pure and Applied Chemistry), with the Chemical Abstracts Service number 173662-97-0. It is a fungicide that is marketed as a racemic mixture (50:50) consisting of the *R*-isomer and the *S*-isomer (Fig. 1). The fungicidal mode of action is inhibition of mitochondrial respiration via binding to complex III.

Fig. 1. Structures of *R*- and *S*-isomers of mandestrobin***R*-Mandestrobin*****S*-Mandestrobin**

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

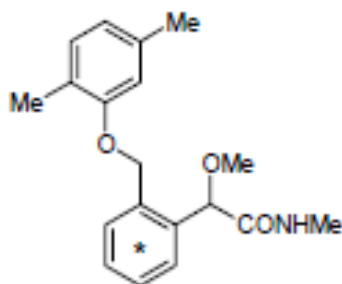
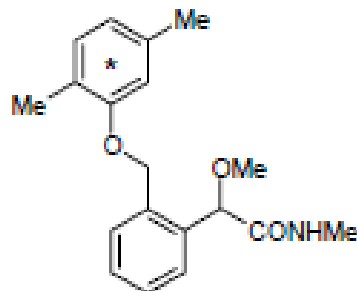
All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with relevant national or international test guidelines, unless otherwise specified. No additional information from a literature search was identified that complemented the toxicological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

Evaluation for acceptable intake

1. Biochemical aspects

The absorption, distribution, metabolism and excretion (ADME) of mandestrobin (code name S-2200) were investigated in rats following the administration of mandestrobin labelled with ^{14}C at either the benzyl or the phenoxy ring in a single oral low dose or a single oral high dose or the administration of radiolabelled mandestrobin in a single oral low dose repeated for up to 14 consecutive days. The rates and routes of elimination were also determined in bile duct-cannulated animals after administration of a single oral low dose. Possible preferential metabolism was investigated by administering single low doses of the *R*-isomer and the *S*-isomer of mandestrobin, labelled uniformly with ^{14}C at the benzyl position.

The positions of the radiolabels in the mandestrobin used in the ADME studies are shown in Fig. 2.

Fig. 2. Position of radiolabels in mandestrobin used in ADME studies**[benzyl- ^{14}C]Mandestrobin****[phenoxy- ^{14}C]Mandestrobin**

* Denotes radiolabelled ring

Source: Kendrick (2012a)

1.1 Absorption, distribution and excretion

In an absorption, distribution and excretion study, Hannover Wistar (CrI) rats (four of each sex) were administered [benzyl-¹⁴C]mandestrobins (purity 98.3%) or [phenoxy-¹⁴C]mandestrobins (purity 98.9%) in a single oral gavage (low) dose of 5 mg/kg body weight (bw) (groups A and B) or [benzyl-¹⁴C]mandestrobins in a single oral gavage (high) dose of 1000 mg/kg bw (group C) for the determination of rates and routes of excretion at various intervals up to 7 days. A further four male and four female rats were given [benzyl-¹⁴C]mandestrobins in a single oral gavage dose of 5 mg/kg bw (group D) or 1000 mg/kg bw (group E) for the determination of plasma concentrations at various intervals up to 120 hours. Biliary excretion was investigated in seven male and seven female rats administered [benzyl-¹⁴C]mandestrobins in a single oral gavage dose of 5 mg/kg bw (group F). An additional group of 16 male and 16 female rats was given [benzyl-¹⁴C]mandestrobins in a single oral gavage dose of 5 mg/kg bw (group G) or 1000 mg/kg bw (group H) for the determination of tissue distribution at various intervals up to 7 days (Table 1). The dosing vehicle was 0.5% aqueous methylcellulose.

Table 1. Animal assignments in ADME studies

Dose group	Label position	Parameter investigated	Dose		Number of animals	
			mg/kg bw	MBq/kg bw	Males	Females
A	Benzyl ring	Excretion balance	5	5	4	4
B	Phenoxy ring		5	5	4	4
C	Benzyl ring		1 000	5	4	4
D	Benzyl ring	Pharmacokinetics	5	10	4	4
E	Benzyl ring		1 000	10	4	4
F	Benzyl ring	Biliary excretion	5	5	7 ^a	7 ^a
G	Benzyl ring	Tissue distribution	5	5	16	16
H	Benzyl ring		1 000	5	16	16

bw: body weight

^a Additional animals were included to ensure that four animals of each sex completed the experimental regimen.

Source: Kendrick, Farrell & Murphy (2012)

Following administration of a single high or low oral dose of racemic mandestrobins labelled with ¹⁴C in either the benzyl or phenoxy ring, the radioactivity was rapidly absorbed. Faecal excretion was the primary route of elimination of the radioactivity. More than 70% of the total excreted radioactivity was eliminated within 48 hours of administration. There did not appear to be any sex-, dose- or radiolabel-related differences in either the rates or routes of excretion (Table 2). In bile duct-cannulated rats, elimination of radioactivity was rapid, with more than 95% of the dose eliminated within the first 24 hours. About 80% was eliminated via the bile, and radioactivity in faeces accounted for less than 2% of the total radioactivity. Elimination via urine was comparable to that of intact animals. Urinary elimination in bile duct-cannulated rats was substantially more rapid than in intact animals, where it was apparently slowed by enterohepatic recirculation.

Absorption of mandestrobins was greater than 90% of the administered dose at 5 mg/kg bw, based on radioactivity recovered in urine and bile. Recoveries of radioactivity were complete at 168 hours (95.6–101%) in all dose groups. After 168 hours, only 0.7–2.1% of the radioactivity remained in carcass and tissues.

For both low and high doses, there were no significant sex differences in the pharmacokinetic parameters. Mean plasma elimination half-lives were approximately 20 hours (low dose) and 27 hours (high dose). The maximum plasma concentration ($C_{\max}$) was observed within 3 and 10 hours post-dosing for the low and high doses, respectively (Table 3).

Table 2. Cumulative recovery of radioactivity in excreta following administration of a single oral low dose of [benzyl-¹⁴C]mandestrobins or [phenoxy-¹⁴C]mandestrobins or a single oral high dose of [benzyl-¹⁴C]mandestrobins to rats at 168 hours or a single oral low dose of [benzyl-¹⁴C]mandestrobins to bile duct-cannulated rats at 72 hours

Matrix	% of administered dose							
	5 mg/kg bw				1 000 mg/kg bw		5 mg/kg bw	
	[benzyl- ¹⁴ C]		[phenoxy- ¹⁴ C]		[benzyl- ¹⁴ C]		[benzyl- ¹⁴ C], bile duct-cannulated rats	
	Males	Females	Males	Females	Males	Females	Males	Females
Urine	18.0	20.6	15.8	21.5	17.2	17.2	18.0	13.7
Faeces	72.9	71.3	73.9	66.5	74.8	72.0	1.43	1.92
Bile	ND	ND	ND	ND	ND	ND	79.7	81.9
Cage wash	4.88	5.99	3.98	6.51	3.65	6.74	1.39	1.35
Cage debris	0.282	0.069	0.172	0.038	0.048	0.117	0.036	0.068
Final cage wash	0.15	0.108	0.071	0.087	0.082	0.357	0.157	0.264
Tissue carcass	2.13	0.974	1.69	1.31	1.22	0.702	0.262	0.388
Total	98.3	99.0	95.6	95.9	97.0	97.1	101	99.6

bw: body weight; ND: not determined

Source: Kendrick, Farrell & Murphy (2012)

Table 3. Mean pharmacokinetic parameters in plasma following oral administration of radiolabelled (benzyl-¹⁴C) mandestrobins to rats

Pharmacokinetic parameters	5 mg/kg bw		1 000 mg/kg bw	
	Males	Females	Males	Females
$t_{1/2}$ (h)	22.52	18.32	24.51	29.4
T_{max} (h)	2.625	1.25	7	9.125
C_{max} (µg eq/g)	0.842	0.829	690	49.2
AUC_{0-120} (h·µg eq/g)	15.64	13.92	1 541	1 262
$AUC_{0-\infty}$ (h·µg eq/g)	16.02	14.08	1 577	1 303
AUC_{0-120} as a % of $AUC_{0-\infty}$	98	99	98	97

AUC_{0-120} : area under the plasma concentration–time curve from 0 to 120 hours; $AUC_{0-\infty}$: area under the plasma concentration–time curve from time 0 to infinity; bw: body weight; C_{max} : maximum concentration; eq: equivalents; $t_{1/2}$: elimination half-life; T_{max} : time taken to reach C_{max}

Source: Kendrick, Farrell & Murphy (2012)

At 1000 mg/kg bw, after T_{max} was achieved, concentrations of radioactivity did not decrease steadily, as was observed following administration of the 5 mg/kg bw dose. Following dose administration at 1000 mg/kg bw in both male and female animals, additional absorption maxima were observed, which were particularly prominent during the first 12 hours post-dosing. The multiple maxima could possibly reflect enterohepatic recirculation, which is supported by the enhanced rapidity of elimination in bile duct-cannulated animals. Systemic exposure (area under the concentration–time curve [AUC]) to mandestrobins-derived radioactivity was about 90- to 100-fold higher in male and female animals receiving the 1000 mg/kg bw dose compared with animals receiving the 5 mg/kg bw dose. As such, exposure was sub-dose-proportional, indicating that saturation of absorption processes

may also have occurred (Table 3). At the terminal time point, plasma radioactivity concentrations were less than or equal to 2% of the concentrations observed at the $C_{\max}$.

Tissue distribution of radioactivity was similar at the high and low doses (Table 4). At the first two time points (0.5 and 2 hours post-dosing for the low-dose groups and 2 and 8 hours post-dosing for the high-dose groups), the majority of radioactivity was detected in the upper half of the gastrointestinal tract. Transit through the gastrointestinal tract was evident over time; at the terminal time point (168 hours), only about 1% of the total radioactivity was associated with tissue and carcass. Excluding the components of the gastrointestinal tract, the highest concentrations of radioactivity were measured in liver and kidneys (up to 9% and 0.4% of the administered radioactivity, respectively). Notable amounts of radioactivity were also detected in fat, pancreas, spleen, ovaries and uterus. Plasma:tissue radioactivity ratios were generally below 1, except for the above-noted organs (Kendrick, Farrell & Murphy, 2012).

Table 4. Tissue distribution of radioactivity in low-dose and high-dose groups at 168 hours after administration

Tissue	Tissue distribution of radioactivity (mean ng equivalents/g)					
	5 mg/kg bw [benzyl- ^{14}C]		5 mg/kg bw [phenoxy- ^{14}C]		1 000 mg/kg bw [benzyl- ^{14}C]	
	Males	Females	Males	Females	Males	Females
Adrenals	21	7	8	ND	ND	ND
Blood	10	3	9	3	732	1 352
Bone	ND	3	ND	ND	ND	ND
Bone marrow	ND	ND	ND	ND	ND	ND
Brain	ND	ND	ND	ND	ND	ND
Eyes	3	1	1	10	ND	ND
Fat	50	19	21	31	593	ND
Hair and skin	8	3	14	8	522	ND
Heart	2	ND	2	2	762	ND
Kidney	34	14	35	19	2 627	1 458
Liver	147	98	130	92	9 033	7 144
Lung	9	ND	6	4	644	ND
Mandibular gland	2	ND	3	2	ND	ND
Muscle (quadriceps)	ND	ND	2	ND	ND	ND
Ovaries	NA	32	NA	46	NA	874
Pancreas	61	20	7	30	1 561	1 225
Pituitary	ND	ND	ND	ND	ND	ND
Plasma	11	2	9	4	170	ND
Red blood cells	9	6	8	2	1 910	2 712
Sciatic nerve	ND	ND	ND	ND	ND	ND
Spinal cord	ND	ND	ND	ND	ND	ND
Spleen	19	5	7	44	607	ND
Testes	ND	NA	ND	NA	ND	NA

Tissue	Tissue distribution of radioactivity (mean ng equivalents/g)					
	5 mg/kg bw [benzyl- ¹⁴ C]		5 mg/kg bw [phenoxy- ¹⁴ C]		1 000 mg/kg bw [benzyl- ¹⁴ C]	
	Males	Females	Males	Females	Males	Females
Thymus	ND	ND	2	6	ND	ND
Thyroid	23	ND	ND	ND	ND	NA
Uterus	NA	62	NA	26	NA	982
Gastrointestinal tract						
Caecum	247	103	300	177	5 547	5 494
Caecum contents	358	132	456	146	8 387	8 775
Large intestine	198	62	162	110	4 179	2 994
Large intestine contents	295	105	312	190	7 380	6 664
Small intestine	775	624	638	526	3 194	655
Small intestine contents	2 050	12	1 241	1 134	1 093	1 780
Stomach	55	41	55	58	17 840	8 373
Stomach contents	118	98	41	114	39 380	23 880

bw: body weight; ND: not detected; NA: not available

Source: Kendrick, Farrell & Murphy (2012)

In an absorption, distribution and excretion study, six groups of Hannover Wistar (CrI) rats (four of each sex per group) were administered [benzyl-¹⁴C]mandestrobins (purity 98.9%) orally by gavage in 0.5% aqueous methylcellulose at 5 mg/kg bw per day for up to 14 consecutive days. The rates and routes of excretion were determined by collecting excreta at 24-hour intervals 1) until 24 hours after the 14th dose and 2) until 336 hours after the 14th dose. Tissue distribution of radioactivity was determined over a time course of up to 336 hours after the 14th daily dose.

During the first 14 days, radioactivity was eliminated primarily in the faeces, with approximately 57.3% and 49.5% of the administered dose eliminated in males and females, respectively. Renal elimination accounted for about 13% and 15% of the administered dose in male and female animals, respectively. Radioactivity recovered from the cage washes accounted for about 6% and 8% of the administered dose in males and females, respectively (Table 5).

Table 5. Recovery of radioactivity in excreta following repeated administration of [benzyl-¹⁴C]mandestrobins at 5 mg/kg bw

Matrix	% of administered dose ^a			
	Days 1–14		Days 14–28	
	Males	Females	Males	Females
Urine	13.25	14.81	1.89	2.07
Faeces	57.25	49.45	9.96	10.56
Cage wash ^b	6.11	7.99	0.56	0.83
Total	76.61	72.25	12.42	13.47

^a Calculated from the sum of 14 administered doses.

^b Includes cage debris, final cage wash and tissues.

Source: Kendrick & Farrell (2012)

During the 14 days after the last dose, concentrations of radioactivity in excreta were generally much lower, given that the vast majority of the administered dose (14 daily doses) had already been eliminated prior to confinement in metabolism cages. Radioactivity was eliminated primarily in the faeces, with about 10% and 10.6% of the administered dose eliminated in males and females, respectively. Renal elimination accounted for approximately 1.9% and 2.1% of the administered dose in male and female animals, respectively (Table 5).

Following the repeated administration of [benzyl-¹⁴C]mandestrobin at 5 mg/kg bw per day to male and female rats for up to 14 days, radioactivity was widely distributed at all of the time points examined (2 hours after one, six, 10 and 14 doses and 168 and 336 hours after the 14th daily dose) (Table 6). Radioactivity was detected in all of the tissues examined on at least one occasion. At 648 hours after dosing (i.e. 336 hours after the final dose), radioactivity was detected in around half of the tissues examined. Excluding the components of the gastrointestinal tract, the highest mean concentrations of radioactivity were measured in the tissues involved in the metabolism and excretion of xenobiotics (i.e. liver and kidney).

As the number of doses progressed, the pancreas, spleen, thyroid, fat, uterus and ovaries became prominent depots for radioactivity. Plasma concentrations, together with all tissue concentrations, declined between 168 and 336 hours after the 14th daily dose. At this time, plasma concentrations in both male and female animals were below the limit of detection, and approximately half or more of the tissues did not contain detectable radioactivity. The observable decline of residues in all tissues indicated no potential for long-term accumulation of radioactivity with repeated daily administration of [benzyl-¹⁴C]mandestrobin at a dose of 5 mg/kg bw per day (Table 6) (Kendrick & Farrell, 2012).

In an absorption, distribution and excretion study, two groups of Hannover Wistar (CrI) rats (four of each sex per group) were administered [benzyl-¹⁴C]mandestrobin, *R*-isomer (purity 99.2%) or *S*-isomer (purity 99.7%), in a single oral gavage dose (in 0.5% aqueous methylcellulose) of 5 mg/kg bw. Rates and routes of excretion were determined at various intervals up to 7 days. Distribution of radioactivity in tissues and organs was determined 7 days after administration.

After 7 days, both isomers were eliminated mainly via faeces: approximately 64–75% of the radioactivity for the *R*-isomer and about 73–80% of the radioactivity for the *S*-isomer. Both isomers were absorbed immediately after administration. The *R*-isomer was almost completely excreted (>95%) after 3 days, whereas excretion of the *S*-isomer was slower, with an almost complete excretion (>94%) after 5 days. Elimination via urine accounted for 22% and 32% of the radioactivity in males and females, respectively, for the *R*-isomer after 3 days and for 15% and 25% of the radioactivity in males and females, respectively, for the *S*-isomer after 5 days. The majority of radioactivity was excreted in faeces, accounting for 73% and 63% of the radioactivity in males and females, respectively, for the *R*-isomer after 3 days and for 77% and 70% of the radioactivity in males and females, respectively, for the *S*-isomer after 5 days. No significant sex differences in the routes of excretion of both isomers were observed. Excretion into expired air was negligible with both isomers and both sexes.

Tissue distribution was generally low (total radioactivity <1%). Residuals were slightly higher in animals given the *S*-isomer, reflecting the slower elimination (Mikata, 2011).

1.2 Biotransformation

The metabolic fate of mandestrobin was investigated in Hannover Wistar (CrI) rats. The test item was administered by gavage to six groups of rats as described in Table 1 of section 1.1. Samples from dose groups A, B, C, F, G and H were pooled per time point, sex and dose group for metabolite isolation and identification by radio-high-performance liquid chromatography (HPLC) and liquid chromatography with mass spectrometry (LC-MS), respectively. Metabolites were determined in urine, faeces, liver, kidney, plasma and bile.

Table 6. Concentrations of radioactivity in organs and tissues following repeated oral administration of ^{14}C -labelled mandestrobin to rats at 5 mg/kg bw per day

Tissue ^a	Concentration of radioactivity (ng equivalents/g)											
	2 hours after 1st dose		122 hours after 1st dose		218 hours after 1st dose		314 hours after 1st dose		480 hours after 1st dose		648 hours after 1st dose	
	–		–		–		2 hours after 14th daily dose		168 hours after 14th daily dose		336 hours after 14th daily dose	
	M	F	M	F	M	F	M	F	M	F	M	F
Liver	4 724	2 025	7 762	4 687	11 760	6 618	10 150	8 868	415	686	115	150
Carcass	215	239	621	603	996	1 041	933	1 165	59	62	86	52
Skin + hair	167	67	301	145	452	302	450	272	93	44	81	40
Bone	66	22	94	55	151	92	134	115	20	10	ND	32
Kidney	1 157	446	1 730	923	3 057	1 094	2 459	1 574	109	91	40	27
Red blood cells	275	67	319	141	431	148	382	208	105	68	38	21
Ovaries	–	874	–	1 813	–	2 360	–	1 123	–	105	–	12
Spleen	169	168	504	724	476	1 319	707	840	29	36	21	11
Uterus	–	1 310	–	3 189	–	4 379	–	4 810	–	90	–	8
Pancreas	571	635	1 019	3 239	1 622	2 878	11 590	4 370	40	67	ND	ND
Plasma	773	227	1 052	513	1 582	582	1 197	868	14	8	ND	ND
Fat	339	270	603	1 124	997	1 151	1 956	803	7	36	ND	ND
Thyroid	322	246	492	249	908	1 201	1 122	686	28	ND	ND	ND
Blood	495	145	686	322	1 031	376	781	571	64	44	7	ND
Adrenals	315	90	462	298	734	427	673	522	13	17	ND	ND
Lung	288	137	473	215	737	417	632	426	32	33	ND	ND
Sciatic nerve	221	ND	301	95	476	223	433	336	38	ND	ND	ND
Pituitary	68	ND	94	91	751	ND	428	261	ND	ND	ND	ND
Muscle (quadriceps)	92	58	145	99	253	167	237	248	ND	6	ND	ND

Tissue ^a	Concentration of radioactivity (ng equivalents/g)											
	2 hours after 1st dose		122 hours after 1st dose		218 hours after 1st dose		314 hours after 1st dose		480 hours after 1st dose		648 hours after 1st dose	
	–		–		–		2 hours after 14th daily dose		168 hours after 14th daily dose		336 hours after 14th daily dose	
	M	F	M	F	M	F	M	F	M	F	M	F
Heart	209	81	301	128	484	185	336	244	8	ND	ND	ND
Mandibular gland	147	51	231	112	366	192	302	227	ND	ND	ND	ND
Testes	131	–	179	–	273	–	229	–	ND	–	ND	–
Thymus	94	50	197	98	280	149	250	171	7	ND	ND	ND
Eyes	76	41	131	59	227	125	212	119	8	ND	ND	ND
Bone marrow	57	ND	195	ND	146	ND	129	92	ND	ND	ND	ND
Spinal cord	33	27	80	35	159	97	102	75	ND	ND	ND	ND
Brain	39	12	71	39	108	45	78	61	ND	ND	ND	ND
Gastrointestinal tract												
Caecum	2 648	8 859	20 270	30 420	33 170	56 130	42 420	71 510	351	486	32	47
Caecum contents	3 234	12 290	34 520	41 870	55 550	78 190	53 210	51 580	465	551	47	65
Large intestine	644	1 466	6 706	10 630	16 680	14 570	20 320	16 980	168	271	9	14
Large intestine contents	160	498	15 550	14 710	34 300	44 160	27 340	36 900	316	622	40	33
Small intestine	21 980	17 450	43 020	37 680	62 130	57 840	95 130	67 350	381	814	61	65
Small intestine contents	44 260	45 220	132 700	132 000	176 400	167 000	228 300	100 600	1 240	1 508	100	98
Stomach	7 592	5 772	19 310	8 914	33 770	17 400	26 010	27 010	30	119	ND	6
Stomach contents	20 250	22 320	54 780	16 440	83 540	31 430	94 490	49 320	44	103	16	35

F: females; M: males; ND: not determined

^a Descending order of magnitude of residues in female tissues sampled 14 days after 14th daily dose (i.e. 648 hours after first dose), excluding gastrointestinal tract.

Source: Kendrick & Farrell (2012)

Mandestrobin was extensively metabolized to numerous metabolites (unchanged parent was found in faeces at only <0.2% of the administered dose at the low dose and <6% at the high dose). No major sex or dose differences were observed, and no major differences in the profiles from intact and bile duct-cannulated animals were apparent. Metabolite profiles generated from the administration of differently labelled molecules (benzyl vs phenoxy) indicated that little cleavage occurred between the two rings. The primary routes of metabolism were by (1) oxidation and subsequent conjugation with glucuronic acid, (2) demethylation with subsequent oxidation or (3) oxidation with subsequent demethylation. The metabolite profiles in urine, faeces and bile are summarized in Table 7.

Table 7. Quantification of metabolites in pooled excreta following administration of a single low or high dose of mandestrobin to rats

Metabolite identity ^a	% of administered dose															
	[benzyl- ¹⁴ C]								[phenoxy- ¹⁴ C]							
	5 mg/kg bw								1 000 mg/kg bw							
	Urine				Bile				Urine				Faeces			
	(cannulated)				(cannulated)				(cannulated)				(cannulated)			
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
	48 h	48 h	24 h	24 h	12 h	24 h	96 h	72 h	48 h	48 h	72 h	48 h	48 h	48 h	72 h	72 h
Mandestrobin (S-2200)	–	–	–	–	–	–	0.09	0.139	–	–	3.92	5.49	–	–	0.08	–
5-CA-S-2200-NHM	3.58	5.70	4.76	4.93	6.86	3.43	19.87	16.57	4.15	4.35	11.84	8.56	2.94	5.48	18.11	15.28
4-OH-S-2200	–	0.03	–	–	–	–	9.47	18.21	–	–	10.45	12.59	–	–	7.09	16.47
4-OH-S-2200-GlucA	0.08	0.01	–	–	33.78	36.67	–	–	–	0.11	–	–	0.02	0.03	–	–
COOH-S-2200 glucuronides	–	–	–	–	20.62	23.76	–	–	–	–	–	–	–	–	–	–
5-COOH-S-2200	1.26	3.25	–	–	1.63	3.38	7.42	8.32	1.78	4.43	10.74	8.71	0.64	2.63	8.05	6.79
5-CA-2-HM-S-2200	0.85	0.65	0.92	0.73	0.10	1.40	4.86	3.97	1.58	0.60	4.74	5.04	0.90	0.68	4.74	4.44
5-CA-2-HM-S-2200-NHM	0.89	0.55	1.59	0.84	1.67	0.49	6.49	0.68	0.61	0.18	2.12	1.51	0.88	0.60	6.37	2.88
De-Xy-S-2200	0.12	0.19	–	–	–	–	1.73	2.75	0.18	0.15	3.29	4.44	–	–	–	0.01
2-COOH-S-2200	0.33	0.64	0.54	0.22	–	–	0.78	0.23	0.58	0.29	–	0.78	0.36	0.86	0.06	0.60
5-CH ₂ OH-S-2200	0.00	–	0.28	0.13	–	–	0.21	0.17	–	0.03	–	1.14	–	–	0.92	0.36
2-CH ₂ OH-S-2200	0.02	0.01	–	–	–	–	0.15	–	–	–	–	0.37	–	–	0.14	–
5-CA-MCBX-NDM	1.79	1.77	3.06	1.58	–	–	0.09	0.06	0.27	0.23	0.96	0.44	1.62	2.19	2.09	0.12
5-CA-S-2200-NDM	0.38	0.60	0.69	0.64	0.04	0.95	–	0.09	0.19	0.36	–	–	0.46	0.74	0.27	–

Metabolite identity ^a	% of administered dose															
	[benzyl- ¹⁴ C]								[phenoxy- ¹⁴ C]							
	5 mg/kg bw								1 000 mg/kg bw							
	Urine		Urine (cannulated)		Bile (cannulated)		Faeces		Urine		Faeces		Urine		Faeces	
	M 48 h	F 48 h	M 24 h	F 24 h	M 12 h	F 24 h	M 96 h	F 72 h	M 48 h	F 48 h	M 72 h	F 48 h	M 48 h	F 48 h	M 72 h	F 72 h
5-CA-2-HM-MCBX	0.87	0.33	0.63	0.14	–	–	0.04	–	0.87	0.28	0.53	–	1.16	0.34	0.29	0.14
DX-CA-S-2200	–	–	–	–	–	–	–	–	0.13	–	–	–	–	–	–	–
5-CA-2-HM-MCBX(-2H)	0.07	0.09	–	–	–	–	–	–	0.79	0.09	–	–	–	0.04	–	–
MCBX	–	–	–	–	–	–	–	–	–	–	0.04	–	–	–	–	–
% of dose identified	10.25	13.81	12.46	9.20	64.69	70.07	51.20	51.20	11.12	11.10	48.62	49.06	8.97	13.58	48.22	47.09
% of dose detected	15.20	18.57	17.42	13.30	79.24	81.41	67.19	64.46	15.68	15.36	70.39	65.43	13.94	19.25	66.74	59.75

–: not detected or below the limit of quantification; bw: body weight; F: females; LC-MS: liquid chromatography with mass spectrometry; M: males

^a Confirmed by LC-MS.

Source: Kendrick, Farrell & Murphy (2012)

In the liver, three metabolites dominated the profiles at both doses: 5-CA-S-2200-NHM, 5-CA-S-2200-NDM and 5-COOH-S-2200. In the kidneys, 5-CA-S-2200-NHM, 5-CA-2-HM-S-2200, 5-CA-MCBX-NDM and 5-COOH-S-2200 were identified. In the plasma, 2-COOH-S-2200, 5-COOH-S-2200, 5-CA-S-2200-NHM and 5-CA-S-2200-NDM were detected (Kendrick, Farrell & Murphy, 2012).

In another biotransformation study, four groups of Hannover Wistar (CrI) rats (four of each sex per group) were administered [benzyl-¹⁴C]mandestrobins (purity 98.9%) orally by gavage (in 0.5% aqueous methylcellulose) at 5 mg/kg bw per day for up to 14 consecutive days. Metabolites were determined by radio-HPLC and LC-MS in urine, faeces, liver, kidney and plasma, from samples pooled per time point, sex and dose group. Metabolites in urine and faeces were determined in pooled samples after a single (0–24 hours after dosing) or repeated administration (312–336 hours after the first dose) of radiolabelled mandestrobins. Metabolites in liver, kidney and plasma were determined at 2 hours after a single dose and repeated doses (314 hours).

In urine, the major metabolites following the administration of both single and repeated doses were 5-CA-S-2200-NHM and 5-CA-MCBX-NDM. Other metabolites following single dosing included 5-COOH-S-2200, 5-CA-2-HM-S-2200-NHM, 5-CA-2-HM-S-2200 and 4-OH-S-2200-GlucA. Following repeated administration, 2-COOH-S-2200, 5-COOH-S-2200 and 4-OH-S-2200-GlucA were especially prominent (Table 8).

In faeces, the major metabolites following both single and repeated daily doses of mandestrobins were 5-CA-S-2200-NHM, 5-CA-2-HM-S-2200-NHM, 5-CA-2-HM-S-2200, 5-CA-MCBX-NDM, 4-OH-S-2200 and 5-COOH-S-2200. Other metabolites identified following single dosing included 5-CA-2-HM-MCBX and 5-CA-S-2200-NDM. Following repeated administration, De-Xy-S-2200 was prominent, particularly in the female profile. Overall, although quantitative differences were apparent in the proportions of some metabolites between male and female profiles, no major qualitative differences were evident. Repeated administration did not significantly affect the profile of the metabolites observed (Table 8).

Table 8. Metabolites in pooled excreta following single or repeated oral administration of radiolabelled mandestrobin to rats at a dose of 5 mg/kg bw

Metabolite identity ^a	% of administered dose							
	Urine				Faeces			
	0–24 hours (single)		312–336 hours (repeated)		0–24 hours (single)		312–336 hours (repeated)	
	M	F	M	F	M	F	M	F
Mandestrobin (S-2200)	ND	ND	ND	ND	0.148	0.097	0.009	0.012
2-COOH-S-2200	0.149	ND	0.047	0.102	0.189	0.046	0.106	0.040
4-OH-S-2200	ND	ND	ND	ND	0.695	0.896	0.547	1.164
4-OH-S-2200-GlucA	0.242	1.048	0.023	0.147	ND	ND	ND	ND
5-CA-2-HM-MCBX	0.105	ND	ND	0.016	0.669	0.139	0.214	0.029
5-CA-2-HM-MCBX(-2H)	0.115	ND	0.026	ND	ND	ND	ND	ND
5-CA-2-HM-S-2200	0.328	0.299	0.063	0.060	1.506	1.150	0.408	0.298
5-CA-2-HM-S-2200-NHM	0.365	0.170	0.060	0.039	2.403	0.521	0.458	0.114
5-CA-MCBX-NDM	0.666	0.475	0.169	0.099	1.157	0.314	0.360	0.128
5-CA-S-2200-NDM	0.141	0.167	0.043	0.044	0.497	0.275	0.179	0.154
5-CA-S-2200-NHM	1.713	1.946	0.209	0.403	6.747	3.693	1.471	1.266
5-COOH-S-2200	0.471	0.535	0.040	0.080	1.715	1.229	0.677	0.830
COOH-S-2200 glucuronide	ND	0.186	0.010	0.051	ND	ND	ND	ND
COOH-S-2200 glucuronide	ND	ND	0.010	ND	ND	ND	ND	ND
COOH-S-2200 glucuronide	0.090	0.412	0.011	0.059	ND	ND	ND	ND
De-Xy-S-2200	ND	ND	ND	ND	0.310	0.446	0.157	1.053
% of dose	6.881	7.741	1.352	1.361	17.64	9.594	5.784	5.529

F: female; LC-MS: liquid chromatography with mass spectrometry; M: male; ND: not detected

^a Confirmed by LC-MS.

Source: Kendrick & Farrell (2012)

In plasma from males administered a single dose, almost double the number of metabolites were identified compared with females. In animals receiving 14 daily doses, a greater number of metabolites could be identified. Although quantitative differences were observed in the proportion of metabolites between males and females, there were no major qualitative differences between sexes or between single and repeated dosing (Table 9).

In the liver, 5-CA-S-2200-NDM and 5-COOH-S-2200 were the major metabolites following a single-dose administration in both aqueous and organic extracts from males and females. Other metabolites in both sexes were De-Xy-S-2200, 5-CA-MCBX-NDM, 5-CA-S-2200-NHM, 5-CA-2-HM-S-2200-NHM, 5-CA-2-HM-S-2200 and 2-COOH-S-2200. Following the repeated administration, a greater number of metabolites were prominent in aqueous liver extract profiles from male and female animals. However, the same known metabolites were the most prominent (De-Xy-S-2200, 5-CA-MCBX-NDM, 5-CA-S-2200-NHM, 5-CA-S-2200-NDM and 5-COOH-S-2200). The next most prominent metabolites included “Unknowns” and 5-CA-2-HM-S-2200-NHM, 5-CA-2-HM-S-2200 and 2-COOH-S-2200. In the organic extract, metabolites De-Xy-S-2200, 5-CA-S-2200-NHM, 5-CA-S-2200-NDM and 5-COOH-S-2200 were the most abundant in both males and females. An additional region of interest in the female metabolic profile was assigned the identity of 5-CA-MCBX-NDM. Therefore, although quantitative differences existed in the proportions of some metabolites, there were

no major qualitative differences in the liver between the sexes. Repeated administration did not significantly alter the metabolic profile (Table 10).

Table 9. Metabolites in plasma following single or repeated oral administration of radiolabelled mandestrobin to rats at a dose of 5 mg/kg bw

Metabolite identity ^a	Concentration in plasma (ng equivalents/g)			
	2 hours (single)		314 hours (repeated)	
	Males	Females	Males	Females
2-COOH-S-2200	22.65	ND	108.5	ND
4-OH-S-2200-GlucA	73.77	57.94	186.7	283.1
5-CA-S-2200-NDM	28.33	ND	25.64	27.14
5-CA-S-2200-NHM	54.86	ND	49.21	81.41
5-COOH-S-2200	116.0	65.43	131.1	194.1
COOH-S-2200 glucuronide	ND	ND	62.57	ND
COOH-S-2200 glucuronide	19.92	ND	14.22	ND
COOH-S-2200 glucuronide	66.54	ND	ND	44.53
UK (Rt 31.3)	ND	ND	66.58	ND
UK (Rt 33.9)	87.63	ND	94.28	91.50
UK (Rt 37.5–38.1)	138.2	<LOQ	147.4	67.50
UK (Rt 38.5–39.3)	81.01	55.04	81.04	57.75
Total concentration	778.2	229.7	1 215	869.8

LC-MS: liquid chromatography with mass spectrometry; LOQ: limit of quantification; ND: not detected; Rt: retention time; UK: unknown

^a Confirmed by LC-MS.

Source: Kendrick & Farrell (2012)

Table 10. Metabolites in liver following single or repeated oral administration of radiolabelled mandestrobin to rats at a dose of 5 mg/kg bw

Metabolite identity ^a	Concentration in liver (ng equivalents/g)							
	2 hours				314 hours			
	Aqueous phase		Organic phase		Aqueous phase		Organic phase	
	Males	Females	Males	Females	Males	Females	Males	Females
2-COOH-S-2200	130.9	ND	ND	ND	172.2	32.15	ND	ND
5-CA-2-HM-S-2200	176.5	54.77	ND	ND	324.3	328.1	ND	ND
5-CA-2-HM-S-2200-NHM	88.25	30.20	ND	ND	320.2	176.4	ND	ND
5-CA-MCBX-NDM	250.7	105.7	ND	ND	1 275	812.0	1 500	580.4
5-CA-S-2200-NDM	1 393	651.2	2 710	1 016	1 331	779.1	3 885	1 787
5-CA-S-2200-NHM	303.3	163.9	548.0	ND	412.9	826.0	1 135	1 790
5-COOH-S-2200	1 127	745.4	1 221	1 238	1 686	1 644	2 114	2 912
De-Xy-S-2200	441.7	332.5	534.5	ND	1 286	2 081	1 073	1 174

Metabolite identity ^a	Concentration in liver (ng equivalents/g)							
	2 hours				314 hours			
	Aqueous phase		Organic phase		Aqueous phase		Organic phase	
	Males	Females	Males	Females	Males	Females	Males	Females
UK (Rt 16.7–16.9)	ND	ND	ND	ND	359.5	212.7	ND	ND
UK (Rt 20.9–21.1)	ND	ND	ND	ND	67.47	136.0	ND	ND
UK (Rt 24.5)	ND	ND	ND	ND	110.8	96.45	ND	ND
UK (Rt 25.3–25.7)	ND	ND	ND	ND	95.67	117.9	ND	ND
UK (Rt 26.7–27.1)	ND	ND	ND	ND	129.9	53.59	ND	ND
UK (Rt 27.9)	ND	ND	ND	ND	138.0	79.14	ND	ND
UK (Rt 29.1–29.3)	274.8	54.77	ND	ND	677.7	158.3	ND	ND
UK (Rt 35.9–36.5)	144.4	84.53	ND	ND	272.9	153.3	ND	ND
UK (Rt 48.1–48.5)	483.9	ND	ND	ND	668.6	ND	ND	ND
Total concentration	4 814	2 223	5 014	2 254	10 070	8 244	10 070	8 244

LC-MS: liquid chromatography–mass spectrometry; ND: not detected; Rt: retention time; UK: unknown

^a Confirmed by LC-MS.

Source: Kendrick & Farrell (2012)

In the kidney following the single oral administration of mandestrobin, males showed a greater number of metabolites compared with females. The major metabolites in the males were 5-CA-2-HM-S-2200-NHM, an unknown region of interest, 5-CA-S-2200-NHM and 5-COOH-S-2200. In females, 5-CA-S-2200-NHM and 5-COOH-S-2200 dominated.

Following repeated oral administration for 14 days, a greater number of metabolites in both male and female profiles were observed. In females, an unknown region of interest and 5-CA-MCBX-NDM were considered of note, in addition to those observed following single dosing (5-CA-S-2200-NHM and 5-COOH-S-2200).

Chromatographic resolution of the kidney metabolic profiles from male animals was much greater. The four most prominent metabolites observed in the female kidney were also prominent in the male profiles.

However, seven additional metabolites were also observed in the males. These were similar to those observed following single administration and were identified as De-Xy-S-2200, 5-CA-2-HM-S-2200-NHM and 5-CA-2-HM-S-2200. Four additional unidentified metabolites were present at concentrations above 100 ng equivalents/g. Overall, in the kidney, there were quantitative and qualitative differences in the metabolites observed between male and female animals and following both single and repeated administration of mandestrobin (Table 11) (Kendrick & Farrell, 2012).

In another study aimed at evaluating the metabolic reactions of (benzyl-¹⁴C)-labelled S- and R-isomers of mandestrobin after a single application, two groups of Hannover Wistar (CrI) rats (four of each sex per group) were administered [benzyl-¹⁴C]mandestrobin, R-isomer (purity 99.2%) or S-isomer (purity 99.7%), in a single oral gavage dose (in 0.5% aqueous methylcellulose) of 5 mg/kg bw. Identification of metabolites was performed by co-HPLC with known standards (ultraviolet absorption retention time compared with that from the refractive index chromatogram) and confirmed using LC-MS. Metabolites were determined in urine and faecal samples collected up to 3 and 4 days after administration, respectively. For both matrices, samples were pooled per time point and sex.

Twelve metabolites were identified and quantified.

Table 11. Metabolites in kidney following single or repeated oral administration of radiolabelled mandestrobin to rats at a dose of 5 mg/kg bw

Metabolite identity ^a	Concentration in kidney (ng equivalents/g)			
	2 hours (single)		314 hours (repeated)	
	Males	Females	Males	Females
2-COOH-S-2200	66.50	ND	72.79	ND
5-CA-2-HM-S-2200	85.22	ND	104.3	ND
5-CA-2-HM-S-2200-NHM	146.9	ND	135.9	ND
5-CA-MCBX-NDM	ND	ND	365.8	207.6
5-CA-S-2200-NDM	ND	ND	64.02	ND
5-CA-S-2200-NHM	310.6	158.3	372.2	719.9
5-COOH-S-2200	179.2	108.3	216.0	417.6
De-Xy-S-2200	83.35	ND	111.0	<LOQ
UK (Rt 24.9)	ND	ND	134.9	ND
UK (Rt 27.1)	ND	ND	157.4	ND
UK (Rt 28.1)	ND	ND	127.1	ND
UK (Rt 32.1–32.5)	ND	ND	130.4	171.4
UK (Rt 34.7–35.5)	105.1	ND	104.3	ND
Total concentration	1 101	402.5	2 371	1 597

LC-MS: liquid chromatography–mass spectrometry; LOQ: limit of quantification; ND: not detected; Rt: retention time; UK: unknown

^a Confirmed by LC-MS.

Source: Kendrick & Farrell (2012)

In the group administered the *R*-isomer, 5-CA-S-2200-NHM was the predominant metabolite in excreta, accounting for 38.6% and 41.2% of the dose in males and females, respectively. The other major metabolites, 5-CA-MCBX-NDM, 4-OH-S-2200, 5-CA-S-2200-NDM and 5-CA-2-HM-S-2200-NHM, were present at 11.8% and 9.5%, 5.5% and 5.0%, 5.8% and 5.6%, and 6.4% and 4.3% of the dose in males and females, respectively. The results showed that the amounts of metabolites in urine and faecal samples between males and females were slightly different, depending on the metabolite (Table 12).

Table 12. Metabolites identified in excreta 3 days (*R*-isomer) or 4 days (*S*-isomer) after administration of radiolabelled mandestrobin to rats at a dose of 5 mg/kg bw^a

Metabolite	% of administered dose			
	<i>R</i> -isomer		<i>S</i> -isomer	
	Males	Females	Males	Females
Urine				
De-Xy-S-2200 (UM-7)	0.6	0.7	0.6	0.7
DX-CA-S-2200 (UM-8)	0.5	0.4	ND	ND
5-CA-2-HM-MCBX (UM-12)	1.0	0.6	1.8	1.8
5-CA-2-HM-S-2200-NHM (UM-14)	1.3	1.2	0.6	0.6
5-CA-2-HM-S-2200 (UM-16)	0.7	0.5	1.1	1.6

Metabolite	% of administered dose			
	<i>R</i> -isomer		<i>S</i> -isomer	
	Males	Females	Males	Females
4-OH-S-2200-GlucA (UM-18)	0.3	4.3	1.3	3.4
5-CA-MCBX-NDM (UM-21)	3.9	4.3	0.6	2.6
2-COOH-S-2200 (UM-23)	0.1	0.1	0.2	0.1
4-OH-S-2200 (UM-25)	0.0	0.6	0.0	0.0
5-CA-S-2200-NHM (UM-26)	5.7	11.7	1.3	1.7
5-CA-S-2200-NDM (UM-27)	0.5	1.1	ND	0.3
5-COOH-S-2200 (UM-28)	0.1	0.5	1.2	3.8
Others ^b	6.9	6.1	6.6	8.2
Total urine	21.6	32.1	15.3	24.8
Faeces				
De-Xy-S-2200 (FM-7)	1.2	1.1	3.1	3.6
DX-CA-S-2200 (FM-8)	ND	0.3	ND	ND
5-CA-2-HM-MCBX (FM-12)	1.1	0.4	1.8	0.9
5-CA-2-HM-S-2200-NHM (FM-14)	5.1	3.1	1.8	0.8
5-CA-2-HM-S-2200 (FM-16)	1.3	1.8	4.7	4.9
4-OH-S-2200-GlucA (FM-18)	0.1	0.2	1.8	0.5
5-CA-MCBX-NDM (FM-21)	7.9	5.2	1.2	0.8
2-COOH-S-2200 (FM-23)	0.7	0.7	1.8	1.3
4-OH-S-2200 (FM-25)	5.5	4.4	23.1	28.4
5-CA-S-2200-NHM (FM-26)	32.9	29.5	7.0	5.7
5-CA-S-2200-NDM (FM-27)	5.3	4.5	1.5	1.7
5-COOH-S-2200 (FM-28)	1.9	3.0	18.0	11.6
Others ^c	6.5	4.5	6.9	4.5
Subtotal	69.5	58.7	72.7	64.7
Unextractable	3.9	4.1	3.8	5.1
Total faeces	73.4	62.8	76.5	69.8

bw: body weight; FM: faecal metabolite; ND: not detected; UM: urinary metabolite

^a Data were obtained from the pooled sample of four rats.

^b Sum of the 16 unidentified metabolites, which were below 3.6% of the dose.

^c Sum of the nine unidentified metabolites, which were below 2.7% of the dose.

Source: Mikata (2011)

In the group administered the *S*-isomer, 4-OH-S-2200 was the predominant metabolite in excreta (faeces), accounting for 23.1% and 28.4% of the dose in males and females, respectively. The other metabolites, 5-COOH-S-2200, 5-CA-S-2200-NHM, 5-CA-2-HM-S-2200 and 4-OH-S-2200-GlucA, occurred in excreta at 19.2% and 15.4%, 8.3% and 7.4%, 5.8% and 6.5%, and 3.1% and 3.9% of the dose in males and females, respectively (Table 12).

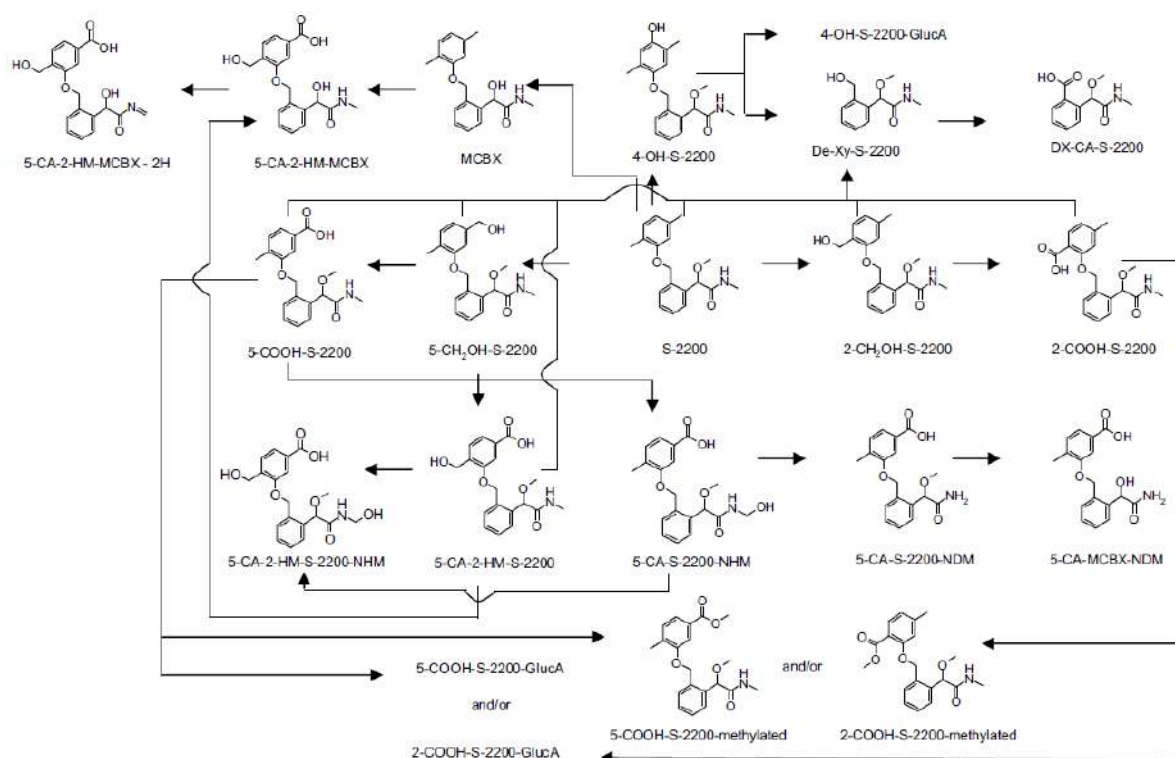
The *R*-isomer was absorbed into the body and metabolized to the predominant metabolite, 5-CA-S-2200-NHM, via 5-COOH-S-2200. Thereafter, 5-CA-S-2200-NHM and its metabolites, 5-CA-2-HM-S-2200-NHM, 5-CA-S-2200-NDM and 5-CA-MCBX-NDM, were rapidly and almost completely

eliminated from the body in urine and faeces. No potential for accumulation in tissues was demonstrated.

The rate of excretion of the *S*-isomer was slower than that of the *R*-isomer. The major metabolite in the *S*-isomer dose group was 4-OH-*S*-2200, and its glucuronide conjugate (4-OH-*S*-2200-GlucA) was detected after enzyme hydrolysis. It is likely that 4-OH-*S*-2200-GlucA is hydrolysed in the small intestine, subjected to enterohepatic circulation and finally excreted as 4-OH-*S*-2200 in faeces. The enterohepatic recirculation slowed excretion of the *S*-isomer-derived radioactivity (Mikata, 2011).

The proposed metabolic pathway in rats is presented in Fig. 3.

Fig. 3. Proposed metabolic pathway of mandestrobin (*S*-2200) in rats



Source: Kendrick, Farrell & Murphy (2012)

2. Toxicological studies

2.1 Acute toxicity

The results of acute oral, dermal and inhalation toxicity studies with mandestrobin, along with the results of dermal and eye irritation and skin sensitization studies, are summarized in Table 13, and the studies are described in more detail below.

(a) Lethal doses

In a limit test, mandestrobin (purity 93.4%) was administered sequentially to two groups of female Wistar rats (three rats per group) via gavage at 2000 mg/kg bw (in 0.5% weight per volume [w/v] aqueous methylcellulose; dosing volume 10 mL/kg bw). Mortality and clinical signs were recorded at 10 and 30 minutes and 1, 2 and 4 hours after treatment and once daily for 14 days thereafter. Body weights were determined on the day of dosing (day 0), day 7 and day 14.

Table 13. Acute toxicity of mandestrobin

Species	Strain	Sex	Route	Purity (%)	Result	Reference
Rat	Wistar	Female	Oral	93.4	LD ₅₀ > 2 000 mg/kg bw	Asano (2010a)
Rat	Wistar	Male and female	Dermal	93.4	LD ₅₀ > 2 000 mg/kg bw	Asano (2010b)
Rat	Wistar	Male and female	Inhalation	93.4	LC ₅₀ > 4.96 mg/L (maximum attainable concentration)	Deguchi (2010)
Rabbit	New Zealand white	Male	Skin irritation	93.4	Not irritating	Ota (2010a)
Rabbit	New Zealand white	Male	Eye irritation	93.4	Mildly irritating	Ota (2010b)
Guinea-pig	Hartley	Female	Skin sensitization	93.4	Not sensitizing (Magnusson & Kligman)	Ota (2010c)

bw: body weight; LC₅₀: median lethal concentration; LD₅₀: median lethal dose

No mortality was noted during the study. Clinical signs appeared from 4 hours after administration and included stains around the anus and liquid stools containing a white compound; the signs disappeared on day 1. Body weight was not affected, and no gross abnormalities at necropsy were found. The acute oral median lethal dose (LD₅₀) of mandestrobin in female rats was greater than 2000 mg/kg bw (Asano, 2010a).

Five male and five female Wistar rats were treated dermally with mandestrobin (purity 93.4%) spread on a gauze pad moistened with water at a dose of 2000 mg/kg bw. The gauze pad was applied to the shaved skin in an area of approximately 4 cm² × 5 cm², covered with an impermeable sheet and held in place with surgical tape for 24 hours, after which it was removed. Mortality and clinical signs were recorded at 10 and 30 minutes and 1, 2 and 4 hours after initiation of treatment and then daily for 14 days. Body weights were recorded on the day of dosing and again at 7 and 14 days post-dosing.

No mortality and no clinical signs were observed. Body weights of the rats were unaffected. No abnormalities were detected at necropsy. The acute dermal LD₅₀ of mandestrobin in rats was greater than 2000 mg/kg bw for both sexes (Asano, 2010b).

Five male and five female Wistar rats were exposed to a 4-hour nose-only dust aerosol atmosphere of mandestrobin (purity 93.4%) at a concentration of 4.96 mg/L (maximum attainable concentration, analytically and gravimetrically determined). The mass median aerodynamic diameter was 3.80 µm, and the geometric standard deviation was 2.65. Mortality and clinical signs were recorded at 0.5, 1, 2, 3 and 4 hours after initiation of exposure, immediately and hourly for up to 2 hours after termination of exposure, and daily for 14 days thereafter. Body weights were recorded on days 0 (before exposure), 1, 3, 7 and 14 during the observation period.

No mortality was noted. Clinical signs included wet fur and stains around the nose in both sexes, which disappeared within 2 hours post-dosing. This finding is considered to be a result of the restraint procedure and not of toxicity related to treatment. Body weight was unaffected by treatment. No abnormalities were detected at necropsy. Therefore, the acute inhalation median lethal concentration

(LC₅₀) of mandestrobin in rats was greater than 4.96 mg/L (the maximum attainable concentration) for both sexes (Deguchi, 2010).

(b) Dermal irritation

Mandestrobin (purity 93.4%) was applied on 6.25 cm² of clipped skin of three male New Zealand white rabbits at a dose of 0.5 g (moistened with 0.4 mL of corn oil) under a semi-occlusive bandage for 4 hours. The animals were monitored for skin reactions of erythema and oedema using the Draize method at 1, 24, 48 and 72 hours after patch removal.

No clinical signs or skin irritation reactions were observed in any animal during the observation period of 72 hours after the removal of the patches (Ota, 2010a).

(c) Ocular irritation

Mandestrobin (purity 93.4%) was tested in six male albino New Zealand white rabbits for eye irritation. Three animals were used for the unwashed group, and the other three for the washed group. A volume of 0.1 mL (0.062 g by weight) was instilled into the conjunctival sac of the right eye of each animal. The treated eye of each of the three animals in the washed group was flushed after 30 seconds to remove the test material, whereas the treated eye of each of the three animals in the unwashed group was not flushed. The animals were monitored for ocular lesions using the Draize scale at 1, 24, 48 and 72 hours after application.

No clinical signs were noted in any of the animals during the experimental period. In the unwashed group, mandestrobin induced very slight conjunctival redness (grade 1), chemosis (grade 1), discharge (grades 1–2) and corneal opacity (grade: intensity 1, area 1) in all three animals. Additionally, congestion in the iris (grade 1) was observed in two of the three animals. All ocular reactions had disappeared by 72 hours after application. The maximum mean total score of the irritant was 10.3 at 24 hours after application. In the washed group, all animals exhibited conjunctival redness (grade 1) and chemosis (grade 1). All ocular reactions had disappeared by 48 hours after application. Washing effect was observed in the eye irrigation test.

Mandestrobin is judged to be mildly irritating to the eye of the rabbit (Ota, 2010b).

(d) Dermal sensitization

In a dermal sensitization study conducted following the Magnusson and Kligman method, 5% mandestrobin (purity 93.4%) in corn oil was injected intradermally into 20 female Hartley guinea-pigs (with and without Freund's Complete Adjuvant). One week later, a topical induction of 25% mandestrobin in acetone was performed for 48 hours. Two weeks after the topical induction, the animals were challenged by topical application of 25% mandestrobin in acetone for 24 hours. The application site was assessed 24 and 48 hours after patch removal. α -Hexylcinnamaldehyde was used as the positive control.

No dermal response was observed in any animal in the treated group (sensitization rate: 0%). In the control group, no skin reactions were noted in any of the 10 animals, whereas in the positive control group (α -hexylcinnamaldehyde), a positive response was recorded.

Mandestrobin is not sensitizing in guinea-pigs (Ota, 2010c).

2.2 Short-term studies of toxicity

The short-term toxicity of mandestrobin was evaluated in mice, rats and dogs.

*(a) Oral administration**Mice*

In a dose selection study, mandestrobin (purity 99%) was administered to four groups of approximately 6-week-old CD-1 mice (main: 10 mice of each sex per group; satellite: five mice of each sex per group) at a dietary concentration of 0, 350, 3500 or 7000 parts per million (ppm) (equal to 0, 49.2, 501.0 and 1036.8 mg/kg bw per day for males and 0, 63.5, 595.4 and 1306.9 mg/kg bw per day for females, respectively) for 14 days. Stability of the test article and achieved concentration were not evaluated. All animals were observed for viability/mortality and clinical signs daily. Feed consumption was recorded daily, and body weight was determined on days -7, 1, 4, 9 and 14. Blood samples were taken at termination of the study for haematology and clinical chemistry, including iron measurements. All animals were subjected to detailed postmortem gross examination. Organ weights were recorded, and an extensive list of tissues was examined histopathologically. The study did not follow a specific guideline and was not performed under GLP, but it was conducted in the spirit of GLP.

There were no decedents during the study, and clinical observations were generally minor findings considered unrelated to treatment with the test article, such as thinning fur and sores/lesions. Females from the main group given 7000 ppm showed a statistically significant increase in body weight gain relative to controls from day 9 to day 15 and from day 1 to day 15. This finding was considered to be of no toxicological relevance, as no effect on body weight was observed. Haematological investigation showed a statistically significantly higher platelet count (25%) and plasma sodium level (2%) relative to controls in males at 7000 ppm. The statistically significantly higher platelet count was mainly due to one animal (#35). This animal also had a high reticulocyte count, lower haemoglobin concentration, lower red blood cell count and lower packed cell volume in comparison with the rest of the group. These findings are likely associated with the clinical observations and pathological findings that indicate injury to the urogenital area of this animal. Microscopically increased haematopoiesis in the bone marrow and spleen were noted. The group mean platelet count for high-dose males was within the 95% reference range of the historical control data.

Overall, these isolated findings were not considered to be toxicologically relevant.

The unsaturated iron binding capacity in high-dose males was 2-fold lower than in controls, and a statistically significant dose-response relationship in males was indicated. However, none of the group mean differences was statistically significantly different from controls as a result of large standard deviations. This finding is not considered to be toxicologically relevant because of the absence of any similar changes in the iron and total iron binding capacity measurements.

A statistically significant increase in relative liver weight (i.e. adjusted to body weight) was observed in males and females given 7000 ppm (11% and 14%, respectively). In females at 3500 ppm, relative liver weight was statistically significantly increased by 14%. Given the absence of changes in biochemistry or microscopic findings indicative of liver damage, the increase in liver weight was considered to be an adaptive change.

Based on the above results, the no-observed-adverse-effect level (NOAEL) for mandestrobin in this study was 7000 ppm (equal to 1036.8 mg/kg bw per day), the highest dose tested (Shaw, 2009a).

In a 13-week study, mandestrobin (purity 93.4%) was administered to four groups of 7-week-old CD-1 mice (12 mice of each sex per group) at a dietary concentration of 0, 1750, 3500 or 7000 ppm (equal to 0, 204, 405 and 807 mg/kg bw per day for males and 0, 252, 529 and 1111 mg/kg bw per day for females, respectively). Achieved concentrations and homogeneity were verified by chemical analysis. All animals were observed for viability/mortality twice daily. Clinical signs were recorded once daily during the treatment. Feed consumption and body weights were recorded weekly. Blood samples were taken at termination of the study for haematology and clinical chemistry. All animals were subjected to detailed postmortem gross examination. Organ weights (adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes and epididymides, thymus, uterus and cervix) were recorded, and an extensive list of tissues was examined histopathologically.

No mortality or clinical signs were noted during the study. No treatment-related effects on feed consumption were observed. No effects on body weight or body weight gain were noted in males. Small, not statistically significant reductions in body weight gain were recorded in females given 1750 and 3500 ppm; the reduction was statistically significant in females given 7000 ppm (Table 14).

Table 14. Key findings of the 13-week dietary toxicity study in mice

Parameter	Males					Females				
	0 ppm	1 750 ppm	3 500 ppm	7 000 ppm	HCD ^a (mean; range) or DR	0 ppm	1 750 ppm	3 500 ppm	7 000 ppm	HCD ^a (mean; range) or DR
Body weight gain, weeks 0–13 (g)	10.5	9.6	8.9	9.7	–	8.3	7.5	7.4	6.1*	–
Feed consumption, mean, weeks 0–13 (g/mouse per day)	5.2	5.0	4.9	5.0	–	4.6	4.5	4.8	4.9	–
Initial body weight (g)	33.1	34.1	33.8	34.7	–	25.2	24.8	24.7	24.6	–
Terminal body weight (g)	43.6	43.7	42.7	44.4	–	33.5	32.3	32.1	30.7	–
Haematology										
Hb (g/dL)	14.6	14.3	13.2**	14.1	13.4; 12.0–15.1	14.6	14.9	14.8	14.1	–
PCV (%)	44.8	44.6	41.6*	44.9	44.5; 38.0–50.4	45.1	45.8	45.8	44.3	–
PLT (10 ⁹ /L)	1 292	1 756**	1 450	1 666*	1 390; 941–1 721	1 555	1 351	1 390	1 526	–
MCHC (g/dL)	32.3	32.1	31.7	31.5	DR*	32.4	32.5	32.3	31.9	–
Clinical pathology										
GLUC (mmol/L)	8.9	9.7	9.3	9.1	–	10.8	10.3	9.3	9.1*	9.7; 7.9–12.0
Cl (mmol/L)	109	109	109	107	DR*	112	112	112	112	–
Urea (mmol/L)	6.4	6.3	6.7	7.3	DR*	7.1	6.8	6.6	6.6	–
T Bili (µmol/L)	2.5	1.8	2.1	1.8	DR*	1.7	1.7	1.7	1.9	–
Organ weights										
Absolute liver weight (g)	2.00	2.11	2.14	2.35***	–	1.58	1.74	1.64	1.81	–
% change	–	5.5	6.9	17.4	–	–	10.1	3.9	14.5	–
Relative liver weight (%)	4.55	4.73	4.95**	5.22***	–	4.65	5.20*	5.02	5.66***	–
% change	–	4.0	8.8	14.7	–	–	11.8	8.0	21.7	–
Histopathology of liver										
No. examined	12	12	12	12	–	12	12	12	12	–
Focal necrosis	2	1	4	5	–	3	2	6	3	–

Parameter	Males					Females				
	0 ppm	1 750 ppm	3 500 ppm	7 000 ppm	HCD ^a (mean; range) or DR	0 ppm	1 750 ppm	3 500 ppm	7 000 ppm	HCD ^a (mean; range) or DR
Inflammatory cell foci	11	11	11	11	–	12	12	12	12	–
Mitotic figures	0	1	0	0	–	0	0	0	2	–
Hepatocyte vacuolation	0	0	0	0	–	0	1	0	0	–
Glycogen vacuolation	8	8	7	9	–	12	10	11	12	–
Pigmented histiocytes	0	2	0	0	–	0	1	0	1	–
Agonal congestion/ haemorrhage	0	0	1	0	–	0	2	0	0	–
Haematopoiesis	0	1	0	0	–	0	0	0	0	–

Cl: chloride; DR: dose–response test; GLUC: glucose; Hb: haemoglobin; HCD: historical control data; MCHC: mean cell haemoglobin concentration; PCV: packed cell volume; PLT: platelets; ppm: parts per million; T Bili: total bilirubin; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (analysis of variance, dose–response and Dunnett's)

^a Historical control data: five studies, 34 animals of same strain and age housed at this laboratory, from 2003.

Source: Beck (2011a)

Mandestrobin administration caused no obvious or adverse effects on haematology or clinical chemistry parameters. Statistical analysis revealed the following statistically significant changes: decreases in haemoglobin concentration and packed cell volume in males given 3500 ppm, and increased platelets in males given 1750 and 7000 ppm. However, these effects were not dose related and in general were within or close to historical control ranges, and therefore they were not considered to be treatment related. The statistically significant dose-responsive reduction in mean cell haemoglobin concentration noted in males was also considered not to be treatment related, as the differences observed were small and not statistically significant for pairwise comparison. In clinical chemistry, a significant decrease in mean glucose was noted in 7000 ppm females, but it was not considered to be of toxicological relevance because, although noted at the highest dose, the value was within the historical control range. Dose-responsive changes were noted for chloride, urea and total bilirubin, but were considered not to be treatment related, as the changes were minor and not statistically significant for any of the individual doses (Table 14).

A statistically significant increase in relative spleen weight was noted in males given 3500 ppm; however, in the absence of a dose–response relationship, the change was not considered to be treatment related. Increases in absolute and relative liver weights were noted in both sexes of all treated groups; however, these increases were not accompanied by any clinical chemistry or histopathological findings. As a result, the observed increased liver weight is considered to be an adaptive change related to mandestrobin metabolism and not an adverse toxic effect.¹ No other treatment-related macroscopic or microscopic findings were noted.

The NOAEL was 3500 ppm (equal to 529 mg/kg bw per day), based on reduced body weight gain in females at 7000 ppm (equal to 1111 mg/kg bw per day) (Beck, 2011a).

¹ The liver weight increase is considered to be due to liver enzyme induction (cytochrome P450 2B [CYP2B]), via activation of the constitutive androstane receptor (CAR) by mandestrobin, as evidenced by mode of action work presented in section 2.6(c) below.

Rats

Mandestrobin (purity 93.4%) was administered to five groups of 7-week-old Wistar rats (12 rats of each sex per group) at a dietary concentration of 0, 800, 4000, 10 000 or 20 000 ppm (equal to 0, 54, 283, 743 and 1545 mg/kg bw per day for males and 0, 62, 320, 788 and 1886 mg/kg bw per day for females, respectively) for 90 days. Achieved concentrations and homogeneity were verified by analysis. All animals were checked for overt clinical signs by daily general cage-side observations. Detailed clinical observations were performed once weekly during the study. Ophthalmological examination was performed on all animals in the control (0 ppm) and high-dose (20 000 ppm) groups in week 12. Body weights and feed consumption were measured weekly during the study. Functional observational battery (FOB) tests were performed weekly on all animals during the study; motor activity assessment was performed in week 12. Blood samples were taken after fasting at termination of the study for assessment of blood biochemical and haematological parameters. Urine analyses were conducted in the first six numbered males and females in week 12. All animals were subjected to detailed postmortem gross examination, and all abnormalities were recorded. Organ weights (adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes and epididymides, thymus and uterus) were recorded. Histological evaluation was performed on an extensive list of organs and tissues.

One female from the 4000 ppm group died; although the cause of death could not be established, microscopic findings suggested that the death was not treatment related. There were no treatment-related clinical signs during the study. There was no treatment-related effect on feed consumption at any dose. Reduced body weight (9% for males and 4% for females) and body weight gain (15% for males and 11% for females) were recorded at the end of treatment for both sexes given 20 000 ppm (Table 15). No treatment-related differences between the controls and any treated group were noted in urine analysis, motor activity or ophthalmological examination. Forelimb grip strength was statistically significantly reduced in male animals in the highest-dose group. In the absence of any other effect, this isolated finding in one sex was considered incidental. Some changes in haematological parameters attained statistical significance at the highest dose; however, the changes were minor and within the range of historical control data.

Table 15. Key body weight, FOB, clinical chemistry and haematological findings of the 90-day dietary toxicity study in rats

Finding	Males						Females					
	0 ppm	800 ppm	4 000 ppm	10 000 ppm	20 000 ppm	HCR ^a or DR	0 ppm	800 ppm	4 000 ppm	10 000 ppm	20 000 ppm	HCR ^a or DR
Body weight (g)												
Week 0	195.3	193.3	188.2	189.9	189.6	–	150.5	145.7	149.7	146.2	149.3	–
Week 13	383.4	397.4	381.2	373.8	349.9	DR**	233.7	225.0	225.6	231.6	223.5	–
Body weight gain (g)												
Weeks 0–13	188.1	204.1	193.0	183.9	160.2	DR**	83.1	79.3	78.6	85.5	74.2	–
Functional observational battery												
Mean forelimb grip strength (kg)	1.023	1.008	1.052	1.070	0.760*	–	0.751	0.787	0.707	0.757	0.742	–
SD	0.270	0.253	0.292	0.149	0.235	–	0.207	0.178	0.205	0.264	0.233	–
Mean hindlimb grip	0.613	0.642	0.625	0.732	0.665	–	0.620	0.612	0.607	0.620	0.633	–

Finding	Males						Females					
	0 ppm	800 ppm	4 000 ppm	10 000 ppm	20 000 ppm	HCR ^a or DR	0 ppm	800 ppm	4 000 ppm	10 000 ppm	20 000 ppm	HCR ^a or DR
strength (kg)												
SD	0.126	0.191	0.178	0.132	0.118	–	0.124	0.157	0.161	0.117	0.373	–
Haematology												
Hb (g/dL)	16.7	16.5	16.5	16.6	16.2**	14.5–18.0	15.7	15.7	15.3	15.7	15.0*	14.2–17.0
MCH (pg)	18.2	18.1	18.0	17.9	17.5*	16.6–19.8	18.8	18.4	18.6	18.8	18.4	–
MCHC (g/dL)	35.5	35.4	34.9	35.1	34.6*	31.5–36.2	34.9	34.3	34.5	34.0	34.2	–
Neutrophils (10 ⁹ /L)	1.3	1.0	1.1	1.2	0.9**	0.5–3.0	0.6	0.5	0.6	0.6	0.6	–
Monocytes (10 ⁹ /L)	0.2	0.2	0.2	0.1	0.1*	0.0–0.3	0.1	0.1	0.1	0.1	0.1	–
Clinical chemistry												
AST (IU/L)	75	65*	66	69	62**	50–87	68	65	64	62	56**	49–103
ALT (IU/L)	46	33*	32*	39	32*	26–69	29	29	32	30	28	–
GGT (IU/L)	2	2	2	2	5**	–	2	2	2	3	4**	–
Na (mmol/L)	145	144*	144	144*	145	132–148	144	143	143	143	144	–
K (mmol/L)	4.7	4.8	4.7	4.7	4.9	–	4.1	4.1	4.1	4.7**	4.7**	3.5–5.0
Ca (mmol/L)	2.71	2.67	2.66	2.71	2.74	–	2.73	2.71	2.71	2.85**	2.88***	2.54–2.91
Total protein (g/L)	69	70	70	71	71	–	71	70	70	74	77**	63–78
Albumin (g/L)	45	45	46	47	47	DR**	48	49	47	50	51*	34–56
Total bilirubin (µmol/L)	2.1	2.0	1.5*	1.4*	1.4**	0.5–2.9	2.1	1.8	1.4**	2.0	2.2	1.0–3.5
Total cholesterol (mmol/L)	2.0	2.2	2.3	2.6**	2.8***	–	1.5	1.7	1.8	2.5***	2.6***	–
Creatinine (µmol/L)	37	35	35	36	36	–	40	40	38	40	34**	26–51

ALT: alanine aminotransferase; AST: aspartate aminotransferase; DR: significant dose–response test; GGT: gamma-glutamyl transferase; Hb: haemoglobin; HCR: historical control range; IU: international units; MCH: mean cell haemoglobin; MCHC: mean cell haemoglobin concentration; ppm: parts per million; SD: standard deviation; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

^a Historical control data from studies performed between March 2005 and January 2008. For haematology and clinical chemistry, 15 different studies were available (203 and 223 animals, respectively).

Source: Beck (2011b)

Total cholesterol was increased (up to 73%) compared with controls, and the increase was statistically significant in both sexes receiving 10 000 and 20 000 ppm. Gamma-glutamyl transferase (GGT) activity was increased by 2–2.5 times compared with controls, and the increase was statistically significant in both sexes given 20 000 ppm. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were statistically significantly decreased in some male treated groups (approximately 18% or less), but there was no dose–response relationship, and the decreased values were within the historical control range. Moreover, the apparent differences may be due to high control values, although they are also within the historical control range. Values for AST and ALT activities in treated males were similar to control female values. Therefore, in this context, the decreases in AST and ALT activities noted in males were not considered to be of any toxicological relevance. A statistically significant decrease in AST activity (18%) was noted in 20 000 ppm females; however, this was also within the historical control range and therefore was not considered to be toxicologically relevant. All other changes in clinical chemistry parameters were mild, within the range of historical control data, inconsistent between sexes and/or with no clear dose–response relationship.

Organ weight effects were noted only in the liver, for which increased absolute and/or relative weights were recorded in males of the 4000, 10 000 and 20 000 ppm groups and females of the 10 000 and 20 000 ppm groups. Mottled liver was also noted in males given 4000, 10 000 and 20 000 ppm. These changes were accompanied by hepatocyte hypertrophy in both sexes at 4000, 10 000 and 20 000 ppm. Incidence and severity increased with dose and correlated with large and/or mottled liver. Hepatocyte hypertrophy was characterized by hepatocytes with increased levels of pale, slightly granular cytoplasm. In the most severely affected livers, hypertrophy was recorded in hepatocytes in all zones of the liver. In less severely affected livers, there was no clear zonal distribution of the enlarged hepatocytes. Agonal congestion/haemorrhage was recorded for some treated males and was considered to be a consequence of the liver hypertrophy by the study author (Table 16).

Table 16. Key macroscopic and microscopic findings of the 90-day dietary toxicity study in rats

Finding	Males					Females				
	0 ppm	800 ppm	4 000 ppm	10 000 ppm	20 000 ppm	0 ppm	800 ppm	4 000 ppm	10 000 ppm	20 000 ppm
Body weight (g)	370.0	381.5	364.5	354.1	339.2	227.1	219.8	220.6	22.9	218.5
Absolute liver weight (g)	8.36	9.03	9.50***	10.18***	12.02***	5.80	5.53	6.08	6.79	8.34***
% change	–	7.9	13.6	21.8	43.8	–	–4.7	4.8	17.0	43.8
Relative liver weight (%)	2.26	2.37	2.61***	2.89***	3.54***	2.55	2.51	2.76	3.05***	3.81***
% change	–	4.9	15.5	27.9	56.6	–	–1.6	8.2	19.6	49.4
Macroscopic pathology: liver										
No. examined	12	12	12	12	12	12	12	11	12	12
Large	0	0	2	7	10	0	0	0	0	4
Mottled	0	0	1	3	5	0	0	0	0	0
Microscopic pathology: liver										
No. examined	12	12	12	12	12	12	12	11	12	12
Hepatocyte hypertrophy ^a										
Grade –	12	12	0	0	0	12	12	7	3	0
Grade 1	0	0	5	0	0	0	0	4	6	0
Grade 2	0	0	7	0	0	0	0	0	3	0

Finding	Males					Females				
	0 ppm	800 ppm	4 000 ppm	10 000 ppm	20 000 ppm	0 ppm	800 ppm	4 000 ppm	10 000 ppm	20 000 ppm
Grade 3	0	0	0	7	0	0	0	0	0	0
Grade 4	0	0	0	5	1	0	0	0	0	3
Grade 5	0	0	0	0	11	0	0	0	0	9
Agonal congestion/haemorrhage										
p ^b	0	0	1	3	5	2	0	0	0	0
Microscopic pathology: thyroid										
Follicular cell hypertrophy ^a										
<i>No. examined</i>	12	12	12	12	12	12	12	11	12	12
Grade –	10	10	6	3	5	10	11	7	7	6
Grade 1	2	2	6	8	5	2	1	4	5	6
Grade 2	0	0	0	1	2	0	0	0	0	0
Microscopic pathology: kidney										
Hyaline droplets ^a										
<i>No. examined</i>	12	12	12	12	12	12	0	0	0	12
Grade –	2	3	1	1	0	12	0	0	0	12
Grade 1	6	6	6	3	2	0	0	0	0	0
Grade 2	4	3	4	7	8	0	0	0	0	0
Grade 3	0	0	1	1	2	0	0	0	0	0
Kidney α_{2u} -globulin-positive reaction by immunohistochemistry ^a										
<i>No. examined</i>	2	0	0	0	2	0	0	0	0	0
Grade –	0	0	0	0	0	0	0	0	0	0
Grade 1	1	0	0	0	0	0	0	0	0	0
Grade 2	1	0	0	0	0	0	0	0	0	0
Grade 3	0	0	0	0	2	0	0	0	0	0

ppm: parts per million; ***: $P < 0.001$ (analysis of variance, dose–response and Dunnett's, Kruskal-Wallis, Jonckheere-Terpstra or Wilcoxon)

^a “–”: finding not present, 1 = minimal, 2 = slight, 3 = moderate, 4 = moderately severe, 5 = severe.

^b P = finding recorded as present (not graded).

Source: Beck (2011b)

The increased liver weights with hepatocyte hypertrophy induced by mandestrobin treatment at and above 4000 ppm are likely a result of the induction of liver enzymes.² Nevertheless, in animals given 10 000 and 20 000 ppm, several clear alterations in blood biochemistry (i.e. increased total cholesterol levels at and above 10 000 ppm and GGT activity at 20 000 ppm) were noted and considered to be evidence of an adverse effect on the liver. Consequently, the liver alterations observed at 10 000 and 20 000 ppm were considered to be toxicologically relevant for humans, but alterations at 4000 ppm were not.

² Mandestrobin increased the activities of 7-pentoxoresorufin *O*-depentylase (PROD, a CYP2B marker) and thyroxine (T₄)-uridine diphosphate glucuronosyltransferase (UGT) in rat liver, with a mode of action identified as being due, at least in part, to CAR activation, similar to phenobarbital (see section 2.6(c) below).

In the thyroid, an increased incidence of follicular cell hypertrophy was noted in animals given 4000, 10 000 and 20 000 ppm. The incidence was higher and the grade was more severe in the males. Follicular cell hypertrophy was characterized by a diffuse increase in the height of the follicular epithelium, generally with a minor reduction in the level of colloid present. However, follicular cell hypertrophy in the rat is generally considered to be an adaptive response to increased thyroid hormone metabolism by the liver and is commonly associated with liver cell hypertrophy. The thyroid follicular cell hypertrophy observed at and above 4000 ppm is consistent with the observed liver alterations, including increased liver weights and hepatocyte hypertrophy. Furthermore, there is evidence in the general literature (Capen et al., 1999; Meek et al., 2003; Cohen et al., 2004) on the biochemical and physiological differences in thyroid function that indicate differences in susceptibility to alteration in thyroid hormone metabolism between rats and humans. Taken together, the thyroid changes induced by mandestrobin are not considered to be toxicologically relevant for humans.³

In the kidney, the severity of hyaline droplets was higher in males given 10 000 or 20 000 ppm; the lesion was characterized as densely eosinophilic, variably sized droplets in the cytoplasm of proximal tubular cells. Immunohistochemistry was performed on two control males and two males given 20 000 ppm, and all were positive for α_{2u} -globulin. The positive intensity for the α_{2u} -globulin was higher in the two males given 20 000 ppm compared with the controls. Hyaline droplets are a common background finding in the kidney of male rats. They generally represent accumulations of α_{2u} -globulin, a naturally occurring male rat protein. This male rat-specific hyaline droplet nephropathy is of little relevance for humans. Therefore, this finding was excluded from consideration for identifying the NOAEL in this study.

The NOAEL was 4000 ppm (equal to 283 mg/kg bw per day), based on increased liver weights accompanied by hepatocyte hypertrophy, increased total cholesterol and thyroid follicular hypertrophy in both sexes at 10 000 ppm (equal to 743 mg/kg bw per day) (Beck, 2011b).

Dogs

In a palatability study, mandestrobin (purity unknown) was administered to two groups of beagle dogs (one of each sex per group) at a dietary concentration of 12 500 or 25 000 ppm (equal to 448 and 1088 mg/kg bw per day for males and 451 and 970 mg/kg bw per day for females, respectively) for 7 days. Animals were observed daily for clinical signs, feed consumption and body weight. No statistical evaluation was performed.

Clinical observations were limited to soft faeces in two animals on day 1. Males were observed to be thin during week 1. These signs are unlikely to be due to treatment and are more consistent with eating and adapting to moistened diet.

Body weights showed no evidence of any adverse treatment-related effect. Three out of four dogs failed to maintain their starting body weight, but this appeared to be due to the addition of water to the diet. The same dogs lost weight during the acclimatization period (week –1) while on moistened control diet. Three out of four animals ate their entire allowance of food throughout the acclimatization and study periods (Shaw, 2008).

In a dose range-finding study, mandestrobin (purity 99%) was administered to four groups of approximately 5-month-old beagle dogs (one of each sex per group) at a dietary concentration of 0, 1000, 10 000 or 20 000 ppm (equal to 0, 45.2, 440.3 and 915.8 mg/kg bw per day for males and 0, 46.7, 478.6 and 992.2 mg/kg bw per day for females, respectively) for 4 weeks. Detailed clinical observations and feed consumption were recorded daily, and body weight was measured twice weekly and on the

³ In the studies by Asano (2012e) and Yamada (2012a) in section 2.6(c) below, the rat-specific mode of action for the thyroid response was identified as being due, at least in part, to hepatic CAR activation and subsequent enhancement of serum T₄ clearance by induced T₄-UGT. Reduced serum T₄ and increased thyroid stimulating hormone (TSH) were measured in the mode of action study in rats (Asano, 2012e) and are the most likely mode of action for the thyroid follicular cell hyperplasia seen in the study by Beck (2011b).

day of necropsy. Blood collection after fasting or before dosing was performed on all animals before the start of treatment and at weeks 2 and 4. Urine analysis was performed on all animals before the start of treatment and at weeks 2 and 4. Ophthalmoscopy was not performed. All animals were subjected to necropsy, and the following organs were weighed: adrenals, brain, epididymides, heart, kidneys, liver, ovaries, pituitary, prostate, spleen, testes, thymus, thyroid and parathyroids, and uterus including cervix. An extensive list of tissues was examined histologically. The study did not follow a specific guideline and was not performed under GLP, although it was conducted in the spirit of GLP. No statistical evaluation was performed.

All animals survived, and no treatment-related clinical signs were observed. Post-dosing observations were limited to soft/liquid faeces and vomiting. These observations were limited to four animals, including two controls and two treated animals. The treated animals were observed with soft faeces on only two occasions (on day 11, 4 hours after feeding, and on day 27, 1 hour after feeding). Body weight was not affected by treatment. Body weight gain was reduced in males fed diets containing 10 000 or 20 000 ppm mandestrobin. No treatment-related effects on feed consumption were observed. No treatment-related effects on haematological parameters were apparent in either week 2 or week 4. Animal 3M had noticeably higher ALT levels relative to controls in weeks 2 and 4. All other treated animals had ALT levels similar to those of controls in weeks 2 and 4. Animal 3M also had higher levels of AST and alkaline phosphatase (ALP) relative to controls in weeks 2 and 4. Both high-dose animals (4M and 8F) had higher levels of ALP in week 4 compared with controls, and 4M also had higher levels of ALP in week 2.

Increased iron in males given 10 000 or 20 000 ppm mandestrobin in the diet in week 4 and decreased unsaturated iron binding capacity in the high-dose male in weeks 2 and 4 and in the high-dose female in week 4 were observed. However, the toxicological relevance of these changes was unclear.

No treatment-related effects on urinary parameters were apparent.

In males, a dose-related increase in organ weights was observed for adrenals, kidney, liver, brain and testes. Pituitary and thyroid/parathyroid weights were also higher than in controls from 10 000 ppm. In females, liver weights were higher than those of controls at 10 000 ppm and above, and kidney and spleen weights were lower than those of controls, in a dose-dependent manner. Uterus, ovaries, adrenals and thymus weights were lower than those of controls at 20 000 ppm. Besides liver, no correlated pathological findings were observed for any of the above-mentioned organs; therefore, the changes in organ weights were considered to be of no toxicological relevance. Macroscopically large liver was recorded in males given 20 000 ppm. The microscopic investigation showed increased hypertrophy (characterized by a ground glass appearance in the midzonal and centrilobular areas) in both sexes fed 20 000 ppm. Given the shortcomings of the study (e.g. low number of animals per group), the study is not considered suitable for the identification of a NOAEL (Shaw, 2009b).

Mandestrobin (purity 93.4%) was administered to four groups of four male and four female beagle dogs (aged 5–6 months) at a dietary concentration of 0, 4000, 12 000 or 40 000 ppm (equal to 0, 90.9, 267.8 and 933.1 mg/kg bw per day for males and 0, 102.7, 304.4 and 820.4 mg/kg bw per day for females, respectively) for 13 weeks. Achieved concentrations and homogeneity were verified by analysis. Detailed clinical observations and feed consumption were recorded daily, whereas body weight was measured weekly. Ophthalmoscopy was performed once pretest and at week 12. A battery of behavioural tests and observations was performed on all animals before initiation of treatment and once weekly thereafter. Blood collection after fasting was performed on all animals before the start of treatment and at weeks 4, 8 and 13 of the administration period. Urine analysis was performed on all animals before the start of treatment and at weeks 3, 7 and 12 of the administration period. Detailed necropsy was performed on all animals after scheduled termination, and the following organs were weighed: adrenals, brain, heart, kidneys, liver, ovaries, pituitary, prostate, spleen, testes, epididymides, thymus, thyroids (with parathyroids) and uterus (including cervix). An extensive list of tissues was examined histologically. Liver and kidneys were also evaluated by electron microscopy.

No mortality and no differences in ophthalmoscopy or urine analysis were noted. Thin appearance was noted in three males and one female given 40 000 ppm mandestrobin in the diet. Clinical signs included red eyes/ears/mouth, sores/lesions and earwax; however, they were considered incidental, as they were either infrequent or noted at a comparable rate in the concurrent control animals. Post-dosing observations were restricted to faecal abnormalities and emesis. Soft, mucoid or liquid faeces were noted sporadically over the dosing phase in males and females given 4000 ppm and above up to 8 hours following presentation of the diets. Emesis was seen in two males given 40 000 ppm in week 1, up to 8 hours following presentation of the diets. Based on their low and irregular incidence, faecal abnormalities and emesis were considered not to be treatment related.

Statistically significant decreases in body weight and body weight gain were recorded in both sexes at 40 000 ppm. Males gained notably less weight than controls over the 13-week period, with individual growth rates for treated animals being variable, ranging from a loss of 7.5% of body weight to a gain of 11.3%. Following 13 weeks of treatment, most females at 40 000 ppm had similar or slightly lower body weights compared with the initial values. Reduced feed consumption was noted only at 40 000 ppm in both sexes, from the fourth week of treatment (Table 17).

Occasional statistically significant differences in mean body temperature were recorded for both sexes at 12 000 ppm and for males at 40 000 ppm. Statistically significant differences in mean heart rate were recorded sporadically for 4000 ppm males and 12 000 ppm females; the effect was dose responsive for females in week 13. However, all these changes were transient, inconsistent and therefore not considered to be treatment related. Weekly neurological examinations revealed incidences of abnormal proprioceptive paw positioning, abnormal righting postural reactions, abnormal hemihopping postural reactions, abnormal wheelbarrowing postural reactions and decreased muscle tone in a few males or females at 40 000 ppm and protrusion of the nictitating membrane in males and females at and above 4000 ppm. In addition, there were single occurrences of abnormal hemihopping/wheelbarrowing postural reactions in one female at 4000 ppm in week 9 and abnormal proprioceptive paw positioning or abnormal hemihopping postural reactions in animals fed 12 000 ppm mandestrobin in week 4. These recorded observations were generally infrequent, occasionally observed in control animals and considered in some cases likely to be a result of the reduced body weight. Overall, no neurotoxicological effect related to mandestrobin was noted.

Increased platelet count was noted in 40 000 ppm males in week 4 and in both sexes at 40 000 ppm in weeks 8 and 13. A statistically significant increase in mean white blood cell count (1.34-fold) was seen in males given 40 000 ppm in week 13, mediated by increases in both neutrophils (1.55-fold) and monocytes (1.83-fold), compared with concurrent controls. It is noted that these changes were within historical control ranges and were comparable to pretest values. At 12 000 ppm and above, increased ALT, ALP and GGT activities were observed in both sexes. Increased AST activity and triglyceride levels and decreased albumin, albumin to globulin ratio, total cholesterol and glucose were observed at 40 000 ppm only, in both sexes (Table 17).

Treatment-related organ weight changes in kidney, brain, spleen, liver, thymus, heart, pituitary, prostate, testes/epididymides and uterus were observed, when compared with concurrent controls (Table 17). In males at 40 000 ppm, absolute and relative spleen weights were increased by 1.48 and 1.92 times, respectively, when compared with concurrent controls. No macroscopic or microscopic correlates were present, and mean values were generally within the historical control reference range (absolute: 21.440–81.380 g; relative: 0.1883–0.6848%; $n = 103$). Relative kidney weights were increased in both sexes (males: 1.30-fold; females: 1.33-fold) at 40 000 ppm, compared with controls. Relative brain weights (1.33-fold, both sexes) were increased at 40 000 ppm, compared with controls. Mean relative pituitary weights were increased in females offered 40 000 ppm. Changes in relative kidney weight, relative brain weight and relative pituitary weight were likely related to the body weight loss in these animals, as the absolute weights were not statistically significantly different, no changes were observed in related clinical chemistry parameters and there were no correlated macroscopic or microscopic findings. Absolute heart weight in males decreased (0.74-fold) at 40 000 ppm. Decreased heart weight was probably related to body weight loss in these animals, as the relative weights were not

Table 17. Key results of the 13-week dietary toxicity study in dogs: haematology, clinical chemistry and organ weights

	Males					Females				
	0 ppm	4 000 ppm	12 000 ppm	40 000 ppm	HCD ^a (mean; range) or DR	0 ppm	4 000 ppm	12 000 ppm	40 000 ppm	HCD ^a (mean; range) or DR
Terminal body weight (kg)	14.07	13.57	13.61	11.16**	—	12.36	11.94	11.93	9.03***	—
Body weight gain, weeks 0–13 (kg)	2.84	2.86	2.55	0.14***	—	3.01	2.38	2.47	−0.57***	—
Feed consumption (g/animal per week)	2 110	2 111	2 111	1 947***	—	2 100	2 109	2 058	1 430***	—
Haematology (week 13)										
WBC (10 ⁹ /L)	9.4	9.6	9.9	12.6*	11.5; 8.0–16.6	9.1	9.6	10.2	10.6	—
Neutrophils (10 ⁹ /L)	4.7	5.1	4.9	7.3**	6.6; 4.2–10.9	4.9	5.2	5.2	5.4	—
Monocytes (10 ⁹ /L)	0.6	0.6	0.6	1.1*	0.5; 0.0–1.1	0.6	0.5	0.5	0.6	—
Platelets (10 ³ /μL)	314	375	366	454	—	285	364	348	481*	—
Blood chemistry (week 13)										
AST (IU/L)	32	37	36	50***	—	30	31	39	47**	—
ALT (IU/L)	37	30	103	215***	—	37	44	121	198**	—
ALP (IU/L)	98	105	171*	288***	—	85	114	195***	297***	—
GGT (IU/L)	4	3	3	10*	—	3	4	5	8*	—
Triglycerides (mmol/L)	0.34	0.43	0.42	0.62**	—	0.38	0.39	0.42	0.67**	—
Albumin (g/L)	36	32	34	27***	—	37	37	35	29***	—

	Males					Females				
	0 ppm	4 000 ppm	12 000 ppm	40 000 ppm	HCD ^a (mean; range) or DR	0 ppm	4 000 ppm	12 000 ppm	40 000 ppm	HCD ^a (mean; range) or DR
A/G	1.8	1.6	1.7	1.0***	—	2.4	2.0	2.0	1.4***	—
Cholesterol (mmol/L)	6.7	5.5	5.6	3.2***	—	6.7	6.1	5.2	4.9*	—
Globulin (g/L)	20	22	20	29**	—	16	18	18	22	DR*
BIL (µmol/L)	2.2	2.2	1.8	2.9	—	2.0	2.0	1.4	2.9	—
GLU (mmol/L)	5.8	5.8	5.9	4.6***	—	5.7	5.9	5.9	5.0*	—
Absolute organ weights (g)										
Kidney	65.7	61.0	63.0	67.7	—	51.7	52.8	51.6	49.3	—
Liver	496.0	504.6	570.1	565.1	—	364.3	451.5***	453.2***	387.5**	—
Brain	82.8	84.3	81.8	85.2	—	78.9	75.8	75.8	71.8	—
Thymus	15.8	13.2	12.8	6.9	—	15.4	15.5	15.2	6.6	—
Spleen	47.2	44.4	46.2	69.9	37.3; 21.4– 81.4	46.1	44.7	53.7	40.6	—
Heart	108.0	100.2	107.7	79.6**	—	90.2	96.3	93.9	71.4	—
Pituitary	0.094	0.097	0.084	0.077	—	0.070	0.077	0.076	0.065	—
Prostate	4.47	3.88	2.96	1.16*	—	—	—	—	—	—
Testes/ epididymides	28.86	21.88	24.51	20.53	—	—	—	—	—	—
Uterus	—	—	—	—	—	4.19	5.62	5.63	2.7	11.3; 1.6– 51.7
Relative organ weights (%)										
Kidney	0.467	0.449	0.465	0.608*	—	0.416	0.447	0.432	0.552*	—
Liver	3.5	3.7	4.2	5.1***	—	2.9	3.8***	3.8***	4.3***	—

	Males					Females				
	0 ppm	4 000 ppm	12 000 ppm	40 000 ppm	HCD ^a (mean; range) or DR	0 ppm	4 000 ppm	12 000 ppm	40 000 ppm	HCD ^a (mean; range) or DR
Brain	0.6	0.6	0.6	0.8**	—	0.6	0.6	0.6	0.8*	—
Thymus	0.112	0.097	0.094	0.060*	—	0.122	0.132	0.128	0.071*	—
Spleen	0.334	0.327	0.336	0.640*	0.188– 0.685	0.367	0.381	0.456	0.463	—
Heart	0.8	0.7	0.8	0.7	—	0.7	0.8	0.8	0.8	—
Pituitary	0.000 67	0.000 72	0.000 62	0.000 65	—	0.000 55	0.000 65	0.000 65	0.000 7**	—
Prostate	0.032	0.029	0.022	0.011*	—	—	—	—	—	—
Testes/ epididymides	0.20	0.16	0.18	0.18	—	—	—	—	—	—
Uterus	—	—	—	—	—	0.034	0.047	0.048	0.030	0.018– 0.466

A/G: albumin/globulin; ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; BIL: bilirubin; DR: dose–response test; GGT: gamma-glutamyl transferase; GLU: glucose; HCD: historical control data; IU: international units; ppm: parts per million; WBC: white blood cells; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (analysis of variance, dose–response and Dunnett's)

^a Historical control data from studies performed between January 1998 and March 2007. For haematology and clinical chemistry, data were obtained from 4–39 different studies and from 33–306 animals.

Source: Beck (2012a)

statistically significantly lower and there were no correlated macroscopic or microscopic findings. Absolute thymus weights (males: 0.44-fold; females: 0.43-fold) and relative thymus weights (males: 0.54-fold, females: 0.58-fold) were reduced for males (particularly animals 13M and 14M) and females given 40 000 ppm, compared with the concurrent controls. Thymus involution/atrophy was noted and characterized by a decreased cortex to medulla ratio, and an increase in tingible body macrophages was noted in some cases. As no microscopic findings were seen at 4000 ppm and no macroscopic or microscopic findings were seen at 12 000 ppm, the macroscopic change in the thymus at 4000 ppm is not considered to be biologically relevant. Increased mean liver weights were noted in males given 12 000 ppm and above and in females given 4000 ppm and above.

Absolute (0.26-fold) and relative (0.34-fold) prostate weights were reduced in males at 40 000 ppm. In addition, a decrease was recorded for two males (6M and 8M) given 4000 ppm and two males (9M and 12M) given 12 000 ppm. The prostate weight of one male (4M) was lower than those of the other animals in the control group. Lower absolute and/or relative testes/epididymides weights were recorded for males (6M, 8M, 10M, 13M, 14M and 16M) given 4000 ppm and above, compared with concurrent controls. A decrease in absolute and relative uterus weights was recorded for two females (29F and 32F) at 40 000 ppm, compared with concurrent controls.

Evidence of delayed sexual maturity in males (prostate, testes/epididymides) and females (ovaries) was noted. The prostate was immature in all males at 40 000 ppm and pubescent in one control male, two males at 4000 ppm and two males at 12 000 ppm. Immaturity was characterized by small, cuboidal acinar cells with no eosinophilic apical cytoplasm and little or no secretion. Pubescence was characterized by the development of a small amount of eosinophilic apical cytoplasm in some acini, with small amounts of secretion. The testes were immature in two males at 40 000 ppm. The testes were pubescent in one or two males from each group, including controls. Severe oligospermia was present in the epididymides of three males at 40 000 ppm, and minimal oligospermia, due to pubescence, was present in one male at 12 000 ppm. Immaturity was characterized by the absence of spermatids within seminiferous tubules and oligospermia in the epididymis. Pubescence was characterized by some spermatids in seminiferous tubules and epididymides. The incidence of pubescence was similar across control and treated groups. The ovaries of two females at 40 000 ppm were immature. The ovaries of all other animals in the study were pubescent. Immaturity was characterized by the absence of secondary or Graafian follicles or corpora lutea. The changes in prostate, testes/epididymides, ovaries and thymus generally correlated with organ weights and macroscopic observations. Decreased uterus weights in two females at 40 000 ppm were related to the body weight losses in these animals, as the absolute and relative weights were within historical control ranges and no gross or histopathological findings were observed; therefore, this finding was not considered to be toxicologically relevant (Table 18).

Table 18. Key results of the 13-week dietary toxicity study in dogs: macroscopic findings and histopathology

Finding	Incidence of finding							
	Males				Females			
	0 ppm	4 000 ppm	12 000 ppm	40 000 ppm	0 ppm	4 000 ppm	12 000 ppm	40 000 ppm
Macroscopic findings								
Animal thin	0/4	0/4	0/4	3/4	0/4	0/4	0/4	1/4
Liver – dark	0/4	0/4	2/4	4/4	0/4	0/4	2/4	4/4
Liver – large	0/4	0/4	3/4	3/4	0/4	0/4	1/4	0/4
Gall bladder – choleliths	0/4	0/4	0/4	4/4	0/4	0/4	0/4	3/4
Gall bladder – distention	0/4	0/4	0/4	2/4	0/4	1/4	0/4	1/4
Prostate – small	0/4	2/4	2/4	4/4	–	–	–	–

Finding	Incidence of finding							
	Males				Females			
	0 ppm	4 000 ppm	12 000 ppm	40 000 ppm	0 ppm	4 000 ppm	12 000 ppm	40 000 ppm
Thymus – small	0/4	1/4	0/4	2/4	0/4	0/4	0/4	1/4
Histopathology								
Liver								
Pigment	0/4	0/4	3/4	4/4	0/4	0/4	3/4	3/4
Periportal/centrilobular fibrosis	0/4	0/4	0/4	4/4	0/4	0/4	0/4	1/4
Centrilobular degeneration	0/4	0/4	2/4	3/4	0/4	0/4	4/4	4/4
Centrilobular hepatocellular swelling	0/4	0/4	3/4	1/4	0/4	3/4	3/4	0/4
Bridging fibrosis	0/4	0/4	0/4	1/4	0/4	0/4	0/4	0/4
Hepatocyte vacuolation	0/4	0/4	1/4	0/4	0/4	0/4	0/4	1/4
Gall bladder								
Choleliths	0/4	0/4	0/4	4/4	0/4	0/4	0/4	1/4
Adherent bile	2/4	0/4	1/4	4/4	0/4	1/4	1/4	2/4
Prostate								
Immature	0/4	0/4	0/4	4/4	–	–	–	–
Pubescent	1/4	2/4	2/4	0/4	–	–	–	–
Mature	3/4	2/4	2/4	0/4	–	–	–	–
Testes								
Immature	0/4	0/4	0/4	2/4	–	–	–	–
Pubescent	2/4	1/4	1/4	1/4	–	–	–	–
Mature	2/4	3/4	3/4	1/4	–	–	–	–
Segmental hypoplasia	1/4	3/4	0/4	1/4	–	–	–	–
Epididymides								
Oligospermia/spermatocytes	0/4	0/4	1/4	3/4	–	–	–	–
Ovaries								
Immature	–	–	–	–	0/4	0/4	0/4	2/4
Pubescent	–	–	–	–	4/4	4/4	4/4	2/4
Thymus								
Involution/atrophy	0/4	0/4	0/4	2/4	0/4	0/4	0/4	2/4
Agonal congestion/haemorrhage	2/4	0/4	1/4	0/4	0/4	0/4	0/4	1/4

ppm: parts per million

Source: Beck (2012a)

Dogs used in 13-week toxicity studies are generally not fully sexually mature at termination, which makes interpretation of prostate, testes and ovary findings in these studies difficult (Hood, 2005). In this study, dogs were 20–24 weeks of age (5–6 months) at the beginning of mandestrobin

administration. At study termination, the dogs were 33–37 weeks old (about 8–9 months). Given that dogs achieve sexual maturity by about 7–10 months, termination in this study occurred approximately at the time dogs would be undergoing puberty, with delayed puberty at the high dose likely being secondary to retarded growth and detected as immaturity. It has been reported that immaturity or delayed development of prostate, testes and ovaries can be associated with stress, weight loss and nonspecific toxicity (Greaves, 2000).

Macroscopic evaluation indicated that three males and one female fed 40 000 ppm mandestrobin were thin. Dark and/or large livers were found in both sexes at 12 000 and/or 40 000 ppm. Abnormal contents were present in the gall bladder of both sexes at 40 000 ppm. Gall bladder was distended in two males and one female given 40 000 ppm and one female given 40 000 ppm (Table 18).

Microscopic evaluation of the liver showed centrilobular hepatocellular swelling in females at 4000 and 12 000 ppm and in males at 12 000 and 40 000 ppm. This correlated with increased liver weights at necropsy. Pigment and centrilobular degeneration were present in males and females fed 12 000 or 40 000 ppm. Pigment was characterized by accumulations of intracellular and intrasinusoidal gold/brown pigment. This pigment is considered to be porphyrin, as it was birefringent under polarized light. Fouchet's and Perls' Prussian Blue stains were negative. Centrilobular degeneration was characterized by decreased size of centrilobular hepatocytes, with a finely vacuolated appearance and often a finely granular gold/brown Schmorl's- and Long Ziehl-Neelsen-positive intracellular pigment (i.e. lipofuscin). Occasional single-cell necrosis of hepatocytes was present. Periportal/centrilobular fibrosis was present in males and females given 40 000 ppm. It was characterized by increased numbers of fibroblasts/fibrocytes in the portal and centrilobular areas. Bridging fibrosis was present in one male fed 40 000 ppm. It was characterized by replacement of centrilobular hepatocytes by fibroblasts/fibrocytes and confluence with periportal/centrilobular fibrosis. Hepatocyte vacuolation was present in one male fed 12 000 ppm and one female given 40 000 ppm (Table 18).

In the gall bladder, choleliths were seen in animals fed diets containing 40 000 ppm mandestrobin. Increased incidence and/or severity of adherent bile compared with controls were seen in males and females given 40 000 ppm (Table 18).

Microscopic findings in controls were generally infrequent, of a minor nature and consistent with the usual pattern of findings in dogs of this strain and age. Thin appearance noted in three males and one female offered 40 000 ppm generally correlated with minimal body weight gain or body weight loss, reduced feed consumption and decreased muscle tone recorded during the FOB. Pathological findings in the liver were consistent with increases in the weight of this organ in mandestrobin-treated males and females and with clinical chemistry changes. Therefore, the hepatic changes observed at 12 000 and 40 000 ppm were considered toxicologically relevant. The increased globulin and total bilirubin levels seen in one male offered 40 000 ppm were considered to be related to the morphological changes noted in the liver and gall bladder of this animal. On the basis that the only liver finding was centrilobular hepatocellular swelling in females and there were no associated changes in plasma enzyme levels, the changes in animals given 4000 ppm were considered not to be toxicologically relevant.

The NOAEL was 4000 ppm (equal to 90.9 mg/kg bw per day), based on increased liver weights accompanied by histopathological changes in the liver and increased ALP activity in blood in both sexes at 12 000 ppm (equal to 267.8 mg/kg bw per day) (Beck, 2012a).

Mandestrobin (purity 93.4%) was administered to beagle dogs (27–34 weeks old) distributed into five groups (four dogs of each sex per group) at a dietary concentration of 0, 200, 800, 4000 or 8000 ppm (equal to 0, 4.3, 19.2, 92.0 and 180.7 mg/kg bw per day for males and 0, 4.5, 20.4, 92.0 and 225.7 mg/kg bw per day for females, respectively) for 52 weeks. Achieved concentrations and homogeneity were verified by analysis. All animals were observed daily. Feed consumption was monitored daily, and body weight measurements were performed weekly. Ophthalmological examination was performed pretest and at week 51. A battery of behavioural tests and observations was performed on all animals before initiation of treatment and once weekly thereafter. Blood collection after fasting was performed on all animals before the start of treatment and at weeks 13, 26 and 51 of

the administration period. Urine analysis was performed on all animals before the start of treatment and at weeks 12, 25 and 51 of the administration period. Bone marrow smears were prepared for all animals but not examined, because no related findings were evident during haematology or histopathology. At necropsy, all animals were subjected to thorough gross examination, and the following organs were weighed: adrenals, brain, heart, kidneys, liver, ovaries, pituitary, prostate, spleen, testes, epididymides, thymus, thyroids (with parathyroids) and uterus (including cervix). Histopathology was performed on an extensive list of tissues.

No mortality and no effects on feed consumption (Table 19) or ophthalmology were noted. Thin appearance was observed in one female at 8000 ppm, and this was considered to correlate with body weight loss and occasional incidences of decreased muscle tone recorded during the FOB.

Lower body weight gain was recorded in females offered diets containing 8000 ppm mandestrobin at 52 weeks, which was due to just one female exhibiting an overall body weight loss of 6.0%. No other treatment-related clinical signs or body weight effects were noted (Table 19).

Overall, there were no findings from any assessment indicative of any neurotoxicological effect of mandestrobin. Occasional differences in body temperature were recorded for males fed at least 800 ppm and females fed at least 200 ppm, and these were dose responsive in males in weeks 15, 18, 28, 34 and 43 and in females in weeks 7, 8, 9, 11 and 15. Differences in heart rate were recorded sporadically for males given at least 200 ppm and females given 800 or 4000 ppm. These changes were transient, inconsistent and not considered to be treatment related. Neurological examinations showed one or more incidences of abnormal proprioceptive paw positioning, abnormal righting postural reactions, abnormal hemihopping postural reactions, abnormal wheelbarrowing postural reactions, abnormal muscle tone, abnormal palpebral closure, protrusion of the nictitating membrane, strabismus/protrusion of the palpebral reflex, bilateral constriction/dilatation of pupil size, abnormal papillary light reflex, increased lacrimation, increased salivation and atypical general reactions. However, these observations were infrequent and occasionally observed in concurrent control animals or before treatment was initiated.

There were no treatment-related effects on urinary parameters. The mean volume of urine voided by females fed diets containing 200 or 800 ppm at week 12 was statistically significantly decreased; however, as the volumes were similar to those measured before treatment, no dose–response relationship was observed and no statistically significant differences were observed in males at week 12 or in either sex at week 25 or 51, these changes were considered to be incidental and not treatment related.

A statistically significant dose-related increased AST activity (2.03-fold) was noted in week 26 for males at 8000 ppm, which was considered not to be treatment related, because it was largely reflective of an increase for one male; values for the other animals in this group were within the historical control range. Furthermore, the plasma AST activity for the same male in week 52 was similar to that determined before treatment. Changes at week 52 for another male given 8000 ppm were considered to be treatment related (see below) (Table 19).

Increased ALT activity was noted in both males and females at 8000 ppm in weeks 13, 26 and 52, with males achieving statistical significance for the dose–response relationship in weeks 13 and 26. ALT activities were increased in one male (8M: weeks 26 and 52) fed 200 ppm, one female (29F: weeks 26 and 52) given 800 ppm and two males (18M and 19M: weeks 13, 26 and 52) and one female (38F: weeks 13, 26 and 52) at 8000 ppm (Table 19).

Statistically significantly higher ALP activities were observed at week 52 (2.73-fold) in males at 4000 ppm and at week 13 (males: 2.83-fold; females: 2.27-fold), week 26 (males: 2.64-fold; females: 2.64-fold) and week 52 (males: 3.59-fold; females: 3.36-fold) in both sexes at 8000 ppm, compared with concurrent controls. The values for one male (10M: weeks 13 and 26) at 800 ppm and two males (13M: week 26; 15M: weeks 13, 26 and 52) at 4000 ppm were above normal expectations (Table 19).

Table 19. Key results of the 1-year dietary toxicity study in dogs

		Males						Females							
		0	200	800	4 000	8 000	HCD ^a		0	200	800	4 000	8 000	HCD ^a	
		ppm	ppm	ppm	ppm	ppm	Mean	Range	ppm	ppm	ppm	ppm	ppm	Mean	Range
Body weight (kg)		14.64	15.22	13.29	14.10	13.70	–	–	12.92	12.71	12.63	13.36	11.05	–	–
Body weight gain, weeks 0–52 (kg)		2.97	3.88	1.82	2.58	2.66	–	–	2.68	2.36	2.57	3.19	0.81 ^b	–	–
Feed consumption (g/animal per week)		2 107	2 110	2 112	2 110	2 111	–	–	2 067	1 920	2 089	1 993	2 094	–	–
Blood chemistry															
AST (IU/L)	w13	32	38	29	34	42	32	20–50	28	28	26	32	36	–	–
	w26	30	30	29	29	61*			26	30	28	23	28		
	w52	29	30	29	27	37			26	24	29	28	30		
ALT (IU/L)	w13	38	42	44	46	136* ^{DR}	–	–	29	36	38	39	79	–	–
	w26	42	48	48	43	113* ^{DR}			31	36	46	31	57		
	w52	40	53	51	47	128			29	35	50	33	59		
ALP (IU/L)	w–1	91	87	127	109	116	–	–	121	99	89	97	101	–	–
	w13	63	68	103	120	178*			91	76	70	91	207*		
	w26	53	52	100	120	140*			75	79	66	78	198**		
	w52	41	43	73	112**	147**			55	64	53	69	185**		
GGT (IU/L)	w13	2	2	3	3	4	–	–	2	3	3	2	3	–	–
	w26	3	5	3	5	3			2	2	4	3	4		
	w52	3	3	4	4	4			3	3	3	4	5* ^{DR}		
Total protein (g/L)	w13	55	55	56	57	57* ^{DR}	–	–	60	58	57	56	55**	57	50–66
	w26	58	58	59	59	58			62	62	60	61	57*		
	w52	58	59	56	59	59			61	61	59	59	57* ^{DR}		

		Males						Females							
		0	200	800	4 000	8 000	HCD ^a		0	200	800	4 000	8 000	HCD ^a	
		ppm	ppm	ppm	ppm	ppm	Mean	Range	ppm	ppm	ppm	ppm	ppm	Mean	Range
Albumin (g/L)	w13	35	36	36	37	36	–	–	39	37	38	37	35* ^{DR}	–	–
	w26	36	35	34	35	33			39	38	36	37	34*		
	w52	35	35	33	37	33			39	37	37	36	34**		
A/G ratio	w13	1.8	1.9	1.8	1.9	1.6	–	–	1.9	1.9	1.9	2.1	1.8	–	–
	w26	1.6	1.6	1.4	1.4	1.4			1.6	1.6	1.5	1.6	1.5		
	w52	1.5	1.5	1.5	1.7	1.3			1.8	1.6	1.6	1.6	1.5		
Cholesterol (mmol/L)	w13	7.1	5.9	6.6	6.9	6.1	–	–	8.3	7.0	6.1	5.9	6.4* ^{DR}	–	–
	w26	6.7	5.7	6.7	6.7	5.8			8.2	6.3*	5.9**	6.6	6.0*		
	w52	6.0	5.1	5.7	6.3	5.5			7.5	6.1	5.5*	5.9	5.4*		
Triglycerides (mmol/L)	w13	0.37	0.28	0.45	0.53	0.48** ^{DR}	0.35	0.17–	0.59	0.63	0.50	0.49	0.51	–	–
	w26	0.33	0.30	0.44	0.49*	0.45		0.71	0.51	0.48	0.48	0.52	0.50		
	w52	0.40	0.36	0.42	0.53	0.52* ^{DR}			0.55	0.60	0.60	0.58	0.52		
Calcium (mmol/L)	w13	2.83	2.79	2.76	2.81	2.80	–	–	2.89	2.88	2.86	2.84	2.70*	2.7	2.35–3.00
	w26	2.66	2.67	2.60	2.66	2.61			2.74	2.76	2.72	2.74	2.59*		
	w52	2.63	2.63	2.57	2.69	2.57			2.71	2.70	2.66	2.66	2.53*		
Absolute organ weights (g)															
Brain		87.4	86.8	84.2	84.3	78.8*	84.7	74.9–	76.6	83.1	77.2	75.8	71.5	–	–
Kidney		73.9	65.0	61.7	60.6*	60.0*	62.8	51.0–	57.3	58.5	58.4	53.1	54.9	–	–
Liver		437	425	409	484	488** ^{DR}	–	–	429	437	437	448	455	–	–
Thymus		11.9	9.92	7.74	8.38	6.72	9.10	3.42–	9.60	9.43	10.9	11.2	7.38	8.2	3.9–13.7

	Males						Females							
	0 ppm	200 ppm	800 ppm	4 000 ppm	8 000 ppm	HCD ^a		0 ppm	200 ppm	800 ppm	4 000 ppm	8 000 ppm	HCD ^a	
						Mean	Range						Mean	Range
Thyroid	1.01	1.14	0.95	1.18	1.20	–	–	1.10	1.28	1.10	1.33	1.38	0.94	0.63–1.39
Spleen	55.7	45.7	39.9	44.6	55.5	55.6	27.9– 81.4	30.7	46.1	39.0	39.6	41.7	54.2	45.8–61.8
Heart	124	121	107	105*	113	110.2	76.9– 142	103	101	97.4	98.8	99.3	–	–
Testes/epididymides	32.3	25.2	24.3*	27.5	25.3	27.7	20.2– 33.5	–	–	–	–	–	–	–
Relative organ weights (%)														
Brain	0.6	0.6	0.6	0.6	0.6	0.64	0.46– 0.82	0.6	0.7	0.6	0.6	0.7	–	–
Kidney	0.50	0.42	0.46	0.43	0.43	0.47	0.39– 0.60	0.44	0.46	0.46	0.40	0.50	–	–
Liver	3.0	2.8	3.1	3.4	3.6**DR	–	–	3.3	3.4	3.5	3.4	4.2*	–	–
Thymus	0.080	0.065	0.057	0.059	0.049	0.069	0.03– 0.11	0.073	0.076	0.088	0.083	0.064	0.07	0.04–0.10
Thyroid	0.007	0.008	0.007	0.008	0.009	–	–	0.009	0.010	0.009	0.010	0.013	0.0069	0.0049– 0.0093
Spleen	0.389	0.298	0.299	0.310	0.407	0.35	0.22– 0.48	0.236	0.359	0.313	0.298	0.371	0.34	0.29–0.42
Adrenals	0.010	0.010	0.010	0.010	0.011	–	–	0.013	0.015	0.017	0.009*	0.017*	0.014	0.010–0.019
Heart	0.8	0.8	0.8	0.7	0.8	0.82	0.71– 0.89	0.8	0.8	0.8	0.7	0.9	–	–
Testes/epididymides	0.220	0.166*	0.186	0.196	0.184	0.18	0.15– 0.20	–	–	–	–	–	–	–
Microscopic findings: liver														
<i>No. examined</i>	4	4	4	4	4			4	4	4	4	4		

	Males						Females							
	0 ppm	200 ppm	800 ppm	4 000 ppm	8 000 ppm	HCD ^a		0 ppm	200 ppm	800 ppm	4 000 ppm	8 000 ppm	HCD ^a	
						Mean	Range						Mean	Range
Hepatocyte hypertrophy	0	0	0	2	3*	–	–	0	0	0	1	4*	–	–
Hepatocyte pigment	1	0	1	3	4	–	–	1	1	0	1	4	–	–
Pigmented macrophages	1	0	0	0	2	–	–	1	0	0	1	2	–	–
Portal fibrosis/bile duct proliferation	0	0	0	0	1	–	–	0	0	0	0	0	–	–
Centrilobular degeneration	0	0	0	0	1	–	–	0	0	0	0	1	–	–
Agonal congestion/haemorrhage	0	0	0	0	1	–	–	0	0	0	0	3	–	–

A/G: albumin/globulin; ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; DR: dose–response test (a significant trend was reported only where none of the pairwise comparisons was significant); GGT: gamma-glutamyl transferase; HCD: historical control data; IU: international units; ppm: parts per million; w: Week; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (analysis of variance, dose–response and Dunnett's)

^a Historical control data were generated from January 1998 to March 2007. For organ weights, the data were obtained from 2–6 studies, with between 3 and 14 animals. For the blood biochemistry parameters, the data were obtained from 2–39 studies, with between 2 and 294 animals.

^b This reduced growth rate was due to just one female exhibiting an overall body weight loss of 6.0%.

Source: Beck (2012b)

Increased GGT activity was seen in one male (8M: week 26) fed diets containing 200 ppm mandestrobin, one female (30F: week 26) given 800 ppm, one male (15M: weeks 13, 26 and 52) offered 4000 ppm and one male (18M: weeks 13 and 52) and two females (38F and 39F: week 52) fed 8000 ppm. The increased GGT activity in females at 8000 ppm attained statistical significance for the dose–response relationship (Table 19).

Total cholesterol was statistically significantly decreased at week 26 in females fed diets containing 200 (0.77-fold), 800 (0.72-fold) or 8000 ppm (0.73-fold) and at week 52 in females offered 800 (0.73-fold) or 8000 ppm (0.72-fold), compared with concurrent controls. As the mean values were similar to those determined before treatment was initiated, the differences at week 26 are considered not to be toxicologically important (Table 19).

Disturbances to ALP (800 ppm only), GGT and/or total cholesterol in animals offered 200 or 800 ppm were considered not to be toxicologically relevant, as they were small in magnitude, inconsistent over time and between sexes and/or lacked a histopathological correlate. However, the increased ALP activities in males offered 4000 ppm at week 52 and in males and females offered 8000 ppm at weeks 13, 26 and 52 were considered to be treatment related. Increased GGT activities in males given 4000 ppm in weeks 13, 26 and 52, in males given 8000 ppm in weeks 13 and 52 and in females given 8000 ppm in week 52 were considered to be treatment related. In addition, increased ALT activity in both sexes at 8000 ppm was considered to be treatment related.

Statistically significantly higher triglyceride levels were observed at week 26 (1.49-fold) in males fed 4000 ppm. This was considered not to be toxicologically relevant, as the mean value was within the historical control range (0.17–0.71 mmol/L). Concentrations were increased in two males (week 13: 13M and 15M; week 26: 15M; week 52: 13M) offered 4000 ppm and three males (week 13: 18M and 20M; week 52: 19M) offered 8000 ppm. The increased triglyceride level for one male offered 4000 ppm and two males offered 8000 ppm at week 13 were considered not to be toxicologically relevant, as the values for these animals at week 52 were similar to those determined before treatment was initiated. However, the increases for one male given 4000 ppm at weeks 13 and 52 and one male fed 8000 ppm at week 52 were considered to be treatment related (Table 19).

Decreased albumin levels were noted in females offered 8000 ppm at weeks 26 and 52. Decreased total protein and calcium levels in females offered 8000 ppm at weeks 13 and 26 and at weeks 13, 26 and 52, respectively, were considered not to be treatment related, but reflective of decreased albumin concentrations (Meuten, 1982). Additionally, all individual values were within the historical control ranges, or differences were similar to those recorded pretreatment. Occasional differences reached statistical significance in other parameters; however, these changes were not considered to be treatment related, as they were slight, within the historical control range or comparable to pretest values, or there were no significant differences between sexes or collection points (Table 19).

Absolute kidney weights were statistically significantly decreased in males offered 4000 ppm (0.82-fold) or 8000 ppm (0.81-fold), compared with concurrent controls. Spleen weights (absolute and relative) were above normal expectations in one male (17M) and one female (38F) at 8000 ppm. The values for one control male (3M) were also high. Absolute brain weights were decreased in males at 8000 ppm (0.90-fold), compared with concurrent controls. Absolute thyroid/parathyroid weights were above normal expectations in one female (36F) fed 4000 ppm and one female (38F) at 8000 ppm. Absolute and relative thymus weights were low in one male (10M) at 800 ppm and two males (17M and 20M) and one female (39F) offered 8000 ppm, compared with concurrent controls (Table 19).

Lower absolute and/or relative testes/epididymides weights were recorded for one male (11M) at 800 ppm and one male (17M) at 8000 ppm, compared with concurrent controls. A statistically significant decrease (0.75-fold) in absolute testes/epididymides weight was seen for males offered 800 ppm, compared with concurrent controls. In addition, the relative testes/epididymides weight for males at 200 ppm was statistically significantly lower (0.75-fold) than for concurrent controls. When compared with concurrent controls, mean relative adrenal weights (0.69-fold and 1.31-fold) were statistically significantly different for females at 4000 and 8000 ppm, respectively. Absolute heart weight was statistically significantly decreased in males at 4000 ppm (0.84-fold), compared with concurrent controls (Table 19).

Organ weight changes in the spleen, thyroid/parathyroid, thymus, testes/epididymides, adrenals and heart were considered not to be toxicologically relevant, as they were inconsistent between the sexes, there were no macroscopic or microscopic correlates, values were within historical control ranges or there was a lack of a clear dose–response relationship. Decreases in absolute kidney weight and brain weight were considered of doubtful toxicological relevance, as the relative weights were not statistically significantly different, all individual values were within historical control limits, no changes were observed in females and no related clinical chemistry parameters or macroscopic or microscopic correlates were present.

Absolute liver weights were increased in males at 4000 ppm (1.11-fold) and 8000 ppm (1.12-fold) and in females at 8000 ppm (1.06-fold), compared with controls. Relative liver weights were increased in males at 4000 ppm (1.13-fold) and 8000 ppm (1.20-fold) and in females at 8000 ppm (1.27-fold), compared with controls. Changes in relative liver weight at 8000 ppm were statistically significant. Dark liver was observed for one male and three females fed 8000 ppm and correlated with findings recorded microscopically. There were no other macroscopic findings suggestive of treatment-related effects (Table 19).

In the liver, hepatocyte hypertrophy was recorded for males and females fed diets containing 4000 or 8000 ppm mandestrobin. Hepatocyte hypertrophy was characterized by hepatocytes in the centrilobular/midzonal area, with increased pale eosinophilic staining cytoplasm. Hepatocyte hypertrophy recorded for females offered 4000 ppm was considered not to be adverse on the basis that no other histopathological findings were recorded and there were no associated changes in blood biochemistry. Increased levels of hepatocyte pigment were recorded for males fed 4000 and 8000 ppm and females fed 8000 ppm. Hepatocyte pigment was characterized by the presence of small golden brown cytoplasmic granules, primarily in hepatocytes in the centrilobular and periportal zones. The incidence of hepatocyte hypertrophy and hepatocyte pigment achieved statistical significance in males and females offered 8000 ppm. There was also a marginal increase in pigmented macrophages in males and females at 8000 ppm. Pigmented macrophages were characterized by macrophages in the portal area and in the hepatic sinusoids, with dark brown cytoplasmic pigment. The hepatocyte pigment and macrophage pigment were identified as lipofuscin using Schmorl's and Long Ziehl-Neelsen stains. Additionally, centrilobular degeneration was recorded for one male and one female offered 8000 ppm. Centrilobular degeneration was characterized by the presence of degenerating hepatocytes in the centrilobular zone, with occasional single-cell necrosis. Portal fibrosis/bile duct proliferation was recorded for one male offered 8000 ppm. It was characterized by the presence of proliferating bile duct cells and fibroblasts, with an overall increase in fibrous tissue in the portal tracts. Agonal congestion/haemorrhage was recorded for one male and three females at 8000 ppm, which correlated with the dark liver observed during the macroscopic examination. However, this finding was considered to be secondary to the hepatocyte hypertrophy, rather than a direct effect of mandestrobin treatment. The increase in lipofuscin in this study was considered to be related to hepatocyte hypertrophy and/or cellular degeneration (Table 19).

The decreased incidence of stomach mineralization in treated males and the increased incidence of sublingual gland inflammatory cell foci in treated females, compared with controls, achieved statistical significance. Both stomach mineralization and inflammatory cell foci in the sublingual salivary gland are commonly seen as background findings. There was no dose-related response for either of the findings, and these statistically significant findings were considered not to be of biological relevance.

The NOAEL was 800 ppm (equal to 19.2 mg/kg bw per day), based on increased relative liver weights, hepatocyte hypertrophy and hepatocyte pigment and disturbances to clinical biochemistry parameters (increased ALP, GGT and triglycerides) in males at 4000 ppm (equal to 92.0 mg/kg bw per day) (Beck, 2012b).

(b) *Dermal application*

Rats

In a study of dermal toxicity, mandestrobin (purity 93.4%) was applied repeatedly for 28 consecutive days to the skin of male and female Wistar rats at a dose of 100, 300 or 1000 mg/kg bw per day for 6 hours per day. Clinical signs and mortality were observed twice daily, before and after the administration. Body weights and feed consumption were measured weekly. Ophthalmoscopy was conducted before the start of administration and in week 4. Haematology and clinical chemistry were conducted at necropsy, after fasting, and urine analysis was performed in week 4. Bone marrow smears were prepared for all animals but not examined, because no related findings were evident during haematology or histopathology. At the end of the study, all animals were terminated and subjected to macroscopic examination. Organs were weighed, and histopathology was performed on an extensive list of tissues.

No mortality or clinical signs were noted during the study.

No effects were noted on body weight, feed consumption, ophthalmoscopy, haematology and clinical chemistry, necropsy, organ weights or histopathology.

As no treatment-related effects were noted, the NOAEL was 1000 mg/kg bw per day, the highest dose tested (Ogata, 2011).

2.3 *Long-term studies of toxicity and carcinogenicity*

Mice

Mandestrobin (purity 93.4%) was administered to four groups of 51 male and 51 female CD-1 mice (6–7 weeks old) at a dietary concentration of 0, 700, 2000 or 7000 ppm (equal to 0, 82.5, 238.8 and 823.9 mg/kg bw per day for males and 0, 99.2, 280.3 and 994.0 mg/kg bw per day for females, respectively) for 78 weeks. In addition to these animals (designated as the “main group”), 12 mice of each sex per group were treated at a dietary concentration of 0, 700, 2000 or 7000 ppm (equal to 0, 88.4, 255.0 and 883.3 mg/kg bw per day for males and 0, 104.0, 325.0 and 1045.1 mg/kg bw per day for females, respectively) for 52 weeks only, for interim sacrifice (designated as the “satellite group”).

Dose, homogeneity and stability in the diet were confirmed by chemical analysis. Animals were checked for viability/mortality twice each day, and clinical signs were observed at least once daily. Body weight and feed consumption were recorded weekly up to week 16 and every 4 weeks thereafter. Blood samples for haematology were collected from all satellite group animals at week 52 and from all main group animals at week 78. A detailed gross necropsy was performed at termination and for all mice found dead or killed in extremis. The following organ weights were measured for all animals in the interim sacrifice group and for at least 10 animals of each sex per group in the main groups: adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes and epididymides, and uterus with cervix. An extensive list of tissues was examined histologically, including gross lesions and tissue masses.

No treatment-related effect on survival was noted (Table 20). The incidences and causes of morbidity and mortality in controls and treated animals were generally similar and consistent with the usual pattern of causes of demise in mice of this strain. Clinical signs in moribund animals generally included hunched posture, sluggishness, pale appearance, laboured/rapid respiration, swollen abdomen and/or thin appearance. Effects on body weight gain were noted only in the 7000 ppm females of the satellite group; however, this effect was not replicated in females in the main dose group and therefore was not considered to be treatment related. No effects on feed consumption were noted. No statistically significant treatment-related effects on haematology parameters were noted at either week 52 (interim) or week 78 (main study). A small number of control and treated animals killed during the treatment period or surviving to termination had elevated white blood cell counts, generally due to increased neutrophils and/or lymphocytes, which generally correlated microscopically with the presence of haemolymphoreticular tumour.

Table 20. Key results of the long-term toxicity and carcinogenicity study in mice

	Males				Females			
	0 ppm	700 ppm	2 000 ppm	7 000 ppm	0 ppm	700 ppm	2 000 ppm	7 000 ppm
Mortality ^a (satellite groups)	2/12	0/12	1/12	2/12	2/12	0/12	1/12	0/12
Survival (%) (satellite groups)	83	100	92	83	83	100	92	100
Mortality ^a (main groups)	13/51	12/51	9/51	13/51	18/51	16/51	16/51	14/51
Survival (%) (main groups)	75	76	82	75	65	69	69	73
Organ weights								
Liver, week 52 (g)	2.34	2.47	2.43	2.59	1.75	1.80	1.93	1.97
% of control	—	5.6	3.8	10.7	—	2.9	10.3	12.6
Liver, week 52 (% of body weight)	4.42	4.67	4.76	5.10**	4.07	4.19	4.41	5.10**
% of control	—	5.7	7.7	15.4	—	2.9	8.4	25.3
Liver, week 78 (g)	2.40	2.55	2.54	2.76 DR*	1.93	2.20	2.32	2.09
% of control	—	6.3	5.8	15.0	—	14.0	20.2	8.3
Liver, week 78 (% of body weight)	4.59	4.85	4.81	5.24**	4.42	4.65	4.96	4.90
% of control	—	5.7	4.8	14.2	—	5.2	12.2	10.9

DR: dose–response test (a significant trend was reported only where none of the pairwise comparisons was significant); ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (analysis of variance, dose–response and Dunnett's)

^a No. of animals that died prior to termination / no. of animals in group.

Source: Beck (2012c)

Liver weight increases were noted in the high-dose males and females. Relative liver weight increases attained statistical significance at both interim and terminal sacrifices in males and at interim sacrifice only in females (Table 20).⁴ The increase in liver weights was considered to be non-adverse in the absence of histological indicators of an adverse effect on the liver.

No macroscopic or non-neoplastic histopathological effects were noted. No treatment-related effect on tumour incidence was noted.

The NOAEL for toxicity was 7000 ppm (equal to 823.9 mg/kg bw per day), the highest dose tested, based on the absence of adverse effects. No carcinogenic potential of mandestrobin was observed (Beck, 2012c).

Rats

Mandestrobin (purity 93.4%) was administered to groups of 50 male and 50 female Wistar rats (6 weeks of age) at a dietary concentration of 0, 400, 2000, 7000 or 15 000 ppm (equal to 0, 21.0, 105.1, 375.6 and 804.3 mg/kg bw per day for males and 0, 26.7, 135.2, 475.0 and 1016.2 mg/kg bw per day for females, respectively) for 104 weeks (main groups: carcinogenicity cohort). In addition to these animals, 20 rats of each sex per group were treated at a dietary concentration of 0, 400, 2000, 7000 or 15 000 ppm (equal to 0, 25.5, 130.3, 448.8 and 991.8 mg/kg bw per day for males and 0, 31.3, 151.4,

⁴ The mode of action for the liver weight increase was considered to be due to liver enzyme induction, via activation of the CAR by mandestrobin, as evidenced by work presented in section 2.6(c) below.

535.3 and 1138.9 mg/kg bw per day for females, respectively) for 52 weeks for interim sacrifice (satellite groups: chronic toxicity cohort).

Doses, homogeneity and stability in the diet were confirmed by analysis. Animals were checked for mortality and clinical signs daily. Body weight and feed consumption were recorded once weekly during acclimatization and up to week 16 and every 4 weeks thereafter. Ophthalmoscopic examinations were performed on all animals before treatment and on control and highest-dose satellite animals in week 50. A FOB was conducted on all rats of the satellite groups in week 51. Blood samples (after fasting) were drawn from all animals of the satellite groups in weeks 13, 26 and 52 and from 10 animals of each sex per main group in weeks 78 and 104. For the chronic toxicity cohort, the full set of haematological and clinical chemistry parameters was determined, whereas for the carcinogenicity cohort, only total and differential white cell count were determined in blood samples. Urine samples were collected from all satellite group animals in weeks 12, 25 and 51. All animals, either found dead or terminated, were subjected to detailed necropsy and collection of tissues. The following organs were weighed: adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes and epididymides, thymus, and uterus and cervix. An extensive list of tissues was examined histologically, including gross lesions and tissue masses.

No treatment-related effects on clinical signs, FOB or ophthalmoscopy were noted. In the carcinogenicity cohort, decreased and dose-responsive mortality was observed in all female treatment groups. In males of the carcinogenicity cohort, the mortality was lower at 400 and 7000 ppm; however, as this was not dose responsive, the decreased mortality was considered not to be treatment related. In the chronic toxicity cohort, body weight suppression was observed in males at 15 000 ppm during the first 24 weeks, accompanied by reduced body weight gain (15%) from start to week 13 and at week 40 (8.1%). Body weight in females at 15 000 ppm was lower than in controls at all measurement intervals (9%). In these females, body weight gain was lower from the start to week 52 (16.4%). In both sexes given diets containing 15 000 ppm, feed consumption was lower over the 52-week treatment period, statistically significantly in females for pairwise comparison and in males for dose-response relationship. The statistically significant increases in body weight, body weight gain and feed consumption in males at 400 ppm were considered incidental, as this finding was not repeated in the carcinogenicity cohort. In the carcinogenicity cohort, decreases in body weight and/or body weight gain in both sexes were observed at 15 000 ppm. In the carcinogenicity cohort, feed consumption decreased in males at 15 000 ppm, attaining statistical significance for pairwise comparison from weeks 1 to 52 and for dose-response relationship from weeks 1 to 104. In females, feed consumption was statistically significantly decreased for dose-response relationship at 15 000 ppm over the 104-week treatment period. However, there was a body weight decrease in females of up to 19% at 2000 ppm (at week 68 and from week 76 onwards), up to 25% at 7000 ppm (at week 24 and from week 40 onwards) and up to 36% at 15 000 ppm (at week 6 and from week 11 onwards). Statistically significant decreases in overall body weight gains (start to week 104) were observed for females fed 2000, 7000 or 15 000 ppm (18.1%, 18.2% and 32.8%, respectively), compared with controls. In males, body weight gain was statistically significantly decreased at 15 000 ppm, by 11% from the start to week 52 and by 18% from the start to week 104, compared with controls (Tables 21 and 22).

Haematology investigation in the chronic toxicity cohort (Table 21) showed a treatment-related change in haemoglobin in males and females at 15 000 ppm and in females at 7000 ppm. However, these changes relative to controls were generally small (~5%), and values were within historical control ranges. In addition, no findings were observed in related end-points, such as red blood cells, reticulocytes or morphological changes in blood-forming organs. Therefore, these decreases were considered not to be of toxicological relevance. All other statistically significant differences between controls and treated groups were consistent with normal variability, small, inconsistent between the sexes, lacking temporal consistency and/or unrelated to dose and therefore considered not to be treatment related. In the carcinogenicity cohort (Table 22), all statistically significant differences in haematological end-points between the controls and treated groups were small, inconsistent between sexes, lacking temporal consistency and/or unrelated to dose and therefore considered to be of no toxicological importance. A small number of control and treated animals killed during the treatment period or surviving to termination had elevated white blood cell counts, generally due to increased

Table 21. Key results of the combined chronic toxicity and carcinogenicity study in rats: chronic toxicity cohort

Parameter	Males						Females							
	0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	HCD		0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	HCD	
						Mean	Range						Mean	Range
Mortality ^a	1/20	1/20	1/20	0/20	0/20	–	–	0/20	0/20	1/20	1/20	0/20	–	–
Survival (%)	95	95	95	100	100	–	–	100	100	95	95	100	–	–
Body weight (g)														
Week 0	184.7	189.1	182.8	180.1	180.0	–	–	140.2	140.9	143.5	141.0	138.7	–	–
Week 52	486.9	532.0*	486.3	470.7	451.5	–	–	275.8	265.6	272.7	268.8	251.9*	–	–
Body weight gain (g)														
Weeks 0–28	251.4	285.2*	249.8	239.6	225.0	–	–	110.6	105.2	105.5	106.6	101.4	–	–
Weeks 0–52	302.5	343.8*	302.7	290.6	271.5	–	–	135.6	124.8	129.4	128.3	113.3**	–	–
Feed consumption (g/animal per week)														
Weeks 1–28	155.7	166.2*	157.0	149.0	148.3	–	–	119.9	115.4	114.9	115.5	111.3* ^{DR}	–	–
Weeks 1–52	154.5	163.9*	155.5	147.5	147.8	–	–	119.4	114.9	114.4	115.1	110.4*	–	–
Haematology (week 52)														
Hb (g/dL)	16.1	16.2	16.1	16.0	15.6**	16.5	15.1– 18.0	15.6	15.4	15.2	14.9***	14.7***	15.6	14.6–16.3
MCH (pg)	17.7	17.5	17.7	17.4	16.9**	–	–	19.0	18.7	18.5*	18.8	18.4**	–	–
MCHC (g/dL)	35.6	35.2	35.2	35.0	34.5**	–	–	35.9	35.4	35.0***	35.2**	34.5***	–	–
PT (s)	22.3	21.3*	21.5	21.1*	20.0***	–	–	21.5	21.9	21.8	21.4	21.5	–	–
LUC (10 ⁹ /L)	0.0	0.0	0.0	0.0	0.1*	–	–	0.0	0.0	0.0	0.0	0.0	–	–
Blood chemistry (week 52)														
ALP (IU/L)	59	52	54	49*	50	–	–	27	28	24	19**	16***	–	–
Calcium (mmol/L)	2.64	2.62	2.63	2.66	2.63	–	–	2.69	2.62**	2.69	2.74*	2.78***	–	–

Parameter	Males						Females							
	0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	HCD		0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	HCD	
						Mean	Range						Mean	Range
Chloride (mmol/L)	105	104*	103***	102***	102***	—	—	103	103	103	103	102** ^{DR}	—	—
Inorganic phosphate (mmol/L)	1.2	1.3	1.3	1.3	1.4**	—	—	0.9	0.9	0.8	0.9	1.0	—	—
Total protein (g/L)	68	69	69	70	69	—	—	72	71	73	75*	74*	—	—
Albumin (g/L)	44	45	45	47*	46	—	—	53	51	52	54	54** ^{DR}	—	—
Globulin (g/L)	24	24	23	23	23	—	—	19	20	20	21*	20	—	—
A/G	1.8	1.9	1.9	2.0*	2.0	—	—	2.8	2.6	2.7	2.6	2.7	—	—
Total bilirubin (µmol/L)	1.7	1.9	1.8	1.7	1.5	—	—	2.8	2.4	2.4	2.7	2.1**	—	—
GGT (IU/L)	2	2	2	2	8***	—	—	2	2	2	2	6***	—	—
Total cholesterol (mmol/L)	2.3	2.3	2.4	2.7	2.9*	—	—	2.0	1.8	2.3	3.1***	3.0***	—	—
Glucose (mmol/L)	6.3	6.1	5.4**	5.7	5.9	—	—	6.1	5.5	5.5*	5.2***	4.8***	—	—
Organ weights														
Absolute liver weight (g)	9.63	11.22**	10.494	10.77*	12.26***	—	—	6.22	5.96	6.41	7.26**	7.71***	—	—
% change			9	12	27					3	17	24		
Relative liver weight (%)	2.01	2.16	2.22*	2.35***	2.81***	—	—	2.33	2.34	2.44	2.83***	3.15***	—	—
% change			10	17	40					5	21	35		
Absolute kidney weight (g)	2.15	2.26	2.099	2.066	2.169	—	—	1.47	1.41	1.42	1.40	1.40	—	—
Relative kidney weight (%)	0.45	0.43	0.44	0.45	0.49**	—	—	0.55	0.55	0.54	0.54	0.57	—	—
% change					10									
Absolute brain weight (g)	2.16	2.19	2.14	2.14	2.06**	—	—	1.96	1.97	1.96	1.93	1.95	—	—
% change					5									
Relative brain weight (%)	0.45	0.42	0.54	0.47	0.47** ^{DR}	—	—	0.74	0.77	0.75	0.75	0.80*	—	—
% change					4							8		

Parameter	Males						Females							
	0	400	2 000	7 000	15 000	HCD		0	400	2 000	7 000	15 000	HCD	
	ppm	ppm	ppm	ppm	ppm	Mean	Range	ppm	ppm	ppm	ppm	ppm	Mean	Range
Gross pathology and histopathology														
Liver														
Large	0	0	0	1	6*	—	—	0	0	1	0	2	—	—
Hepatocellular eosinophilia/hypertrophy	0	0	0	15***	20***	—	—	0	0	0	17***	15***	—	—
Hepatocyte vacuolation	1	3	1	3	6	—	—	0	2	3	4*	2	—	—
Agonal congestion/haemorrhage	2	1	1	5	10	—	—	1	0	1	1	1	—	—
Thyroid														
Follicular cell hypertrophy	1	0	1	9**	18***	—	—	0	0	0	9***	15***	—	—

A/G: albumin/globulin; ALP: alkaline phosphatase; DR: dose–response relationship (a significant trend was reported only where none of the pairwise comparisons was significant); GGT: gamma-glutamyl transferase; Hb: haemoglobin; HCD: historical control data; IU: international units; LUC: large unstained cells; MCH: mean cell haemoglobin; MCHC: mean cell haemoglobin concentration; ppm: parts per million; PT: prothrombin time; *: $P < 0.005$; **: $P < 0.01$; ***: $P < 0.001$ (analysis of variance, dose–response and Dunnett's)

^a No. of animals that died prior to termination / no. of animals in group.

Source: Beck (2012d)

Table 22. Key results of the combined chronic toxicity and carcinogenicity study in rats: carcinogenicity cohort

Parameter	Males						Females							
	0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	HCD (%)		0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	HCD (%)	
						Mean	Range						Mean	Range
Mortality ^a	18/50	8/50	18/50	9/50	14/50	–	–	17/50	15/50	14/50	10/50	9/50	–	–
Survival (%)	64	84	64	82	72	–	–	66	70	72	80	82	–	–
Body weight (g)														
Week 0	188.3	187.6	188.7	186.4	188.9	–	–	144.0	141.7	143.4	143.8	144.4	–	–
Week 104	611.5	613.4	570.6	584.5	538.5***	–	–	363.6	353.8	323.2**	323.2**	292.5***	–	–
Body weight gain (g)														
Weeks 0–52	327.5	329.8	331.7	314.5	291.8***	–	–	139.9	143.5	130.7	120.9***	110.8***	–	–
Weeks 0–104	424.0	426.5	384.4	396.7	349.2***	–	–	220.0	214.5	180.1**	179.9**	147.9***	–	–
Feed consumption (g/animal per week)														
Weeks 1–52 ^b	152.2	151.2	153.6	150.2	144.6*	–	–	113.5	114.2	113.1	110.6	107.2* ^{DR}	–	–
Weeks 1–104 ^b	148.4	147.4	149.4	147.2	141.9* ^{DR}	–	–	113.6	114.1	112.8	111.6	108.1* ^{DR}	–	–
Haematology (week 104)														
WBC (10 ⁹ /L)	5.9	4.7**	4.8*	5.3	5.1	–	–	3.4	3.5	4.1	3.5	4.3	–	–
Lymphocytes (10 ⁹ /L)	3.9	3.1*	3.3	3.3	3.5	–	–	2.1	2.0	2.1	2.0	2.0	–	–
Eosinophils (10 ⁹ /L)	0.1	0.1	0.1	0.1	0.1	–	–	0.1	0.1	0.1	0.1	0.4*	–	–
Organ weights														
Absolute liver weight (g)	11.51	11.87	11.17	13.10	11.85	–	–	6.989	7.262	7.340	7.226	7.748	–	–
Relative liver weight (%)	2.051	2.029	2.042	2.215	2.334*	–	–	2.153	2.038	2.472*	2.446*	2.753***	–	–

Parameter	Males							Females						
	0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	HCD (%)		0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	HCD (%)	
						Mean	Range						Mean	Range
Absolute kidney weight (g)	2.420	2.456	2.445	2.694	2.335	–	–	1.677	1.695	1.675	1.560	1.601	–	–
Relative kidney weight (%)	0.433	0.421	0.448	0.456	0.461*	–	–	0.520	0.481	0.569	0.526	0.574*	–	–
Absolute brain weight (g)	2.24	2.20	2.25	2.22	2.23	–	–	2.02	2.00	2.00	2.02	2.02	–	–
Relative brain weight (%)	0.40	0.38	0.41	0.38	0.44	–	–	0.63	0.57	0.69	0.69	0.73**DR	–	–
Macroscopic findings: liver														
Large	2	4	4	7	9	–	–	0	2	2	2	3	–	–
Microscopic non-neoplastic findings														
<i>Liver</i>														
Hepatocellular eosinophilia/hypertrophy														
Decedents	1	1	1	2	2	–	–	1	4	2	7*	5*	–	–
Terminal kill	6	10	12	28****	35****	–	–	15	18	30****	36****	37****	–	–
All animals	7	11	12	30****	37****	–	–	16	22	21**	43****	42****	–	–
Hepatocyte vacuolation														
Decedents	6	2	11	3	12*	–	–	5	3	1	0	4	–	–
Terminal kill	22	37	26	35*	33**	–	–	15	10	18	29*	34****	–	–
All animals	28	39*	37	38	45*	–	–	20	13	19	29	38*	–	–
- Minimal	24	35	33	31	35	–	–	19	12	19	29	36	–	–

Parameter	Males							Females						
	0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	HCD (%)		0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	HCD (%)	
						Mean	Range						Mean	Range
- Slight	3	2	3	7	9	—	—	1	0	0	0	2	—	—
- Moderate	1	2	1	0	1	—	—	0	1	0	0	0	—	—
Bile duct hyperplasia														
Decedents	1	1	0	0	1	—	—	3	6	4	3	1	—	—
Terminal kill	2	5	7	4	4	—	—	10	17	18	26**	18	—	—
All animals	3	6	7	4	5	—	—	13	23	22	29**	19	—	—
<i>Thyroid</i>														
Follicular cell hypertrophy														
Decedents	0	0	0	0	0	—	—	0	0	0	0	0	—	—
Terminal kill	0	2	0	0	10**	—	—	0	0	0	0	3	—	—
All animals	0	2	0	0	10**	—	—	0	0	0	0	3	—	—
Follicular cell hyperplasia														
Decedents	0	0	0	1	4*	—	—	0	1	1	0	0	—	—
Terminal kill	4	11	5	4	9	—	—	1	0	3	3	4	—	—
All animals	4	11	5	5	13*	—	—	1	1	4	3	4	—	—
<i>Kidney</i>														
Papillary mineralization														
Decedents	2	3	2	3	3	—	—	10	4	3	6	4	—	—
Terminal kill	7	8	7	10	13	—	—	17	13	25	30*	26	—	—
All animals	9	11	9	13	16	—	—	27	17	28	36	30	—	—

Parameter	Males							Females						
	0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	HCD (%)		0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	HCD (%)	
						Mean	Range						Mean	Range
Pelvic mineralization														
Decedents	7	4	8	0	8	—	—	14	12	8	8	7	—	—
Terminal kill	22	30	19	30	26	—	—	30	29	34	36	36	—	—
All animals	29	34	27	30	34	—	—	44	41	42	44	43	—	—
Corticomedullary mineralization														
Decedents	0	0	0	0	0	—	—	0	0	3	1	4*	—	—
Terminal kill	0	1	0	0	0	—	—	6	7	14*	16*	14	—	—
All animals	0	1	0	0	0	—	—	6	7	17*	17*	18**	—	—
- Minimal	0	1	0	0	0	—	—	6	6	12	15	13	—	—
- Slight	0	0	0	0	0	—	—	0	1	2	1	1	—	—
Epididymis														
Oligospermia														
Decedents	1	3	1	6	3	—	—	—	—	—	—	—	—	—
Terminal kill	0	1	1	2	7*	—	—	—	—	—	—	—	—	—
All animals	1	4	2	8	10*	—	—	—	—	—	—	—	—	—
Testis														
Interstitial cell hyperplasia	2	0	1	1	0	—	—	—	—	—	—	—	—	—
Ovary														
Sex cord stromal hyperplasia														
Decedents	—	—	—	—	—	—	—	0	0	0	1	0	—	—

Parameter	Males						Females							
	0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	HCD (%)		0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	HCD (%)	
						Mean	Range						Mean	Range
Terminal kill	—	—	—	—	—	—	—	3	8	5	5	5	—	—
(%)	—	—	—	—	—	—	—	6	16	10	12	10	27.1	2–48% ^c
All animals	—	—	—	—	—	—	—	3	8	5	6	5	—	—
Neoplastic microscopic findings														
<i>Testis</i>														
Interstitial cell adenoma	0	0	0	2	3	—	—	—	—	—	—	—	—	—
(%)	0	0	0	4	6	2.3	0–6 ^d							
<i>Ovary</i>														
Sex cord stromal tumours	—	—	—	—	—	—	—	2	0	1	4	6** ^{DR}	—	—
(%)								4	0	2	8	12	See text	
Combined sex cord stromal tumour and hyperplasia ^e	—	—	—	—	—	—	—	5	8	6	8	9	—	—
(%)	—	—	—	—	—	—	—	10	16	12	16	18	—	—

DR: dose–response relationship (a significant trend was reported only where none of the pairwise comparisons was significant); HCD: historical control data; ppm: parts per million; WBC: white blood cells; *: $P < 0.005$; **: $P < 0.01$; ***: $P < 0.001$ (analysis of variance, dose–response and Dunnett's)

^a No. of animals that died prior to termination / no. of animals in group.

^b Week 48 excluded from female calculation of feed consumption due to animals fed ad libitum.

^c Data generated from 2000 to 2008, from eight studies.

^d Data generated on Han Wistar rats from 2000 to 2008, from eight studies.

^e The number of animals exhibiting both hyperplasia and tumour is counted as 1.

Source: Beck (2012d)

neutrophils and/or lymphocytes, which generally correlated microscopically with the presence of haemolymphoreticular tumours.

In the chronic toxicity cohort, the activity of GGT was statistically significantly increased in both sexes fed diets containing 15 000 ppm mandestrobin at week 13 (males: 1.5-fold; females: 2-fold), at week 26 (males: 2.5-fold; females: 1.5-fold) and at week 52 (males: 4-fold; females: 3-fold), compared with controls. Total cholesterol was higher at week 13 in animals fed 7000 ppm (males: 29%; females: 38%) and 15 000 ppm (males: 47%; females: 56%), at week 26 in females fed 7000 ppm and 15 000 ppm (50% and 65%, respectively) and at week 52 in females fed 7000 ppm (55%) and in both sexes fed 15 000 ppm (males: 26%; females: 50%), compared with controls. Therefore, the consistent increases seen in females given 7000 ppm and in both sexes fed 15 000 ppm were considered to be toxicologically relevant. As no disturbances were observed on subsequent occasions, the change in males fed 7000 ppm at week 13 was considered not to be toxicologically relevant (Table 21).

All other statistically significant differences between control and treated groups were consistent with normal variability (i.e. within historical control ranges, which, however, are available only for weeks 13 and 26), small, inconsistent between the sexes, lacking temporal consistency and/or unrelated to dose and therefore considered to be incidental.

Urine analysis showed an increased mean volume of voided urine in males fed diets containing 400 ppm at weeks 12 and 51 and in males given 15 000 ppm at week 51. Quantitative measurements indicated that excretion was approximately 64–71% higher. However, as there were no similar effects in females or overt differences in week 25, these changes were considered to be incidental and not treatment related. Increased urine output correlated with a decrease in specific gravity.

In the chronic toxicity cohort, a statistically significant increase in relative kidney weights (10%) was noted in males at 15 000 ppm. A statistically significant decrease in absolute brain weight (5%) was noted in males at 15 000 ppm. In contrast, relative brain weight was increased at 15 000 ppm in males and females, by 4% and 8%, respectively. Changes in kidney and brain weights were considered not to be toxicologically relevant as they were small, there were no macroscopic or microscopic correlates and they were related to body weight suppression. Increased liver weights were noted in treated males at and above 2000 ppm and in females at and above 7000 ppm. In males, absolute liver weights at 2000, 7000 and 15 000 ppm were increased by 9%, 12% and 27%, respectively. In females, absolute liver weights at 7000 and 15 000 ppm were increased by 17% and 24%, respectively. Relative liver weights in males at 2000, 7000 and 15 000 ppm were increased by 10%, 17% and 40%, respectively; in females at 7000 and 15 000 ppm, relative liver weights were increased by 21% and 35%, respectively (Table 21). Mean relative ovary weights were statistically significantly increased (26%) in females at 15 000 ppm. This finding is considered to be incidental, as there was no similar change seen for the females in the carcinogenicity cohort at the terminal kill, and it was not associated with any pathological correlates. Other absolute and relative organ weight changes were considered not to be biologically relevant, as they were small in magnitude, not dose dependent, inconsistent between sexes, spontaneous background changes due to normal interanimal variability and/or lacking a histopathological correlate.

From the macroscopic investigation, a statistically significantly increased incidence of large liver was recorded in males at 15 000 ppm. Large liver was also noted in two females at 15 000 ppm, in one female at 2000 ppm and in one male at 7000 ppm, which generally correlated with findings seen microscopically. Other statistically significant differences between groups for the macroscopic findings, such as mottled liver and red mandibular lymph node, which correlated with agonal congestion/haemorrhage microscopically, were sporadic and not dose related. Increased incidences and severity of hepatocellular eosinophilia/hypertrophy were noted in animals at 7000 and 15 000 ppm and were characterized by enlarged hepatocytes with increased amounts of eosinophilic cytoplasm. This was considered to be an adaptive change associated with mandestrobin metabolism. A minor increase in hepatocyte vacuolation in males at 15 000 ppm, characterized by variable numbers of small to large cytoplasmic vacuoles within scattered hepatocytes, was also recorded, but, according to the study author, the toxicological relevance of this change remained unclear (Table 21). The effects on liver

weight were associated with the blood biochemistry and pathological changes and therefore were considered to be toxicologically relevant.

In the thyroid, increased incidences and severity of follicular cell hypertrophy in animals at 7000 and 15 000 ppm were noted (Table 21). Follicular cell hypertrophy was characterized by follicles with columnar epithelium with increased amounts of apical cytoplasm, with or without a decrease in follicular colloid. This is generally considered to be an adaptive change due to increased thyroid hormone metabolism in the liver and is commonly associated with liver cell hypertrophy.

In the carcinogenicity cohort, statistically significantly increased relative kidney weights were noted at 15 000 ppm for both sexes (males: 7%; females: 10%). Changes in kidney weights were considered not to be toxicologically relevant, as they were small and likely related to body weight suppression. The relative brain weight was increased at 15 000 ppm for both sexes (males: 10%; females: 16%); in females, changes achieved statistical significance. Changes in brain weights were considered not to be toxicologically relevant, as there were no macroscopic or microscopic correlates and they were likely related to body weight suppression. In males, a statistically significant increase in relative liver weight (14%) was observed at 15 000 ppm. In females, statistically significant relative liver weight increases of 15%, 14% and 28% were seen at 2000, 7000 and 15 000 ppm, respectively (Table 22).

From the macroscopic investigation, large liver was variably recorded in males at 7000 and 15 000 ppm, although this was not statistically significant (Table 22).

Microscopic evaluation in all animals showed, in the kidney, an increased papillary mineralization (not statistically significant) in females and males at and above 7000 ppm; however, there was no clear dose–response relationship. Papillary mineralization was characterized by small amounts of dark basophilic mineral within the renal papillary tubules. A significant increase in minimal corticomedullary mineralization in females at and above 2000 ppm was noted. In the absence of other correlated biological changes, this effect was considered to be of minimal toxicological relevance. Corticomedullary mineralization was characterized by small amounts of dark, basophilic material within the tubules and tubular epithelium at the corticomedullary junction (Table 22).

In the liver, increased hepatocellular eosinophilia/hypertrophy in animals at 2000 ppm and above, characterized by enlarged hepatocytes with increased amounts of pale eosinophilic cytoplasm, was noted. Increases in hepatocellular eosinophilia/hypertrophy were statistically significant in males at 7000 and 15 000 ppm and in females at and above 2000 ppm. There was no associated increase in hepatic tumours noted in the study. Increased hepatocyte vacuolation was observed in males (unscheduled and terminal kill) at all doses, achieving statistical significance at 400 and 15 000 ppm, but with no clear dose–response relationship. The increased incidence at all doses was of minimal severity. At 7000 and 15 000 ppm, an increase in slight hepatocyte vacuolation was noted. In females, an increase was observed at 7000 and 15 000 ppm, with the value at the highest dose being statistically significant. Hepatocyte vacuolation was characterized by variable numbers of small to large cytoplasmic vacuoles within scattered hepatocytes. According to the study author, the toxicological relevance of this change in this study was unclear. Bile duct hyperplasia was slightly increased in males at and above 2000 ppm and variably in treated females. This finding was considered not to be toxicologically relevant, as it was not observed in a dose-dependent manner and there was no associated bile duct epithelial degeneration or necrosis, fibrosis or progression to bile duct neoplasia (Table 22).

In the thyroid gland, increased follicular cell hypertrophy and hyperplasia were noted in males and, to a lesser extent, in females at 15 000 ppm; this increased incidence was statistically significant in males at 15 000 ppm. Follicular cell hypertrophy was characterized by follicles with columnar epithelium with increased amounts of apical cytoplasm, with or without a decrease in follicular colloid. This was not accompanied by an increase in thyroid follicular cell tumours and is known to be associated with hepatocellular hypertrophy (Table 22).

In the ovary, a minor increase in sex cord stromal hyperplasia was noted in treated females, but not in a dose-dependent manner and also without statistical significance. Sex cord stromal hyperplasia was characterized by a hyperplastic lesion composed of mixtures of granulosa, theca, luteal or Sertoli

cells within the ovarian parenchyma, with minimal involvement of the surface epithelium. The incidence in treated animals was within current historical background data (Table 22).

Increased oligospermia was recorded in the epididymis of males at 15 000 ppm; however, the finding was primarily noted unilaterally. This was considered to be an age-related change due to the predominantly unilateral nature and, in the absence of any other male reproductive tract findings, not associated with generalized hormonal disruption or mandestrobin toxicity (Table 22).

A reduction in the incidence of benign mammary fibroadenomas and a statistically significant reduction in the incidence of pituitary tumours (adenoma and carcinoma) were noted in females at 15 000 ppm. This correlates with the reduction in fatal tumours observed in female decedents at 15 000 ppm.

An increased incidence of sex cord stromal tumours was recorded in the ovary of females at 7000 and 15 000 ppm. These increased incidences were not statistically significant when analysed using pairwise analysis, but were positive for tests (Peto) of an increasing dose–response relationship. The incidence of sex cord stromal tumour in the ovary exceeded the laboratory historical control range for this strain of rats. However, it is also noted that the incidence in the concurrent control animals exceeded or was close to the upper range of the historical controls (Tables 23 and 24). Simultaneous occurrences (multiplicity) of sex cord stromal tumour were found in two rats at the highest dose of 15 000 ppm.

It is noted that the tumours occurred at doses at which body weight gain was reduced by more than 20%. In addition, higher survival rates of 80% and 82% in females at 7000 and 15 000 ppm, respectively, were observed. This may have contributed to the higher numbers of tumours. In this respect, it is noted that in the historical control data provided, the tumours occurred at a survival greater than 70%.

A dose-related increase in interstitial cell adenomas was noted in the testis of males. The increase was statistically significant in a trend test, but no dose was statistically significantly increased in pairwise comparisons.

The NOAEL for toxicity was 400 ppm (equal to 26.7 mg/kg bw per day), based on effects on body weight and liver (histopathological changes and increased liver weights) in females at 2000 ppm (equal to 135.2 mg/kg bw per day). The NOAEL for carcinogenicity was 7000 ppm (equal to 375.6 mg/kg bw per day), based on an equivocal increase in the incidence of tumours (ovarian sex cord stromal adenomas and testicular interstitial cell adenomas) at the highest dose (Beck, 2012d).

Table 23. First set of historical control data for sex cord stromal hyperplasia and tumours

Parameter	Study 1	Study 2	Study 3	Study 4	Study 5	Study 6	Study 7	Study 8	Total average
Number of animals examined	70	50	50	50	50	50	100	80	480
Survival (%)	78	74	72	74	72	37	45	58	64
Sex cord stromal hyperplasia	31/70	14/49	1/50	24/50	1/50	13/50	26/100	16/59	126/478
(%)	44	28	2	48	2	26	26	27	26.3
Sex cord stromal tumour	1/70	0/49	0/50	2/50	0/50	0/50	0/100	0/59	3/478
(%)	1.4	0	0	4.0	0	0	0	0	0.63

Source: Yamada & Miyata (2012)

Table 24. Second set of historical control data for sex cord stromal hyperplasia and tumours

Parameter	Study 1	Study 2	Study 3	Study 4	Study 5	Study 6	Study 7	Total average
Completed year	2000	2001	2003	2004	2005	2007	2008	2000–08
Pathologist ^a	A	B	B	C	A	D	D	A, B, C, D
Number of animals examined	70	50	50	50	100	60	100	480
Survival (%)	78	74	72	72	79	58	45	68
Sex cord stromal hyperplasia	31/70	14/49	1/50	1/50	31/64	15/59	26/100	120/442
(%)	44	29	2	2	48	27	26	27.1
Sex cord stromal tumour	1/70	0/49	0/50	0/50	2/64	0/59	0/100	3/442
(%)	1.4	0	0	0	3.1	0	0	0.68

^a The pathologist for the carcinogenicity study on mandedrobin is not the same as the pathologists for studies 1–7 in this table.

Source: Beck (2012d)

2.4 Genotoxicity

Mandedrobin was negative for genotoxicity in three in vitro assays (gene mutation, chromosomal aberration and forward mutation assay) in the presence and absence of metabolic activation and in an in vivo assay (mouse bone marrow micronucleus assay). The results of the genotoxicity tests are summarized in Table 25 and described briefly below.

Table 25. Results of genotoxicity studies performed with mandedrobin

Type of study	Organism/cells	Dose/concentration range	Purity (%)	Result	Reference
In vitro					
Gene mutation (Ames test)	<i>Salmonella typhimurium</i> TA98, TA100, TA1535 and TA1537 and <i>Escherichia coli</i> WP2uvrA	–S9: 9.77–5 000 µg/plate +S9: 39.1–5 000 µg/plate	93.4	Negative ±S9	Kitamoto (2010a)
Chromosomal aberration assay	Chinese hamster lung cells (CHL/IU)	–S9: 3.91–80.0 µg/mL +S9: 100–150 µg/mL	93.4	Negative ±S9	Kitamoto (2010b)
HPRT forward mutation assay	Chinese hamster V79 cells	–S9: 1.0–50.0 µg/mL +S9: 8.0–144.0 µg/mL	93.4	Negative ±S9	Wollny (2010)
In vivo					
Micronucleus assay	Mouse (CD-1) bone marrow erythrocytes (gavage)	500–2 000 mg/kg bw	93.4	Negative	Kitamoto (2010c)

bw: body weight; HPRT: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × g supernatant fraction from rat liver homogenate

(a) *In vitro*

Mandestrobin (purity 93.4%) was tested in two separate experiments for its mutagenic potential on *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* strain WP2uvrA using the preincubation method at concentrations up to 5000 µg/plate in the absence and presence of S9 mix. Cytotoxicity in strains TA100, TA1535 and TA1537 was seen at and above 313 µg/plate without S9 mix and at and above 625 µg/plate with S9 mix. No cytotoxicity was observed for strains WP2uvrA and TA98 with or without S9 mix. Precipitation of the test compound was observed at and above 1250 µg/plate with and without S9 mix. There was no significant dose-related increase in the number of revertant colonies, with or without S9 mix. Positive controls showed a marked increase in the number of revertant colonies. Mandestrobin was not mutagenic under the test conditions (Kitamoto, 2010a).

Mandestrobin (purity 93.4%) was tested for its clastogenic potential in Chinese hamster lung cells (CHL/IU). Based on the solubility limit in dimethyl sulfoxide (DMSO), the highest concentration was set at 1000 µg/mL in the medium. Precipitates were seen in the medium at the beginning of treatment at and above 125 µg/mL and at the end of treatment at and above 500 µg/mL. In a preliminary cytotoxicity assay, marked growth inhibition was seen under all treatment conditions (6-hour exposure ±S9 and 24-hour exposure –S9). In the main test, when cells were exposed for 6 hours, no increase in the incidence of aberrant cells (structural, numerical or polyploid) in any treatment groups without S9 was observed. A suggestive dose-related increase in cells with structural aberrations was seen in the presence of S9 in cells treated for 6 hours. The increase at the highest concentration (3.5%) fell outside of the historical range for the laboratory (3%), but was considered to be of limited biological relevance, as it occurred at a concentration at which a greater than 55% inhibition of cell growth was observed, and cytotoxicity can lead to artefactual clastogenic results. When CHL/IU cells were treated for 24 hours without S9 mix, the negative result of the previous experiment was confirmed. In the presence of S9 mix, a repeat 6-hour treatment resulted in negative findings through a dose series of 100–150 µg/mL. Positive and negative controls gave expected results. Mandestrobin showed little potential to induce chromosomal aberrations under the test conditions (Kitamoto, 2010b).

Mandestrobin (purity 93.4%) was tested in a gene mutation (*HPRT* locus) assay in Chinese hamster V79 cells. In a preliminary cytotoxicity assay, cell survival after 4 hours of treatment was reduced to 16.3% at 15.6 µg/mL without S9 mix and to less than 10% at 250 µg/mL with S9 mix. After 24 hours of treatment, cell survival was reduced to less than 10% at 62.5 µg/mL without S9. Three independent experiments were performed. In experiment I, cells were exposed for 4 hours with and without metabolic activation (S9 mix). Nine doses from 0.25 to 12.0 µg/mL were chosen for treatment without S9 mix, and the six treatments from 1.0 to 10.0 µg/mL were selected for evaluation. Six doses from 8.0 to 172.0 µg/mL were chosen for treatment with S9 mix, and the five treatments from 8.0 to 128.0 µg/mL were selected for evaluation. In experiment IA, cells were exposed for 4 hours with metabolic activation. Seven doses from 16.0 to 172.0 µg/mL were chosen for treatment with S9 mix, and the five treatments from 16.0 to 144.0 µg/mL were selected for evaluation. In experiment II, cells were treated for 24 hours without metabolic activation and for 4 hours with metabolic activation. Nine doses from 1.88 to 70.0 µg/mL were chosen for treatment without S9 mix, and the five doses from 7.5 to 50.0 µg/mL were selected for evaluation. Seven doses from 16.0 to 172.0 µg/mL were chosen for treatment with S9 mix, and the five doses from 16.0 to 144.0 µg/mL were selected for evaluation. Cytotoxic effects occurred at 8.0 µg/mL and above without metabolic activation (4 hours of treatment), at 144 µg/mL and above with metabolic activation (4 hours of treatment) and at 50.0 µg/mL and above without metabolic activation (24 hours of treatment).

No relevant and reproducible increase in mutant colony number per 10⁶ cells was noted in the main experiments up to the maximum concentration. Isolated increases in the mutation frequency, exceeding the threshold of 3 times the mutation frequency of the corresponding solvent control, occurred occasionally, but were not reproduced in the parallel cultures under identical conditions and were based on the relatively low solvent controls. Compared with the corresponding negative controls, the 3-fold threshold was not exceeded. In addition, the mean values of mutation frequency between the first and the second cultures at 8.0 µg/mL (experiment I with metabolic activation), 32.0 µg/mL (experiment IA with metabolic activation) and 144.0 µg/mL (experiment II with metabolic activation)

were below the threshold and were within the historical control range. Furthermore, linear regression analysis (least squares) found no significant dose-dependent trend in the mutation frequency, indicated by a *P*-value below 0.05 in all of the experimental groups.

Appropriate positive controls showed a distinct increase in induced mutant colonies. Mandestrobin did not induce gene mutations at the *HPRT* locus in V79 cells (Wollny, 2010).

(b) *In vivo*

Mandestrobin (purity 93.4%) was tested for its clastogenic potential in an in vivo chromosomal aberration assay in mouse (CD-1) bone marrow. A range-finding toxicity assay was conducted in five mice of each sex per group administered a single mandestrobin dose (10 mL/kg bw) of 0 (0.5% aqueous methylcellulose), 500, 1000 or 2000 mg/kg bw by gavage. Mice were observed for clinical signs and mortality immediately after dosing, at 2.5 hours after dosing and daily for 2 days. Body weights were recorded once daily for 2 days. No abnormal signs were observed in any animals, but a slightly decreased body weight gain was noted in males at 2000 mg/kg bw. However, it was concluded that there were no substantial differences in sex response, and 2000 mg/kg bw was chosen as the highest test dose.

In the micronucleus test, mandestrobin was administered by gavage in 0.5% w/v aqueous methylcellulose to five male mice per group at a dose of 0, 500, 1000 or 2000 mg/kg bw; bone marrow smears were prepared 24 hours later. An additional group of five animals received mandestrobin at 2000 mg/kg bw, and smears were prepared 48 hours after treatment. The positive control material was cyclophosphamide. The incidence of micronucleated cells in 2000 polychromatic erythrocytes (PCEs) was scored for each animal, and the incidence of PCEs in 1000 erythrocytes (PCEs and normochromatic erythrocytes [NCEs]) was also recorded. Observations were performed using a microscope.

No dose-related increase in the incidence of micronuclei was noted. The positive control showed an appropriate increase in micronucleus formation. Although no change in the PCE/NCE ratio was found, a slight decrease in body weight gain at 2000 mg/kg bw indicated systemic absorption. Additionally, the exposure of bone marrow to mandestrobin was seen in the rat ADME studies.

In conclusion, mandestrobin did not cause an increase in the number of micronucleated PCEs in male mice when tested up to a dose of 2000 mg/kg bw (Kitamoto, 2010c).

2.5 Reproductive toxicity

(a) *Multigeneration studies*

In a dose range-finding study, groups of 10 Wistar rats of each sex were fed mandestrobin (purity 93.4%) at a dietary concentration of 0, 5000, 10 000 or 20 000 ppm (equal to 0, 244.1, 498.5 and 1032.5 mg/kg bw per day for males and 0, 316, 667.8 and 1229.0 mg/kg bw per day for females, respectively). Rats were mated after 4 weeks of exposure, and females were allowed to complete gestation and lactation while being maintained on treated diet. Adult males were terminated after gestation in the females was complete. At 4 days postpartum, litters were standardized to eight pups, and surplus pups were necropsied, then discarded. Adult females were terminated at 21 days postpartum, at weaning of the pups. Pups were terminated at 21 days of age.

No mortality or clinical signs were observed. In males, statistically significant decreases in body weight and body weight gain were noted in the 20 000 ppm group during the dosing period. In females, statistically significant decreases in body weight or body weight gain were noted in the 20 000 ppm group from day 28 of dosing through the gestation period to the lactation period. In females at 10 000 ppm, a statistically significant decrease in body weight or body weight gain was noted on day 28 of dosing and day 20 of gestation (Table 26).

Table 26. Key findings in the preliminary range-finding reproductive toxicity study in rats

	Males				Females			
	0 ppm	5 000 ppm	10 000 ppm	20 000 ppm	0 ppm	5 000 ppm	10 000 ppm	20 000 ppm
Parental animals (F₀)								
<i>Body weight (g)</i>								
Before mating (day 0)	217.0	215.9	217.1	216.2	162.6	159.1	160.2	161.2
Before mating (day 28)	324.4	328.9	320.0	302.2*	211.8	205.2	199.3**	201.6*
Gestation day 20	379.3	383.4	374.4	346.8*	329.4	318.4	312.5*	284.3**
Lactation day 21	(day 62)	(day 62)	(day 62)	(day 62)	267.5	277.4	267.8	244.3**
<i>Feed consumption (g/animal per day)</i>								
Before mating (day 7)	17.6	18.0	17.0	13.1**	13.6	11.7**	12.0*	10.1**
Before mating (day 28)	18.4	18.7	18.4	18.1	13.9	13.7	12.2**	13.0
Gestation day 20	17.4	17.9	18.0	17.3	19.7	18.2	19.3	16.7**
Lactation day 21	(day 62)	(day 62)	(day 62)	(day 62)	61.8	57.7	50.3*	36.8**
<i>Necropsy findings</i>								
Number of animals examined	10	10	10	10	10	10	10	10
Liver dark brownish change	0	0	0	10	0	0	1	8
Liver enlargement	0	0	5	10	0	0	1	7
Thyroid enlargement	0	1	1	4	0	0	1	1
<i>Organ weights</i>								
Absolute liver weight (g)	10.8	13.4**	14.7**	16.3**	12.0	14.1	16.4**	19.8**
% change	—	24	36	51	—	17	37	65
Relative liver weight (%)	2.85	3.48**	3.93**	4.71**	4.50	5.11	6.13**	8.15**
% change	—	22	38	65	—	—	36	81
Absolute thyroid weight (mg)	22.4	29.9*	30.1*	33.4**	18.2	18.3	20.8	19.6
% change	—	33	34	49	—	—	14	8
Relative thyroid weight (%)	5.92	7.76	8.14*	9.76**	6.82	6.62	7.78	7.94
% change	—	—	37	65	—	—	14	16
Absolute ovary weight (mg)	—	—	—	—	93.9	84.7	82.6	64.8**
% change	—	—	—	—	—	—	—	30

	Males				Females			
	0 ppm	5 000 ppm	10 000 ppm	20 000 ppm	0 ppm	5 000 ppm	10 000 ppm	20 000 ppm
Relative ovary weight	—	—	—	—	35.11	30.46	30.84	26.55**
% change	—	—	—	—	—	—	—	24
Absolute uterus weight (g)	—	—	—	—	0.503	0.490	0.301**	0.229**
% change	—	—	—	—	—	—	40	55
Relative uterus weight (%)	—	—	—	—	0.18	0.17	0.11**	0.094**
% change	—	—	—	—	—	—	39	48
<i>Histopathology</i>								
Number of animals examined	10	10	10	10	10	10	10	10
Liver								
Brown pigment in bile duct, focal (grades 1–3)	0	0	1	6	0	0	5	9
Brown pigment deposition in perilobular hepatocytes (grade 1)	0	0	0	3	0	0	2	6
Periductular inflammatory cell infiltration, focal (grade 1)	0	0	1	4	0	0	3	8
Eosinophilic foci of altered hepatocytes (grade 1)	0	0	0	1	0	0	0	1
Centrilobular hypertrophy, hepatocyte (grades 1–3)	0	4 (gr. 1)	10 (gr. 1–3)	10 (gr. 1–3)	0	6 (gr. 1–2)	10 (gr. 1–2)	10 (gr. 2–3)
Bile duct proliferation (grades 1–2)	0	0	0	1	0	0	3	5
Ovary								
Decreased vacuolation in interstitial gland (grade1)	—	—	—	—	0	0	1	7
Uterus								
Atrophy (grade 1)	—	—	—	—	0	0	2	10
Thyroid								

	Males				Females			
	0 ppm	5 000 ppm	10 000 ppm	20 000 ppm	0 ppm	5 000 ppm	10 000 ppm	20 000 ppm
Diffuse hypertrophy of follicular cells (grade 1)	0	0	2	5	0	0	0	0
<i>Reproductive performance</i>								
Copulation index (%)	100	100	100	100	100	100	100	100
Fertility index (%)	100	100	100	100	100	100	100	100
<i>Litter examination</i>								
Implantation sites/dam	—	—	—	—	13.5	12.1	11.5	11.0
Offspring at birth/dam	—	—	—	—	13.0	10.4	10.0	10.5
Live offspring at birth/dam	—	—	—	—	13.0	10.4	10.0	10.1*
Viability index (M/F) (day 0)	—	—	—	—	100	100	100	96.5*
Weaning index	—	—	—	—	100	100	100	95
Offspring (F₁)								
<i>Pup body weight (g)</i>								
Day 0	5.7	6.0	5.9	5.6	5.4	5.8	5.6	5.2
Day 21	52.5	49.4	45.0*	26.6**	50.5	47.7	43.4*	25.5**
<i>Pup organ weights on weaning</i>								
Terminal body weight (g)	53.1	49.9	44.9**	26.3**	50.5	48.8	44.3*	25.8**
Absolute brain weight (g)	1.459	1.437	1.438	1.258**	1.424	1.417	1.390	1.228**
% change	—	—	—	14	—	—	—	14
Relative brain weight (%)	2.76	2.89	3.22*	5.04**	2.83	2.91	3.15	5.03**
% change	—	—	16	83	—	—	—	78
Absolute thymus weight (mg)	202.2	194.1	172.2	75.6**	208.2	206.4	174.0	80.1**
% change	—	—	—	63	—	—	—	38
Relative thymus weight (% × 10 ⁻³)	380.6	390.2	380.7	280.4**	411.2	423.8	390.1	297.7**
% change	—	—	—	26	—	—	—	28
Absolute spleen weight (g)	0.248	0.210	0.194*	0.091**	0.228	0.210	0.189	0.088**
% change	—	—	22	63	—	—	—	61

	Males				Females			
	0 ppm	5 000 ppm	10 000 ppm	20 000 ppm	0 ppm	5 000 ppm	10 000 ppm	20 000 ppm
Relative spleen weight (%)	0.46	0.41	0.43	0.33**	0.45	0.42	0.42	0.33*
% change	—	—	—	29	—	—	—	27
Absolute uterus weight (mg)	—	—	—	—	36.91	37.98	31.38	25.18**
% change	—	—	—	—	—	—	—	32

F: females; Fo: parental generation; F₁: first filial generation; gr.: grade; M: males; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

Source: Hoshino (2010)

In males, statistically significant increases in absolute and relative liver weights were observed at 5000 ppm (absolute 24%; relative 22%), 10 000 ppm (absolute 36%; relative 38%) and 20 000 ppm (absolute 51%; relative 65%). In females, statistically significant increases in absolute and relative liver weights were observed at 10 000 ppm (absolute 37%; relative 36%) and 20 000 ppm (absolute 65%; relative 81%). Statistically significant increases in absolute and relative thyroid weights were observed in males at 10 000 ppm (absolute 34%; relative 37%) and 20 000 ppm (absolute 49%; relative 65%). In females, statistically significant decreases in absolute and relative uterus weights were noted at 10 000 ppm (absolute 40%; relative 39%) and 20 000 ppm (absolute 55%; relative 48%). In addition, decreased absolute and relative ovary weights were noted at 20 000 ppm (absolute 30%; relative 24%). However, no treatment-related changes in reproductive function, such as mating ability, fertility, pregnancy, parturition or nursing behaviour, were noted in females (Table 26).

Dark brownish change in the liver was noted in one female in the 10 000 ppm group and in 10 males and eight females in the 20 000 ppm group. Enlargement of the liver was noted in five males and one female in the 10 000 ppm group and in 10 males and seven females in the 20 000 ppm group. Enlarged thyroid was noted in one, one and four males and in zero, one and one female in the 5000, 10 000 and 20 000 ppm groups, respectively (Table 26).

Histopathology investigation in adults revealed brown pigment in the bile ducts in one male and five females at 10 000 ppm and in six males and nine females at 20 000 ppm. Brown pigment deposition in the perilobular hepatocytes was noted in two females at 10 000 ppm and in three males and six females at 20 000 ppm. Focal periductular inflammatory cell infiltration was noted in one male and three females at 10 000 ppm and in four males and eight females at 20 000 ppm. Proliferation of the bile ducts was noted in three females at 10 000 ppm and in one male and five females at 20 000 ppm. Eosinophilic focus of the altered hepatocytes was noted in one male and one female at 20 000 ppm. Centrilobular hypertrophy of the hepatocytes was noted in four, 10 and 10 males and six, 10 and 10 females at 5000, 10 000 and 20 000 ppm, respectively. Diffuse hypertrophy of the follicular thyroid cells was noted in two males at 10 000 ppm and in five males at 20 000 ppm. Hypertrophy in the thyroid follicular cells in this study is considered to be an adaptive change caused by the induction of hepatic enzymes. Among eight animals with enlarged thyroids at necropsy, diffuse hypertrophy of the follicular cells was noted in two males at 20 000 ppm. In the other six animals, vacuolation of the follicular cells was noted. It has been reported that vacuolation of thyroid follicular cells is a spontaneous lesion in BrlHan:WIST@Jcl (GALAS) rats (Shimoi et al., 2001). In the present study, this change was also noted in one female in the control group. Therefore, vacuolation of the follicular cells was considered to be incidental. In the ovary, decreased vacuolation in the interstitial gland was noted in one female at 10 000 ppm and in seven females at 20 000 ppm. Uterus atrophy was noted in two females at 10 000 ppm and in 10 females at 20 000 ppm (Table 26).

Mating performance and fertility appeared unaffected at all doses. A statistically significant decrease in the viability index on day 0 was noted in the 20 000 ppm group. Furthermore, a decrease in weaning index (not statistically significant) was noted in the 20 000 ppm group, and this change was

induced by the death of four F₁ animals after the culling in one dam. A statistically significant decrease in the number of live offspring at birth (and as a consequence also in the number of live offspring on day 4 before culling) was noted in the 20 000 ppm group. However, the number of live offspring at birth in the control group was above the mean of the background range of the test facility, and that in the 20 000 ppm group was slightly below the mean of the background range. Accordingly, the decrease in the number of live offspring at birth in the high-dose group was considered to be an incidental change attributable to the increased number of live offspring at birth in the control group and was judged to be toxicologically irrelevant (Table 26).

Pups at 10 000 ppm and 20 000 ppm showed statistically significant decreases in body weight or body weight gain after postnatal day (PND) 4 (before culling) in males and females, which increased in magnitude up to weaning. At necropsy, no abnormal changes attributable to the test substance were noted in either sex. At 20 000 ppm, statistically significant decreases in absolute brain, thymus and spleen weights in both sexes and in absolute uterus weights in females were observed. In addition, absolute spleen weights were also statistically significantly decreased in the mid-dose male pups (10 000 ppm). Furthermore, statistically significant decreases in relative weights of thymus and spleen were noted in both sexes at 20 000 ppm. Statistically significant increases in relative brain weights (in mid- and high-dose males as well as in high-dose females) and in relative uterus weights (in high-dose females) were considered to result from excessive lower body weight at necropsy (Table 26).

A top dietary concentration of 10 000 ppm was considered appropriate for the main study (Hoshino, 2010).

The main two-generation rat reproductive toxicity study was conducted with mandestrobin (purity 93.4%) at dietary concentrations of 0, 1000, 3000 and 10 000 ppm (equal to 0, 47.77, 145.7 and 511.7 mg/kg bw per day for males and 0, 65.68, 200.3 and 672.0 mg/kg bw per day for females, respectively). Groups of 26 male and 26 female Wistar rats were allocated to treatment groups (F₀ generation) and were exposed to treated diets 10 weeks prior to mating and then during gestation and lactation, until scheduled termination. At the end of the lactation period (21 days postpartum), 24–26 male and female pups were selected to become the F₁ parental animals. These animals were again permitted a 10-week premating phase starting at approximately 28 days of age and then mated; females were allowed to rear their litters to form the F₂ generation. The F₂ animals were monitored until weaning, and the study was then terminated. Exposure to treated diet continued throughout the study, including the premating, mating, gestation and lactation periods of the F₀ and F₁ generations. The F₂ pups were indirectly exposed to test substance until weaning (day 21 postpartum).

Mortality and clinical signs were checked at least once daily for all animals. Body weights and feed consumption were determined weekly throughout the study until termination for males except during the mating period. For females, body weight and feed consumption were recorded weekly during the premating periods, on days 0, 7, 14 and 20 postcoitum and on days 0, 4, 7, 14 and 21 postpartum. The age and body weight at which vaginal opening or preputial separation occurred were recorded for F₁ generation weanlings selected for breeding the F₂ generation. Sperm analysis was performed for all males per group (F₀ and F₁ animals), comprising assessment of motility, morphology and sperm count. A record of mating of females was made by daily examination of the vaginal smears for spermatozoa and/or appearance of a vaginal plug throughout the pairing period. The day on which evidence of mating was observed was considered to be day 0 postcoitum. Towards the end of the gestation period (gestation days [GDs] 21–25), females were examined twice daily for signs of parturition. Females without litters by GD 25 were killed and necropsied on GD 26. Day 0 of lactation was the day on which a female had delivered all its pups. All F₀ and F₁ males were terminated 5 and 6 weeks postcoitum, respectively. All F₀ and F₁ females were terminated on day 21 of lactation. On PND 4, litter size was randomly adjusted to eight pups (equal sex ratio, in principle). Litters with fewer than eight pups were maintained as they were. The pups culled at the litter size adjustment were subjected to necropsy. The following organ weights were recorded from all F₀ and F₁ parent animals on day 21 postpartum or shortly thereafter: brain, pituitary, thyroid, liver, kidney, adrenal, spleen, testis, epididymis, prostate, seminal vesicle, ovary and uterus. Full histopathological examination was carried out on the following organs/tissues of

all males and females in the control and 10 000 ppm groups: pituitary, adrenal, testis, epididymis, seminal vesicle, coagulating gland, prostate, ovary, fallopian tube, uterus and vagina. In addition, the livers of males and females and the thyroids of males in the F₀ and F₁ generations and the kidneys of F₀ females were also examined. Because treatment-related changes were seen in livers of top-dose F₀ and F₁ males and females, in thyroids of top-dose F₀ and F₁ males and in adrenals of top-dose F₁ females, the thyroids and livers of F₀ and F₁ animals and adrenals of F₁ females in the 1000 and 3000 ppm groups were also examined histologically.

In parental animals from the F₀, F₁ and F₂ generations, no clinical signs were noted during the study.

In F₁ animals, one female (#60425-6) died on PND 13 in the 10 000 ppm group. In F₂ animals, one male and one female (#20411-43, #70404-58) died on PND 5 in the 10 000 ppm group. Before litter size adjustment (PND 4), death was observed sporadically in a few pups in all dose groups, including the control groups for both F₁ and F₂ pups.

In F₀ males, body weight gain was suppressed at 10 000 ppm, with significant differences in body weight and body weight gain throughout the observation period. Reduced feed consumption was also noted in males at 10 000 ppm from pre-mating days 28 to 63 of dosing (Table 27).

Table 27. Key findings of the two-generation reproductive toxicity study in rats (first generation)

Finding	Males				Females			
	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm
F₀ parental animals								
Dose (mg/kg bw per day)								
Premating (weeks 0–2)	0	56.15	166.3	559.1	0	62.48	195.3	628.5
Postmating/gestation	0	43.27	132.0	452.1	0	60.19	186.2	602.8
Lactation	–	–	–	–	0	162.8	511.0	1 634.6
Feed consumption (g/animal per day)								
Premating (week 10, ending day 70)	17.8	17.5	17.3	17.0	12.9	12.3	12.4	12.5
Premating (mean over weeks 4–9)	18.0	17.8	17.4	16.6**	12.7	12.4	12.9	12.4
Lactation (day 7)	–	–	–	–	41.8	39.1	41.5	36.0*
Lactation (day 14)	–	–	–	–	47.6	45.2	48.3	44.3
Lactation (day 21)	–	–	–	–	60.4	58.2	60.0	58.8
Body weight (g)								
Before mating (day 0)	185.8	185.7	185.9	185.8	134.6	135.8	135.9	135.8
Before mating (day 70)	393.8	391.2	386.2	369.9**	221.1	225.4	225.1	220.4
Gestation day 20	438.7	437.7	436.1	415.0**	326.1	327.0	329.2	324.3
Lactation day 21	(day 119)	(day 119)	(day 119)	(day 119)	268.1	272.7	273.8	273.9
Body weight gain (g)								
Premating (days 0–70)	208.0	205.5	200.3	184.1**	86.5	89.7	89.2	84.6
Gestation (days 0–7)					24.2	21.0	21.8	20.8
Gestation (days 0–14)					48.2	44.0	43.9	42.7**

Finding	Males				Females			
	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm
Gestation (days 0–20)	252.9 (days 0–119)	252.0 (days 0–119)	250.2 (days 0–119)	229.2** (days 0–119)	103.3	101.0	103.3	102.0
Reproductive performance								
Number of pairs	–	–	–	–	26	26	26	26
Mating index (%)	–	–	–	–	100	100	100	96.2 (25/26)
Mean precoital time (days)	–	–	–	–	2.9	3.2	2.5	3.6
Fertility index (%)	–	–	–	–	96.2 (25/26)	100	100	96.0 (24/25)
Gestation index (%)	–	–	–	–	100	100	100	100
Gestation duration (days)	–	–	–	–	22.1	22.0	22.0	22.0
Implantations (mean)	–	–	–	–	13.0	12.2	13.2	13.0
Live young at birth	–	–	–	–	12.2	11.7	12.2	12.3
Birth index (%)	–	–	–	–	93.78	95.86	92.64	95.40
Organ weights								
Absolute liver weight (g)	12.65	13.04	14.12**	15.53**	10.42	11.45*	12.24**	17.24**
% change	–	–	12	23	–	10	17	65
Relative liver weight (%)	2.87	2.97	3.23**	3.73**	3.89	4.21*	4.47**	6.30**
% change	–	–	12	30	–	8	15	75
Absolute kidney weight (g)	2.536	2.545	2.555	2.499	1.987	1.992	1.961	2.095**
% change	–	–	–	–	–	–	–	5
Relative kidney weight (%)	0.577	0.580	0.585	0.599	0.742	0.731	0.717	0.766**
% change	–	–	–	–	–	–	–	3
Absolute thyroid weight (mg)	23.75	24.04	25.29**	28.97**	20.98	21.90	21.67	21.55
% change	–	–	6	22	–	–	–	–
Relative thyroid weight (%)	5.45	5.48	5.78*	7.02**	7.85	8.00	7.93	7.88
% change	–	–	6	29	–	–	–	–
Absolute ovary weight (mg)	–	–	–	–	91.34	90.35	97.48	82.33
Relative ovary weight (%)	–	–	–	–	34.14	33.21	35.66	30.06*
% change	–	–	–	–	–	–	–	12
Absolute uterus weight (g)	–	–	–	–	0.45	0.54	0.51	0.38*
% change	–	–	–	–	–	–	–	16
Relative uterus weight (%)	–	–	–	–	0.167	0.198	0.187	0.137**
% change	–	–	–	–	–	–	–	18
Gross findings								
No. of animals examined	26	26	26	26	25	26	26	24
Kidney: greenish change, cortex	0	0	0	0	0	0	0	5*

Finding	Males				Females			
	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm
Liver: dark brownish change	0	0	0	0	0	0	0	24**
Liver: enlargement	0	0	0	0	0	0	0	20**
Histopathology								
<i>Liver (no. examined)</i>	26	26	26	26	25	26	26	24
Brown pigment, bile duct/periportal								
Grade 1	0	0	0	9**	0	0	0	10**
Grade 2	0	0	0	0	0	0	0	7
Grade 3	0	0	0	0	0	0	0	3
Brown pigment deposition, hepatocyte, perilobular								
Grade 1	0	0	0	0	0	0	0	4*
Cell infiltration, inflammatory, focal, periductular								
Grade 1	0	0	0	4*	0	0	0	10**
Proliferation, bile duct								
Grade 1	0	0	0	0	0	0	0	5**
Grade 2	0	0	0	0	0	0	0	4
Grade 3	0	0	0	0	0	0	0	3
Hypertrophy, hepatocyte, diffuse								
Grade 1	0	0	15**	20**	0	6*	17**	1**
Grade 2	0	0	0	4	0	0	0	21
Grade 3	0	0	0	0	0	0	0	2
<i>Thyroid (no. examined)</i>	26	26	26	26	0	0	0	0
Hypertrophy, follicular cell diffuse								
Grade 1	0	0	0	4*	—	—	—	—
F₁ pups								
Body weight (g)								
PND day 0	5.8	5.8	5.8	5.8	5.5	5.5	5.4	5.5
PND day 7	16.2	15.9	16.3	14.7*	15.7	15.5	15.7	14.2*
PND day 14	32.5	32.0	32.3	28.0**	31.6	31.2	31.4	27.1**
PND day 21	52.0	51.1	50.3	43.8**	50.3	49.5	48.5	41.9**
Before mating (day 91)	390.7	391.6	381.7	353.0**	224.0	229.5	225.7	211.8
Gestation day 20	465.5	464.1	452.5	428.0**	340.8	349.5	346.3	328.9
Lactation day 21	(day 147)	(day 147)	(day 147)	(day 147)	277.4	289.9*	290.1*	279.0
Body weight gain (g)								
PND day 7	6.2	6.0	6.2	5.2**	6.0	5.8	6.0	5.0**
PND day 14	22.5	22.0	22.2	18.4**	21.9	21.5	21.6	17.9**
PND day 21	42.0	41.2	40.2	34.3**	40.5	39.9	38.8	32.7**

Finding	Males				Females			
	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm
F₁ pup development								
Day of cleavage of balanopreputial gland (males)	41.1	41.8	42.0	42.7**	—	—	—	—
Day of vaginal opening	—	—	—	—	31.0	30.9	31.0	32.5*
Body weight (g) at cleavage of balanopreputial gland/vaginal opening	173.96	181.38	179.62	167.17	94.96	96.46	95.5	89.33**

bw: body weight; F₀: parental generation; F₁: first filial generation; grades: 1, minimal; 2, mild; 3, moderate; PND: postnatal day; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

Source: Matsuura (2012)

In F₁ animals at 10 000 ppm, although birth weights were almost the same as those in the control group, postnatal body weight gain was suppressed in males and females before weaning, and statistically significant differences in body weight and body weight gain were noted in both sexes from PNDs 7 to 21 (Table 27). After weaning, suppressed body weight gain continued in males until the terminal necropsy, with statistically significant differences in body weight and body weight gain at almost all measurement points. In females, statistically significantly lower body weights also continued after weaning. However, a statistically significant difference in body weight gain was observed only on PND 28 (data not shown). Statistically significantly lower feed consumption was observed sporadically in males and females at 10 000 ppm (data not shown).

In F₂ animals at 10 000 ppm, statistically significant differences in body weight gain were noted in both sexes from PNDs 7 to 21 and in body weight from PNDs 14 to 21 (Table 28).

Table 28. Key findings of the two-generation reproductive toxicity study in rats (second generation)

Finding	Males				Females			
	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm
F₁ animals								
Dose (mg/kg bw per day)								
Premating (weeks 0–2)	0	84.73	254.5	881.2	0	90.11	247.9	929.3
Postmating/gestation	0	47.77	145.7	511.7	0	65.68	200.3	672.0
Lactation	—	—	—	—	0	163.4	504.6	1 687.5
Reproductive performance								
Number of pairs	—	—	—	—	25	26	26	24
Mating index (%)	—	—	—	—	100	100	100	100
Mean precoital time (days)	—	—	—	—	2.6	2.6	2.3	2.0
Fertility index (%)	—	—	—	—	100	96.2	92.3	100
Gestation index (%)	—	—	—	—	100	100	100	100
Gestation duration (days)	—	—	—	—	22.2	22.0	22.0	21.8**
Implantations (mean)	—	—	—	—	13.8	13.5	13.1	13.2
Live young at birth	—	—	—	—	13.0	12.9	12.5	12.3

Finding	Males				Females			
	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm
Birth index (%)	—	—	—	—	93.96	95.29	95.44	93.04
Organ weights at weaning								
Body weight (g)	52.0	51.4	50.5	44.0**	50.6	49.8	48.8	41.6**
Absolute spleen weight (g)	0.266	0.248	0.232*	0.199**	0.252	0.250	0.232	0.200**
% change	—	—	13	25	—	—	—	21
Relative spleen weight (%)	0.51	0.48	0.45*	0.44**	0.49	0.50	0.47	0.48
% change	—	—	12	14	—	—	—	—
Absolute thymus weight (mg)	204.1	199.0	203.4	167.8**	208.8	206.2	208.5	172.1**
% change	—	—	—	18	—	—	—	18
Relative thymus weight ($\times 10^{-3}$ %)	392.9	387.5	403.2	380.1	413.0	414.28	426.5	412.4
Absolute uterus weight (mg)	—	—	—	—	40.38	40.38	42.70	34.60*
% change	—	—	—	—	—	—	—	14
Relative uterus weight ($\times 10^{-3}$ %)	—	—	—	—	80.0	81.9	87.13	84.14
Organ weights for F₁ parental animals								
Absolute liver weight (g)	13.86	14.00	14.62	15.97**	11.14	12.30**	13.33**	16.96**
% change	—	—	—	15	—	10	20	52
Relative liver weight (%)	2.97	3.00	3.216**	3.74**	4.01	4.25	4.59**	6.10**
% change	—	—	8	26	—	—	14	52
Absolute thyroid weight (mg)	25.32	25.12	26.02	32.41*	23.61	22.06	20.62	21.03
% change	—	—	—	28	—	—	—	—
Relative thyroid weight ($\times 10^{-3}$ %)	5.45	5.39	5.75	7.72**	8.49	7.65	7.12	7.55
% change	—	—	—	42	—	—	—	—
Absolute ovary weight (mg)	—	—	—	—	97.10	95.83	98.25	84.40**
% change	—	—	—	—	—	—	—	13
Relative ovary weight (%)	—	—	—	—	35.09	33.03	33.84	30.28**
% change	—	—	—	—	—	—	—	14
Absolute spleen weight (g)	0.688	0.709	0.701	0.733	0.612	0.693	0.634	0.661
% change	—	—	—	—	—	—	—	—
Relative spleen weight (%)	0.14	0.15	0.15	0.17**	0.22	0.24	0.21	0.23
% change	—	—	—	21	—	—	—	—

Finding	Males				Females			
	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm
Gross findings								
Liver: dark brownish change	0/25	0/26	0/26	0/24	0/25	0/25	0/24	20/24**
Liver: enlargement	0/25	0/26	0/26	0/24	0/25	0/25	0/24	6/24**
Histopathology								
<i>Liver (no. examined)</i>	25	26	26	24	25	25	24	24
Brown pigment, bile duct/periportal								
Grade 1	0	0	10**	22**	0	0	11**	5**
Grade 2	0	0	0	2	0	0	0	16
Grade 3	0	0	0	0	0	0	0	3
Brown pigment deposition, hepatocyte, perilobular								
Grade 1	0	0	0	0	0	0	0	5*
Cell infiltration, inflammatory, focal, periductular								
Grade 1	0	0	0	24*	0	0	3	24**
Proliferation, bile duct								
Grade 1	0	0	0	0	0	0	0	7**
Grade 2	0	0	0	0	0	0	0	8
Grade 3	0	0	0	0	0	0	0	1
Hypertrophy, hepatocyte, diffuse								
Grade 1	0	0	6*	19**	0	4*	18**	11**
Grade 2	0	0	0	4	0	0	0	13
<i>Adrenals (no. examined)</i>	25	0	0	24	25	25	24	24
Hypertrophy, cortical cell, fascicular zone								
Grade 1	0	—	—	0	0	0	0	5*
F₂ animals								
Body weight (g)								
PND day 0	5.8	5.7	5.9	5.8	5.5	5.5	5.6	5.4
PND day 7	16.0	15.8	15.9	15.0	15.5	15.3	15.5	14.3
PND day 14	32.9	31.7	32.4	29.6**	32.1	31.0	31.8	28.5**
PND day 21	53.5	51.6	52.2	46.3**	51.4	50.3	50.4	44.5**
Body weight gain (g)								
PND day 7	6.1	5.8	6.0	5.4**	5.9	5.6	5.7	5.1**
PND day 14	23.0	21.7	22.4	20.0**	22.5	21.3	22.0	19.4**
PND day 21	43.6	41.7	42.2	36.7**	41.9	40.7	40.6	35.4**
F₂ pup organ weights at weaning								
Absolute thymus weight (mg)	212.0	204.9	201.5	182.5**	210.7	209.0	205.3	179.0**

Finding	Males				Females			
	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm
% change	—	—	—	14	—	—	—	15
Relative thymus weight ($\times 10^{-3}$ %)	395.0	392.7	384.13	388.0	406.4	408.9	401.9	400.5
Absolute spleen weight (g)	0.273	0.253	0.245	0.209**	0.274	0.262	0.234	0.203**
% change	—	—	—	24	—	—	—	26
Relative spleen weight (%)	0.50	0.48	0.46	0.44**	0.52	0.50	0.46*	0.45**
% change	—	—	—	12	—	—	12	14

bw: body weight; F₂: second filial generation; grades: 1, minimal; 2, mild; 3, moderate; PND: postnatal day; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

Source: Matsuura (2012)

No treatment-related changes were observed in the estrous cycles in females of any treated group of both the F₀ and F₁ generations. All mating pairs in each group in both generations showed evidence of copulation by 14 days after the start of mating, except for one pair in the 10 000 ppm group in the F₀ generation. Therefore, the test compound was judged to have no effect on mating ability.

In F₀ and F₁ animals, no treatment-related changes were observed in the mating ability or fertility of males or females of any treated group. In the F₀ generation, all the mating pairs of each group, except for one pair in the 10 000 ppm group, copulated during the first or second estrous stage of females within 13 days after the start of mating. There were no statistically significant differences in the copulation index, in the number of estrous stages without copulation or in the days until copulation between the control and treated groups. In the F₁ generation, there were only one and two non-pregnant females at 1000 and 3000 ppm, respectively, and no statistically significant difference was found in the fertility index between the control and treated groups.

In both F₀ and F₁ generations, no treatment-related changes suggesting effects on spermatogenesis were detected in any treated group. All parameters, including sperm motility, spermatid counts, sperm counts and the incidences of abnormal sperm and tailless sperm, were comparable between the control and treated groups, with no statistically significant differences. Furthermore, no abnormalities were observed in males that had no copulation or did not impregnate their mating partners, except for one male in the control group of the F₀ generation, which showed low sperm motility of 40%.

No treatment-related differences were found in the number of implantations or offspring born alive, birth index or gestation index between the control and treated groups in both the F₀ and F₁ generations. In the F₁ generation, the gestation length in the 10 000 ppm group (21.8 days) was statistically significantly shorter than in the control group (22.2 days) (Table 28). However, there was no treatment-related change in gestation duration for F₀ dams (Table 27). In F₁ dams, the difference from the control group in gestation duration was small, and the gestation duration of all dams in the 10 000 ppm group was 21 or 22 days, which is within the normal range (21–23 days) of this strain. Moreover, the mean gestation duration in the 10 000 ppm group was almost equal to the lower limit of the historical control range of the test facility (mean duration: 21.9–22.2 days; range of individual duration: 21–23 days; 2002–2009), whereas that in the control group was equal to the upper limit of the historical control range. Therefore, the statistically significant difference observed in the gestation duration in the 10 000 ppm group was considered to be a variation within the normal range and was judged to be of no toxicological relevance.

In F₁ and F₂ pups, no treatment-related changes were detected in the number of offspring born and born alive, sex ratio, live birth index, viability index on days 4, 7 and 14, and weaning index (Tables 27 and 28).

External examination of pups revealed no anomalies in F₁ or F₂ pups of any group, including the pups that died.

At 10 000 ppm, preputial separation (cleavage of balanopreputial gland) in F₁ males and vaginal opening in F₁ females showed a statistically significant delay of 1.6 and 1.5 days on average, compared with the control group (Table 27). The body weights in the 10 000 ppm group on the corresponding days were lower than those in the control group in both sexes (males, 96.1% of the control value; females, 94.1% of the control value), and a statistically significant difference was observed in females. In males, besides the slight (not statistically significant) body weight decrease at cleavage, body weight was consistently statistically significantly lower than that of controls from culling to day 91 after birth (~15%). Similarly, body weight gains were also statistically significantly depressed, but less severely. Females showed the same pattern. It is noted that no observations or examinations suggested effects on any reproductive function, including estrous cycle, sperm parameters and histopathological findings. Overall, this slight delay in sexual maturation in both sexes was considered to be related to growth retardation.

Pathological examination revealed treatment-related changes in the liver of both sexes and the thyroid of males in both generations and the adrenals of F₁ females. At necropsy, dark brownish change and enlargement of the liver were noted in F₀ and F₁ females in the 10 000 ppm group (Tables 27 and 28).

Liver weights were increased in males in the 3000 and 10 000 ppm groups and in females in the 1000 ppm group and above in both generations. In addition, increases in thyroid weights were observed in F₀ males in the 3000 and 10 000 ppm groups and in F₁ males in the 10 000 ppm group, as were decreases in ovary weights in F₀ and F₁ females and uterus weights in F₀ females in the 10 000 ppm group (Tables 27 and 28). In F₀ animals, treatment-related increases in absolute and relative liver weights were observed in males at 3000 ppm (absolute 12%; relative 12%) and 10 000 ppm (absolute 23%; relative 30%). In females, absolute and relative liver weights were increased at 1000 ppm (absolute 10%; relative 8%), 3000 ppm (absolute 17%; relative 15%) and 10 000 ppm (absolute 65%; relative 75%), compared with controls. In females, absolute and relative kidney weights were statistically significantly decreased at 10 000 ppm (absolute 5%; relative 3%). In males, absolute and relative thyroid weights were increased with statistical significance at 3000 ppm (absolute 6%; relative 6%) and 10 000 ppm (absolute 22%; relative 29%), compared with controls. In females at 10 000 ppm, relative ovary weights were decreased by 12% compared with controls, and absolute and relative uterus weights were decreased by 16% and 18% compared with controls, respectively (Table 27). In addition, significantly higher relative weights of the brain and seminal vesicle were observed in the 10 000 ppm group. However, the absolute weights of both organs were comparable to those in the control group, with no statistically significant differences; therefore, these changes were considered to be due to the lower body weights at necropsy in this group and were judged to be of no toxicological relevance.

In F₁ pups at weaning, statistically significant decreases in the absolute and relative weights of the spleen were noted in males at 3000 ppm (absolute 13%; relative 12%) and 10 000 ppm (absolute 25%; relative 14%). In addition, at 10 000 ppm, statistically significantly lower absolute weights (18%) were observed in the thymus in both sexes and in the spleen (21%) and uterus (14%) in females (Table 28), as were statistically significantly higher relative weights of the brain in both sexes in the 10 000 ppm group. However, the changes were judged to be of no toxicological relevance, as absolute organ weight change was not accompanied by relative organ weight change and vice versa; therefore, they were considered to be due to the lower body weights at necropsy in this group.

In F₁ adults, males had statistically significantly increased absolute and relative liver weights (absolute 15%; relative 26%) at 10 000 ppm and relative liver weight (8%) at 3000 ppm. Females showed increased absolute liver weight at 1000, 3000 and 10 000 ppm, by 10%, 20% and 52%, respectively, whereas relative liver weights were increased at 3000 ppm and 10 000 ppm by 14% and 52%, respectively. In males, absolute and relative thyroid weights were statistically significantly increased at 10 000 ppm by 28% and 42%, respectively. In females, absolute and relative ovary weights were statistically significantly decreased by 13% and 14%, respectively, at 10 000 ppm. In addition, in males, statistically significantly higher relative weights of several other organs (brain, spleen, kidney,

adrenal, testis, seminal vesicle and epididymis) were observed in the 10 000 ppm group. However, the absolute weights of these organs were comparable to those in the control group, with no statistically significant differences; therefore, these changes were considered to be due to the lower body weights at necropsy in this group and were judged to be of no toxicological relevance. Furthermore, in females, there were statistically significantly lower absolute brain weight in the 10 000 ppm group and relative brain weights in the 1000 and 3000 ppm groups, relative pituitary weights in the 3000 and 10 000 ppm groups, and relative left adrenal weight in the 3000 ppm group. However, they were changes observed only in absolute or relative weight, not both, with a lack of clear dose dependency; therefore, they were judged not to be treatment related (Table 28).

In F₂ pups at weaning, statistically significant decreases in the absolute and relative weights of the spleen were noted in males and females at 10 000 ppm (males: absolute 24%, relative 12%; females: absolute 26%, relative 14%). Moreover, the spleen weights of females in the 3000 ppm group also tended to be lower than those in the control group, and a statistically significant difference was noted in the relative weights (12%). In addition, statistically significantly decreased thymus weights were observed in males and females, by 14% and 15%, respectively, in the 10 000 ppm group (Table 28), and statistically significantly higher relative brain weights were seen in both sexes in the 10 000 ppm group. However, these changes were observed only in the absolute or relative weight, not both, and the corresponding relative or absolute weights of these organs were similar to those of the control group; therefore, they were considered to be due to the lower body weights at necropsy in this group and were judged to be of no toxicological relevance.

In F₁ females, ovarian follicle count revealed a statistically significant increase in the number of medium-sized follicles in the 10 000 ppm group. In addition, although there was no statistically significant difference, the number of large follicles also tended to increase in the 10 000 ppm group. However, the number of small follicles, which include primordial follicles, and the total number of ovarian follicles in the 10 000 ppm group were comparable with those in the control group, with no statistically significant differences. Moreover, the percentage of ovarian follicles at each developmental stage in the 10 000 ppm group was almost equal to that in the control group, indicating no effects on oogenesis. In addition, there were no treatment-related changes suggesting impairment of ovarian function in any reproductive observations, including estrous cycles and the number of implantations, and no histopathological changes were detected in the ovaries in this group. Consequently, the increase in number of medium-sized ovarian follicles was judged to be incidental and of no toxicological relevance.

In histopathological examination, findings included brown pigment in the bile duct/periportal area in F₀ males and females in the 10 000 ppm group and F₁ males and females in the 3000 and 10 000 ppm groups, focal periductular inflammatory cell infiltration in F₀ males and females and F₁ males in the 10 000 ppm group and F₁ females in the 3000 and 10 000 ppm groups, and brown pigment deposition in the peribiliary hepatocytes and proliferation of the bile duct in F₀ and F₁ females in the 10 000 ppm group. The brown pigments were considered to be porphyrin pigments, because the pigment stained dark blue with Schmorl and negative for Hall and Berlin blue and had birefringence, according to an examination using a polarizing lens. Diffuse hypertrophy of the hepatocytes was also observed in males in the 3000 and 10 000 ppm groups and in females in the 1000 ppm group and above in both generations. However, this change, accompanied by increased liver weights, was considered to be a physiological adaptation to the repeated administration of the test compound, and only the adaptive changes were observed in F₀ and F₁ females in the 1000 ppm group, without any other change. In addition, hypertrophy of the follicular cells of the thyroid was considered to be secondary to the hepatocellular hypertrophy observed in F₀ males in the 10 000 ppm group, and hypertrophy of the cortical cells in the fascicular zone of the adrenal was suggestive of a stress-related change in F₁ females in the 10 000 ppm group. Besides, greenish change in the cortex and increased kidney weights without histopathological changes were observed in F₀ females in the 10 000 ppm group. In contrast, no histopathological changes were detected in the reproductive organs (including the ovary and uterus) of males or females in either generation.

Mandestrobien showed no evidence of an effect on fertility or reproductive function. Among offspring, there were no treatment-related changes in the viability index, number of offspring or birth

weights, although postnatal body weight gain was suppressed in both sexes of F₁ and F₂ offspring in the 10 000 ppm group. A slight delay in sexual maturation attributed to growth retardation was noted in both sexes (vaginal opening in F₁ females and preputial separation in F₁ males) in the 10 000 ppm group. Slightly lower spleen weights at weaning were also observed in F₁ male offspring and F₂ female offspring in the 3000 and 10 000 ppm groups and in F₂ male offspring in the 10 000 ppm group; however, they completely recovered to the control level in F₁ adult animals. No treatment-related changes were found in any external features, clinical signs or necropsy findings at weaning of F₁ or F₂ offspring (Table 28).

The NOAEL for parental toxicity was 1000 ppm (equal to 47.77 mg/kg bw per day), based on increased liver weights, diffuse liver hypertrophy, brown pigment in the bile duct/periportal area in both sexes in the F₁ generation and periductular inflammatory cell infiltration in F₁ females at 3000 ppm (equal to 145.7 mg/kg bw per day).

The NOAEL for offspring toxicity was 1000 ppm (equal to 47.77 mg/kg bw per day), based on decreased spleen weights in F₁ male and F₂ female pups.

The NOAEL for reproductive toxicity was 10 000 ppm (equal to 511.7 mg/kg bw per day), the highest dose tested (Matsuura, 2012).

(b) Developmental toxicity

Rats

In a dose range-finding developmental toxicity study, seven Wistar rats of each sex per dose group were administered mandestrobin (purity 93.4%) orally by gavage (in 0.5% w/v methylcellulose) at a dose of 0, 250, 500 or 1000 mg/kg bw per day during GDs 6–19. Females were cohabited with males on a one-to-one basis for mating. Presumed-pregnant females (GD 0) were allocated to each dose group. All animals were examined twice daily to detect dead or moribund animals. Additionally, animals were observed immediately after dosing and at 0.5, 1, 2 and 4 hours after dosing for signs of reaction to treatment. Body weight was recorded on GDs 4, 6, 7, 8, 9, 12, 15, 17, 19 and 20. Feed consumption was recorded for GDs 4–5, 6, 7, 8, 9–11, 12–14, 15–16, 17–18 and 19. A necropsy was performed on GD 20. The ovaries and the uteri were examined for pregnancy status, gravid uterus weight, number of corpora lutea and the number and intrauterine position of implantations (subdivided into live fetuses, early intrauterine deaths, late intrauterine deaths and dead fetuses). Individual fetal and placental weights were recorded, and fetuses were examined externally and sexed. No claim of GLP compliance was made, but the study was conducted in accordance with current requirements.

All treated animals showed piloerection on GDs 6–7. On GD 7, piloerection was seen in all treated animals at half an hour post-dosing for up to 1 hour post-dosing only. No significant adverse signs of maternal or embryo/fetal toxicity were observed at any of the doses.

No treatment-related effects on body weight or feed consumption were observed. In the caesarean section examination, there were no adverse effects on uterus weight, numbers of pregnancies, corpora lutea and implantations, the mean incidence of preimplantation and postimplantation losses, or litter size.

There was no effect of treatment on sex ratio, mean litter weight, mean placental weight or mean fetal weight. In the 250 mg/kg bw per day dose group, there were two malformed fetuses in two litters; one fetus had a severely reduced orbit of the eye, and the other fetus had no patent anal opening. There was one malformed fetus in the 500 mg/kg bw per day dose group, with a severely displaced umbilical opening associated with absent muscle tissue. At 1000 mg/kg bw per day, the incidence of the variation haematoma of the lower jaw was higher than in the control group, but this was an isolated finding and was not considered likely to be an adverse effect of treatment. Overall, there was no effect of treatment on the mean incidence of external fetal variations and malformations.

Based on the results of this study, a top dose of 1000 mg/kg bw per day was recommended for the main developmental toxicity study (Rhodes, 2009a).

In the main developmental toxicity study, mandestrobin (purity 93.4%) was administered in 0.5% aqueous methylcellulose to groups of 24 presumed pregnant Wistar rats by gavage at a dose of 0, 100, 300 or 1000 mg/kg bw per day from GDs 6 to 19. All females were observed twice daily for mortality and signs of toxicity. Feed consumption was recorded for GDs 3–5, 6, 7, 8, 9–11, 12–14, 15–16, 17–18 and 19. Body weights were recorded on GDs 3, 6, 7, 8, 9, 12, 15, 17, 19 and 20. On GD 20, all dams were terminated. Postmortem examination, including gross macroscopic examination of all internal organs, with emphasis on the uterus, uterine contents, position of fetuses in the uterus and number of corpora lutea, was performed, and the data were recorded. All organs or tissues showing grossly visible abnormalities were preserved. The uteri (and contents) and placentae of all females with live fetuses were weighed at necropsy. Fetuses were removed from the uterus, sexed, weighed individually and examined for gross external abnormalities. Approximately one half of the fetuses in each litter, selected by systematic sampling, were dissected, and the viscera were examined. They were then eviscerated, the carcasses were processed to stain the ossified skeleton by the Alizarin technique, the cartilage was processed with Alcian Blue staining and the skeletons were examined. The remaining fetuses were placed in Bouin's solution. At examination, the head was removed, and the coronal sections of the head were examined. The remaining portion of the fetus was examined by dissection and preserved, with the head sections.

No mortality was observed in the study. Post-dosing observations showed mouth rubbing (and very occasionally salivation) at 300 and 1000 mg/kg bw per day immediately after dosing only, but not at 0.5 and 1 hour post-dosing. This post-dosing observation was seen with increasing frequency at 300 mg/kg bw per day from GD 9 onwards and at 1000 mg/kg bw per day from GD 7 onwards. This observation was considered a common finding in rodents in oral (gavage) prenatal developmental toxicity studies and likely is related to taste aversion rather than evidence of toxicity.

Feed consumption was statistically significantly lower for the 1000 mg/kg bw per day group on GDs 6, 7 and 8 (days 1, 2 and 3 of dosing). Over the entire dosing period, mean feed consumption was similar in all groups. Mean body weight gain and mean gravid uterus weight adjusted for body weight were unaffected by treatment. There was no adverse effect of treatment on mean uterine/implantation data (Table 29).

Table 29. Key findings of the developmental toxicity study in rats

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
Maternal body weight (g), day 20	303.2	300.2	309.9	300.4
Maternal body weight gain, days 6–20 (%)	41.4	39.2	43.3	38.4
Feed consumption (g/rat per day)				
Day 6	19.1	17.8	18.6	16.7*
Day 7	20.5	19.2	19.4	17.7**
Day 8	20.2	19.7	20.3	17.7*
Days 6–18	21.4	20.8	21.0	20.5
Number of females mated	24	24	24	24
Number of non-pregnant females	0	1	0	0
Number of pregnant females (%)	24 (100)	23 (95.8)	24 (100)	24 (100)
Number of females with live fetuses	24	23	24	24
Mean number of early intrauterine deaths/dam (number of dams affected)	0.3 (7)	0.8 (10)	0.4 (8)	0.7 (14*)
% postimplantation loss/dam (dams affected)	2.8 (7)	8.6 (10)	4.4 (10)	6.9 (14*)

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
Mean litter size	10.5	10.0	11.2	10.0
Mean fetal weight (g)	3.75	3.77	3.77	3.83
Mean placental weight (g)	0.48	0.5	0.48	0.5
Sex ratio (% males)	50.0	47.3	53.8	55.6
Total number of fetuses with external or visceral variations, <i>n</i> (%)	77 (30.9)	78 (35.7)	103 (37.4)	101 (42.4)
Litter incidence	24/24	23/23	24/24	24/24
Fetuses with external or visceral malformations, <i>n</i> (%)	0	0	0	2 (0.7)
Litter incidence	0	0	0	2/24
Kidney, severely increased pelvic cavitation	0	0	0	2/24
Total number of fetuses with skeletal variations, <i>n</i> (%)	122 (94.8)	108 (93.7)	128 (96.4)	120 (99.2)
Total number of fetuses with skeletal malformation, <i>n</i> (%)	0	0	1 (0.8)	3 (2.9)
Litter incidence	—	—	1/24	3/24 DR* F+
Rib cartilage shortened	0/24	0/23	0/24	1/24
Sternebrae, cleft xiphoid cartilage	0/24	0/23	0/24	1/24
Vertebral cervical arch and centrum, additional ossification site fused	0/24	0/23	1/24	0/24
Vertebral cervical arch, additional cartilaginous ventral plate fused	0/24	0/23	0/24	1/24
Total number of fetuses with malformations, <i>n</i> (%)	0	0	1 (0.4)	5 (2.1)
Litter incidence	—	—	1/24	5/24* F+

bw: body weight; DR: significant dose–response test; F+: Cochran-Armitage and Fisher's exact (upper tail); *: $P < 0.05$; **: $P < 0.01$

Source: Rhodes (2012a)

There were no treatment-related effects on the number of pregnancies, the mean numbers of corpora lutea or implantations or the mean incidence of preimplantation and postimplantation losses. The number of dams with early intrauterine deaths or postimplantation loss at 1000 mg/kg bw per day showed an apparent statistically significant increase; however, both mean number of early intrauterine deaths and mean percentage of postimplantation loss showed no dose–response relationship or statistical significance.

There was no effect of treatment on sex ratio, mean litter weight, mean placental weight or mean fetal weight. Malformations were noted in one fetus in the group receiving 300 mg/kg bw per day and five fetuses from five litters in the group receiving 1000 mg/kg bw per day. In the fetus from the 300 mg/kg bw per day group, vertebral cervical arch and centrum, additional ossification site fused was noted. Among the five fetuses with malformation in the 1000 mg/kg bw per day group, one had cleft xiphoid cartilage in the sternebrae, one shortened rib cartilage and one additional cartilaginous vertebra plate fused in the vertebral cervical arch, and two had severely increased pelvic cavitation in the kidney. Historical control data for this latter visceral malformation indicated that this abnormality is a rare finding (Table 30).

Table 30. Historical control data (Charles River) for kidney, severely increased pelvic cavitation

	% fetal incidence		No. of studies	No. of litters	No. of fetuses	Years covered
	Mean	Range				
1	0.33	0–0.8	3	65	598	Unknown
2	0.13	0–0.8	6	113	1 064	2002–2011
3	0.04 (cumulative)	–	–	–	2 243	2002–2011

Source: Rhodes (2012a)

The NOAEL for maternal toxicity was 300 mg/kg bw per day, based on reduced feed consumption at 1000 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 300 mg/kg bw per day, based on an increase in a limited number of visceral and skeletal malformations at 1000 mg/kg bw per day (Rhodes, 2012a).

Rabbits

In a dose range-finding (non-GLP-compliant) developmental toxicity study, mandestrobin (purity 93.4%) was administered in 0.5% aqueous methylcellulose by gavage to presumed-pregnant female New Zealand white rabbits (seven per group) at a dose of 0, 250, 500 or 1000 mg/kg bw per day from GD 7 to GD 28. Observations were recorded at least 6 times per day during the dosing period. Feed consumption was recorded daily from GD 3 to GD 29, and body weights were measured on GDs 3, 7, 8, 9, 12, 15, 17, 19, 22, 25, 28 and 29. Females were terminated on GD 29, and a gross examination was performed. Gravid uterine weight was measured. The ovaries and uteri were removed and examined, and the following data were recorded: pregnancy status, gravid uterus weight, number of corpora lutea and the number and intrauterine position of implantations. Individual fetal and placental weights were recorded, and fetuses were examined externally and sexed by internal gonadal inspection.

One animal of the 1000 mg/kg bw per day dose group presented the clinical observation “head tilt”; however, veterinary examination revealed no treatment-related effects. Mean body weight, mean body weight gain and mean gravid uterus weight were unaffected by treatment.

A statistically significant dose-response relationship for body weight loss among animals treated at 500 and 1000 mg/kg bw per day was observed on the first day of dosing. However, this was considered not to be treatment related owing to the body weight loss seen prior to treatment in these groups. Mean feed consumption at the high dose was very slightly lower than that of controls throughout the study, but was statistically significantly lower only on the first day of dosing. There was no effect of treatment on sex ratio, mean litter weight or mean placental weight. In the high-dose group, mean fetal weight was slightly, but not statistically significantly, lower than that of controls, and this was considered to be a result of the higher litter size at this dose.

There was no effect of treatment on the mean incidence of external fetal variations and malformations. In the high-dose group, there was one malformed fetus with spina bifida, severely malformed head structures and severely flexed forelimb wrist joints. This was an isolated finding and was not considered to be related to treatment by the study author.

Although statistical significance was not reached, there were more fetuses with variations in the high-dose group (slightly enlarged bilateral eye bulge, upper incisor not erupted).

On the basis of this study, a top dose of 1000 mg/kg bw per day was recommended for the main developmental toxicity study (Rhodes, 2009b).

In the main developmental toxicity study, mandestrobin (purity 93.4%) was administered (in 0.5% aqueous methylcellulose) by gavage to 24 presumed-pregnant female New Zealand white rabbits

per group at a dose of 0, 100, 300 or 1000 mg/kg bw per day from GD 7 to GD 28. Observations were recorded at least twice daily during the dosing period and at least once daily during the pre-dosing and post-dosing period. Feed consumption was measured daily from GDs 3 to 29, and body weights were recorded on GDs 3, 7, 8, 9, 12, 15, 17, 19, 22, 25, 28 and 29. Females were terminated on GD 29, and a gross examination was performed. Gravid uterine and placental weights were measured, and fetuses were removed by caesarean section. The number of corpora lutea, number of implantations, number of live fetuses, and number and intrauterine position of embryonic/fetal deaths (including time of resorptions) were recorded. Each live fetus was weighed and examined externally. The heads and hearts of approximately half of the fetuses in each litter were fixed in Bouin's solution. Serial sections of the head were examined and preserved in 10% neutral buffered formalin. Several coronal slices of each heart were made to reveal the internal structure. These heart sections were examined and then preserved in neutral buffered formalin. All fetuses were dissected and sexed, and the viscera were examined. The remaining skeletons were examined for skeletal abnormalities.

No maternal animals died during the study period. One animal in the 300 mg/kg bw per day group aborted its pregnancy on GD 20 (Table 31). This abortion was not considered to be an effect of treatment because it was in one animal only and was not seen at the high dose. Clinical observations were generally unremarkable and showed no dose-related trend. Group mean body weight gains during the study were highly variable, with no marked adverse effect seen. Mean feed consumption at all doses was slightly lower than in controls, and this was statistically significant at 300 and 1000 mg/kg bw per day. These slight differences from controls are considered unlikely to be indicative of significant systemic toxicity to the pregnant rabbit because in the pre-dosing period, feed consumption at 1000 mg/kg bw per day was also slightly lower than in controls, and the difference was comparable with that during the treatment phase.

Table 31. Key findings of the developmental toxicity study in rabbits

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	HCD ^a	
					Mean	Range
Number of females inseminated	24	24	24	24	—	—
Number of non-pregnant females	1	1	2	6	—	—
Number of pregnant females (%)	23 (95.8)	23 (95.8)	22 (91.7)	18 (75.0)	—	—
Accidental death	0	0	0	0	—	—
Abortion/premature delivery	0	0	1	0	—	—
Total litter resorptions	0	0	0	1	—	—
Number of litters for evaluation	23	23	21	17	—	—
Uterine data^b						
Mean number of corpora lutea	10.3	10.1	10.0	9.7 (9.4)	10.4	10.0–11.9
Mean number of implantations	8.9	9.4	8.8	8.8 (8.4)	8.6	7.6–9.4
Mean % preimplantation loss	15.0	7.7	12.9	9.1 (13.0)	17.3	9.5–27.6
Mean number of intrauterine deaths early	0.6	0.9	0.8	0.9 (0.9)	0.58	0.4–0.8
Mean number of intrauterine deaths late	0.2	0.3	0.2	0.3 (0.3)	0.2	0.1–0.3
Mean number of dead fetuses	0.0	0.0	0.0	0.1 (0.1)	0.0	0.0

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	HCD ^a	
					Mean	Range
Mean % postimplantation loss	8.7	13.6	11.0	13.3 (18.1)	9.1	7.1–14.2
Number of dams affected	10	17*	11	11	–	–
Mean number of live fetuses	8.0	8.2	7.8	7.5 (7.1)	7.8	6.9–8.7
Maternal body weight (kg)						
Day 7	3.32	3.28	3.31	3.44	–	–
Day 29 (surviving does)	3.83	3.72	3.70	3.87	–	–
Number of implantations/doe	8.9	9.4	8.8	8.8	–	–
Resorption and fetal death (%)	8.7	13.6	11.0	13.3	–	–
Mean litter size (live fetuses)	8.0	8.2	7.8	7.5	7.8	6.9–8.7
Mean fetal weight (g)						
Males	44.4	43.9	43.0	45.9	–	–
Females	45.1	43.4	41.7	45.4	–	–
Sex ratio (% males)	49.1	50.3	57.6	50.0	49.7	45.1–50.7
Fetuses with external or visceral malformations, <i>n</i> (%)	7 (3.3)	4 (1.5)	2 (1.2)	2 (1.7)	(2.6)	–
Litter incidence	6/23	3/23	2/21	2/17	–	–
Fetuses with external or visceral variations, <i>n</i> (%)	98 (55.3)	85 (46.0)	80 (49.4)	54 (43.0)	(48.7)	–
Litter incidence	22/23	23/23	21/21	15/17	–	–
Fetuses with skeletal malformation, <i>n</i> (%)	3 (1.5)	4 (1.7)	7 (4.9)	2 (1.6)	(2.7)	–
Litter incidence	2/23	3/23	6/21	2/17	–	–
Fetuses with skeletal variations, <i>n</i> (%)	148 (79.8)	142 (75.0)	114 (70.8)	110 (85.8)	(73.9)	–
Litter incidence	22/23	23/23	21/21	17/17	–	–
Total number of fetuses with malformations, <i>n</i> (%)	8 (4.3)	6 (3.2)	9 (5.5)	3 (2.3)	–	–
Litter incidence	6/23	4/23	7/21	3/17	–	–

bw: body weight; *n*: number; *: $P < 0.05$ (Cochran-Armitage and Fisher's exact, upper tail)

^a Historical control data were derived from six embryo/fetal studies performed with New Zealand white rabbits at Covance from April 2004.

^b The animal with total embryo/fetal loss was excluded from the analysis; values in parentheses contain this doe in the analysis.

Source: Rhodes (2012b)

The number of pregnancies was 23, 23, 22 and 18, and there were 23, 23, 21 and 17 surviving females with litters on GD 29 at 0, 100, 300 and 1000 mg/kg bw per day, respectively (Table 31). The distribution of non-pregnant animals in the high-dose group ($n = 6$) was considered a chance event by the study author and unrelated to treatment, as any increase in very early postimplantation losses would have been seen as an increase in this parameter in the mean data. Implantation in rabbits occurs at days 7–7.5 after insemination, at the beginning of the dosing period.

One animal receiving 1000 mg/kg bw per day (number 93) had total embryo/fetal loss; this isolated incidence was not considered to be an effect of treatment by the study author. The mean numbers of corpora lutea and implantations and the mean incidence of preimplantation and postimplantation losses showed no effect of treatment. The number of dams with postimplantation loss at 100 mg/kg bw per day showed an apparent statistical significance; however, all data were within expected ranges, and this was not considered to be biologically relevant.

Mean litter size was unaffected by treatment (Table 31).

There was no effect of treatment on sex ratio, mean litter weight, mean placental weight or mean fetal weight.

Overall, there was no effect of treatment on the incidence of fetal variations and malformations, which were all within expected ranges for this strain of rabbit (Table 31). Malformations were noted in eight fetuses from six litters in the control group, six fetuses from four litters in the group receiving 100 mg/kg bw per day, nine fetuses from seven litters in the group receiving 300 mg/kg bw per day and three fetuses from three litters in the group receiving 1000 mg/kg bw per day.

In conclusion, administration of mandestrobin by oral gavage to pregnant rabbits elicited no systemic toxicity to the maternal rabbits. There was no evidence of embryo or fetal toxicity at any dose tested. Based on these observations, the NOAELs for both maternal and embryo/fetal toxicity were 1000 mg/kg bw per day, the highest dose tested (Rhodes, 2012b).

2.6 *Special studies*

(a) *Neurotoxicity*

Acute neurotoxicity

In a preliminary acute neurotoxicity study, mandestrobin (purity 93.4%) was administered to groups of three Wistar rats of each sex by a single oral gavage dose of 0, 300, 1000 or 2000 mg/kg bw in 0.5% w/v aqueous methylcellulose. Observations were made at 1, 2, 3, 4, 5, 6, 7 and 8 hours after treatment. Body weights were determined on the day of dosing prior to treatment and were similar across all groups. On the day following dosing, animals were euthanized.

No mortality was noted. Clinical signs included statistically significantly higher mean defecation count (1.3 counts) and number of urine pools (1.0 pool) for males at 2000 mg/kg bw at approximately 1 hour following dosing on study day 0. However, these findings were transient, the magnitude of change relative to the control group was small and there were no correlating findings for the 2000 mg/kg bw males. In males from the control group, a urine pool count of 0.7 was reported at 8 hours. Therefore, these changes were not considered test compound related. No neurotoxic effects were noted.

On the basis of these results, doses of 500, 1000 and 2000 mg/kg bw were selected for evaluation of the acute neurotoxic potential of mandestrobin. It was also determined that the time at which FOB and locomotor activity would be observed in the definitive study was 8 hours post-dosing on study day 0 (time of peak effect) (Herberth, 2011a).

In the main acute neurotoxicity study, groups of 12 Wistar rats of each sex were given a single oral gavage dose of mandestrobin (purity 93.4%) in 0.5% w/v aqueous methylcellulose at 0, 500, 1000 or 2000 mg/kg bw. Clinical observations were performed once daily on all animals, except on days of FOB assessments. Body weights were recorded at least weekly. FOB and locomotor activity testing were carried out during the week of acclimation, at the time of peak effect (8 hours post-dosing) on study day 0, and again on days 7 and 14. On day 15, animals were terminated and submitted for neuropathology.

No mortality, clinical signs or macroscopic or microscopic findings were noted at necropsy. Body weights were unaffected by treatment. Home cage, handling, open field, sensory, neuromuscular and physiological parameters were unaffected by test compound administration.

In locomotor activity, statistically significantly lower mean overall total and ambulatory counts in the 2000 mg/kg bw males and statistically significantly lower mean total and ambulatory counts in the 1000 and 2000 mg/kg bw males during the second subinterval (11–20 minutes) were noted at the time of peak effect on study day 0, but were unaffected on study day 7 or 14. There were no other findings to corroborate this effect, which was therefore attributed to transient systemic toxicity at the high dose, rather than typical neurotoxicity. No other statistically significant changes were noted in 1000 mg/kg bw males during the other subintervals; therefore, the decreases were attributed to better habituation observed in this group and not to mandestrobin administration. Statistically significantly lower mean overall ambulatory counts were noted for 2000 mg/kg bw females at the time of peak effect on study day 0, primarily due to reductions in ambulatory counts during the first two subintervals (0–10 and 11–20 minutes). Although there were no differences in total motor activity counts noted for these animals on study day 0, similar reductions in ambulatory counts were noted for the 2000 mg/kg bw males at this evaluation; therefore, these reductions were attributed to mandestrobin administration. No other mandestrobin-related effects on locomotor activity were noted in 2000 mg/kg bw females on study day 7 or 14 (Table 32).

During FOB assessments, on study day 7, home cage parameter observations recorded statistically significantly fewer females in the 1000 mg/kg bw group sitting or standing normally in the home cage; however, this behaviour was not observed at 2000 mg/kg bw. Therefore, this decrease was not attributed to mandestrobin administration. On study day 7, open field parameter observations recorded a statistically significant decrease in the number of rearing counts for 2000 mg/kg bw females. This decrease was attributed to a single female in this group that did not rear during the evaluation, whereas rearing counts for the remaining females in the 2000 mg/kg bw group were similar to those observed in the control group. The number of rearing counts of the corresponding female was similar to that of animals in the control group on study days 0 and 14. Therefore, the decrease observed on study day 7 was not attributed to mandestrobin administration.

Table 32. FOB and locomotor activity findings of the acute neurotoxicity study in rats

End-point	Day	Min	Males				Females			
			0 mg/kg bw	500 mg/kg bw	1 000 mg/kg bw	2 000 mg/kg bw	0 mg/kg bw	500 mg/kg bw	1 000 mg/kg bw	2 000 mg/kg bw
Home cage parameter: Mean no. of animals sitting or standing normally	0	–	5	6	3	3	6	6	4	2
	7	–	2	4	5	2	8	4	2 [#]	4
	14	–	3	5	3	5	5	7	9	8
Open field parameter: Mean no. of animals rearing	0	–	6.5	7.3	6.8	5.5	12.3	7.9	9.2	8.8
	7	–	9.7	8.7	10.5	8.0	15.8	12.8	12.0	11.4*
	14	–	10.6	12.3	13.2	11.4	17.0	16.6	15.1	16.9
Locomotor activity counts: Total counts	0	0–10	1 146	1 094	1 052	979	1 275	1 283	1 186	1 115
		11–20	570	568	379 ⁺	300 ⁺	482	409	467	435
		21–30	258	194	136	131	145	214	168	126
		31–40	118	177	121	118	120	261	99	89
		41–50	82	143	106	127	168	209	219	152

End-point	Day	Min	Males				Females			
			0 mg/kg bw	500 mg/kg bw	1 000 mg/kg bw	2 000 mg/kg bw	0 mg/kg bw	500 mg/kg bw	1 000 mg/kg bw	2 000 mg/kg bw
Locomotor activity counts: Ambulatory counts		51–60	94	93	124	63	149	159	177	125
	Cum.	–	2 268	2 267	1 918	1 718 ⁺	2 339	2 536	2 316	2 042
	0	0–10	363	331	318	286	445	460	414	348
		11–20	121	100	49 ⁺	31 ⁺	105	87	97	84
		21–30	33	20	8	10	16	46	16	11
		31–40	2	25	21	6	20	66	9	16
		41–50	1	16	18	8	26	22	31	17
		51–60	2	6	9	1	11	27	19	5
	Cum.	–	522	498	424	343 ⁺	623	708	587	480 ⁺

bw: body weight; Cum: cumulative; #: $P < 0.05$ (Fisher's exact test); *: $P < 0.05$ (Dunnett's test); +: $P < 0.05$ (sequential linear trend test)

Source: Herbert (2011b)

The NOAEL for neurotoxicity was 1000 mg/kg bw, based on decreased overall locomotor activity (total and/or ambulatory counts) at 2000 mg/kg bw. The NOAEL for systemic toxicity was 2000 mg/kg bw, the highest dose tested (Herberth, 2011b).

Subchronic neurotoxicity

In a dose range-finding study, mandestrobin (purity 93.4%) was given to four groups of five Crl:WI(HAN) rats of each sex per group at a dietary concentration of 0, 1500, 5000 or 15 000 ppm (equal to 0, 132, 430 and 1200 mg/kg bw per day for males and 0, 135, 430 and 1305 mg/kg bw per day for females) for 28 days. All animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily, and detailed physical examinations were performed approximately weekly. Individual body weights were recorded twice weekly, and feed consumption was recorded weekly. At the scheduled termination, complete necropsies were conducted, and the brain was collected from all animals.

All animals survived. There were no test substance-related clinical observations or macroscopic findings in any of the treated groups. A marginally lower body weight (2–4%) was noted in both sexes given 15 000 ppm during the first week of treatment. A statistically significantly lower body weight gain (15%) was recorded in both sexes given 15 000 ppm during the first week of treatment (days 0–3). However, no effect on body weight was observed through the end of dosing, as body weights for the 15 000 ppm groups were similar to those of the vehicle control group. Therefore, these body weight changes were not considered to be adverse. A marginally, but not statistically significantly, reduced feed consumption was reported in males given 15 000 ppm during the first week of treatment. It is noted that feed consumption was similar to that of controls from day 7 to 28. There were no treatment-related effects evidenced by the macroscopic investigation, and no effect on brain weight.

The NOAEL for systemic toxicity was 15 000 ppm (equal to 1200 mg/kg bw per day), the highest dose tested (Hosako, 2011a). Based on the results of the study, the dose of 15 000 ppm was considered appropriate for subsequent neurotoxicity testing.

In a subchronic neurotoxicity study, mandestrobin (purity 93.4%) was administered orally to 12 Wistar rats of each sex per group at a dietary concentration of 0, 1500, 5000 or 15 000 ppm (equal to 0, 99, 338 and 1024 mg/kg bw per day for males and 0, 122, 415 and 1223 mg/kg bw per day for

females, respectively) for 13 weeks. All animals were observed twice daily for mortality and moribundity. Detailed physical examinations, feed consumption and body weights were recorded once weekly, beginning 1 week prior to test diet administration. Ophthalmoscopy was performed in all animals during acclimatization and in week 12. A FOB, including locomotor activity testing, was performed during acclimatization and in weeks 1, 3, 7 and 12. At termination of the study, all animals were euthanized and submitted for neuropathology.

No mortality or clinical signs were noted. One male in the 5000 ppm group and one female in the control group were found in the incorrect cages and were euthanized and sent to necropsy on study day 58. No treatment-related effects were noted during ophthalmoscopy or neuropathology.

Test compound-related, statistically significantly reduced mean body weight gains were noted for males offered diet containing 15 000 ppm of the test compound during study days 0–7, 14–21 and 28–35. As a result, the mean body weight gain of males in the high-dose group was somewhat lower (not statistically significantly) than in the control group over the entire treatment period (study days 0–91), and mean body weights in the high-dose group were 4.8–9.3% lower than in the control group beginning on study day 7 and continuing until the end of the treatment period; differences in mean body weight were statistically significant on study days 35–56. Mean body weight gains were unaffected by test compound exposure thereafter. Mean body weight gain for males offered diet containing 15 000 ppm of the test compound was statistically significantly higher than in the control group during study days 77–84; however, this was transient, and an increase in mean body weight gain was not considered toxicologically relevant. Mean body weights and body weight gains for males offered diet containing 1500 and 5000 ppm of the test compound and for females at all test compound concentrations were unaffected by test compound exposure. Statistically significant differences from the control group were transient, did not occur in a dose-related manner, did not affect mean body weights and/or did not affect overall mean body weight gains (Table 33).

Statistically significantly reduced mean feed consumption (evaluated as g/animal per day and g/kg bw per day) was noted for males offered diet containing 15 000 ppm of the test compound during study days 0–7 (g/animal per day and g/kg bw per day), days 21–28 (g/animal per day) and days 28–35 (g/animal per day). In addition, slightly lower (not statistically significantly) mean feed consumption (g/animal per day) was noted during study days 7–21. These results corresponded to reduced mean body weight gains noted for these males during this period and were considered to be test substance related (Table 33).

Table 33. Body weight, body weight gain and feed consumption in a subchronic neurotoxicity study in rats

End-point	Study day(s)	Males				Females			
		0 ppm	1 500 ppm	5 000 ppm	15 000 ppm	0 ppm	1 500 ppm	5 000 ppm	15 000 ppm
Mean body weight (g)	0	190	190	188	188	133	129	130	132
	35	344	340	338	313*	199	192	196	201
	42	359	353	353	327*	204	197	204	205
	49	380	374	374	345*	215	204	212	212
	56	389	383	384	353*	219	208	215	216
	91	435	430	427	400	234	228	234	234
Mean body weight gain (g)	0–7	38	38	37	29**	15	18	18	19
	7–14	42	41	41	39	16	16	19	19
	14–21	32	32	32	26*	18	13**	12**	16

End-point	Study day(s)	Males				Females			
		0 ppm	1 500 ppm	5 000 ppm	15 000 ppm	0 ppm	1 500 ppm	5 000 ppm	15 000 ppm
Mean feed consumption (g/animal per day)	21–28	21	20	18	16	7	8	9	9
	28–35	21	19	21	15**	9	9	9	6
	0–91	245	239	237	211	101	99	104	102
	0–7	22	22	21	19**	15	15	15	16
	7–14	23	23	23	22	15	15	16	17**
	14–21	24	23	23	22	16	16	16	17
	21–28	24	23	23	21*	16	15	16	17
	28–35	25	23	24	22**	17	16	18	16

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)

Source: Herberth (2012)

The only statistically significant difference during home cage observations was recorded in the 1500 ppm group and consisted of a lower number of females that were alert and oriented towards the observer and a higher number of females that were sitting or standing normally. However, these transient findings were not considered to be abnormal in the home cage, had no dose–response relationship and therefore were not considered to be treatment related.

Handling, sensory and physiological parameters were unaffected by test compound administration. During open field parameter observations, a lower mean time to first step was recorded in all treated females during study week 1, but was not considered to be treatment related by the study author, as this was a small magnitude of change, findings were noted only during the week 1 assessment, there was no dose–response relationship and no effect was observed in males. A higher mean rearing count was also noted in 5000 ppm males during study week 3, but was also not considered to be treatment related, as there was no dose–response relationship. Reduced forelimb grip strength was recorded in 15 000 ppm females during study week 7, but was not considered to be treatment related, as these results were not noted during study weeks 3 and 12 and there were no additional effects on related parameters. In addition, statistically significantly lower mean rotarod performance during week 1 and lower mean forelimb grip strength during week 3 were noted in 1500 ppm females, but were not considered to be treatment related, as no dose–response relationship was evident. Locomotor activity patterns (mean ambulatory and total motor activity counts) were unaffected by test diet consumption. Statistically significant differences were limited to lower mean total counts in 15 000 ppm females during 51–60 minutes in study week 1, higher mean cumulative total counts in 15 000 ppm males in study week 7, and higher mean ambulatory counts in 15 000 ppm females during 21–30 minutes in study week 12. However, findings were transient or did not affect mean overall counts, and no remarkable shifts in the pattern of habituation occurred in any of the test compound–exposed groups when the animals were evaluated in study weeks 1, 3, 7 and 12. Therefore, they were considered not to be treatment related.

The NOAEL for systemic toxicity was 5000 ppm (equal to 338 mg/kg bw per day), based on transient decreases in body weight, body weight gain and feed consumption at 15 000 ppm (equal to 1024 mg/kg bw per day). The NOAEL for neurotoxicity was 15 000 ppm (equal to 1024 mg/kg bw per day), the highest dose tested, based on the absence of neurotoxic effects (Herberth, 2012).

(b) Immunotoxicity

In a dose range–finding immunotoxicity study, mandestrobin (purity 93.4%) was administered to four groups of Han Wistar rats (eight females per group) at a dietary concentration of 0, 1500, 5000 or 15 000 ppm (equal to 0, 135, 436 and 1340 mg/kg bw per day, respectively) for 28 days. A concurrent

positive control group (group 5) was offered the basal diet on a comparable regimen. The assessment of immunotoxicity was based primarily on the results of a splenic antibody-forming cell (AFC) assay to assess the T cell-dependent antibody response (TDAR) to sheep red blood cells (sRBCs). All TDAR group animals (groups 1–5) were immunized with an intravenous injection of sRBCs on study day 24. A positive control group (group 5) received cyclophosphamide monohydrate (CPS) at a dose of 50 mg/kg bw per day on study days 24 through 27. All animals were observed twice daily for mortality and moribundity. Detailed clinical observations were performed during pretest and once weekly during the study. Following 28 days of dose administration, all animals were euthanized, and blood samples for possible immunoglobulin M (IgM) antibody analysis (AFC assay) were collected. At the scheduled necropsy, the spleens from all animals were weighed.

All animals survived to the scheduled necropsy. There were no test compound-related clinical observations or macroscopic findings in any of the test compound-treated groups. Test compound-related lower body weights, body weight gains (3–7%) and feed consumption were noted at 15 000 ppm during the first week of test diet administration and attributed to unpalatability. However, body weights and feed consumption were similar to those of the vehicle control group throughout the remainder of the study. Statistically significantly lower mean body weight gains compared with the vehicle control group were noted in the positive control group from study days 24 to 28. As a result, lower mean body weight (5.3%) compared with the vehicle control group was noted, and lower mean cumulative body weight gain was noted from study days 0 to 28. Lower body weights are consistent with the known effects of CPS.

No test compound-related changes in absolute or relative adrenal gland, brain, spleen or thymus weight were observed when compared with the vehicle control group. There were no test compound-related effects on spleen cell number or the response to sRBCs, as measured by AFC IgM specific activity (AFC/ 10^6 spleen cells) and total spleen activity (AFC/spleen), at any dose tested.

In the absence of mandestrobin-related effects on the AFC response, the NOAEL for the humoral immune response in the TDAR groups was considered to be 15 000 ppm (equal to 1340 mg/kg bw per day), the highest dose tested. No adverse effects were observed for the general toxicity parameters evaluated (Hosako, 2011a).

In the main immunotoxicity study, mandestrobin (purity 93.4%) was administered to four groups of 10 female Han Wistar rats at a dietary concentration of 0, 1500, 5000 or 15 000 ppm (equal to 0, 147, 471 and 1419 mg/kg bw per day, respectively) for 28 days. The assessment of immunotoxicity was based primarily on the results of a splenic AFC assay to assess the TDAR to sRBCs. A positive control group (group 5) received CPS at a dose of 50 mg/kg bw per day on study days 24 through 27. All animals (groups 1–5) were immunized with an intravenous injection of sRBCs on study day 24. Animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily, and detailed physical examinations were performed approximately weekly. Individual body weights were recorded twice weekly, and feed consumption was recorded weekly. Following 28 days of dose administration, all animals were euthanized. Blood samples for possible serum IgM antibody analysis were collected from all animals at the scheduled necropsy. Complete necropsies were conducted on all animals, and the spleen, thymus, lymph nodes, Peyer's patches and adrenal glands were collected from all animals. Spleen, thymus and adrenal gland weights were recorded. Liver and kidney weights were not recorded.

No mortality or clinical signs were noted during the study.

Body weights were unaffected by test compound administration. However, a statistically significantly higher mean body weight gain was noted at 15 000 ppm (study days 3–7), and higher mean cumulative body weight gains were noted at 5000 ppm (study days 0–7, 0–10 and 0–14) and 15 000 ppm (study days 0–10 and 0–14), compared with the vehicle control group. Differences in body weight gain were probably due to biological variability and were not considered related to test compound administration owing to the lack of a clear dose-response trend, the transient nature and the fact that the cumulative body weight gains at the end of the study (study day 28) were not affected at either

dietary concentration. Statistically significantly lower mean body weight gains and cumulative body weight gains were noted in the positive control group from study days 24 to 28 and 0 to 28, respectively, compared with the vehicle control group. These lower body weight gains were consistent with the known effects of CPS. Feed consumption was unaffected by test compound administration.

There were no mandestrobin-related gross observations. A small thymus was noted in 9/10 animals in the positive control (CPS) group at the scheduled necropsy; however, these findings were consistent with the known effects of CPS and correlated with the lower thymus weights.

Higher relative spleen weights were recorded at 15 000 ppm; however, absolute spleen weights were not affected. There were no mandestrobin-related effects on adrenal gland or thymus weights. There were no statistically significant effects on spleen cell number, and mandestrobin did not significantly suppress the humoral immune response to the T cell-dependent antigen sRBCs when evaluated as either specific activity (AFC/10⁶ spleen cells) or total activity (AFC/spleen). The positive control agent produced satisfactory results.

The NOAEL for immunotoxicity was 15 000 ppm (equal to 1419 mg/kg bw per day), the highest dose tested, based on the absence of mandestrobin-related effects on the AFC response. No adverse effects were observed for general toxicity (Hosako, 2011b).

(c) Mechanistic studies

Several in vivo and in vitro mechanistic studies were performed (as summarized in Table 34) to investigate the mode of action of the liver and thyroid effects of mandestrobin and to address possible hormonal effects of mandestrobin.

Table 34. Summary of mechanistic studies with mandestrobin

Study	Species/test cells	Results	Reference
In vivo studies			
Short-term study	Rat (males and females)	<i>Liver</i> : ↑ in liver weight with diffuse, hepatocellular hypertrophy, proliferation of liver smooth endoplasmic reticulum, ↑ in liver CYP2B and T ₄ -UGT activities and ↑ in replicative DNA synthesis in a dose-dependent and reversible manner <i>Thyroid</i> : hypertrophy, ↓ in serum T ₄ levels and ↑ in TSH (findings were reversible)	Asano (2012e)
Short-term study	Mouse (males)	↑ in liver weight and induction of CYP2B activity	Yamada (2012b)
In vitro studies			
Steroidogenesis assay	Human adrenocortical NCI-H295R cell line	No influence on testosterone or estradiol production	Kubo (2012)
Reporter gene assays	hERα-HeLa-9903 and hAR-HeLa 4-11 cell lines	Mandestrobin and its metabolites (5-COOH-S-2200, 4-OH-S-2200, 5-CH ₂ OH-S-2200 and 5-CA-S-2200-NHM) did not show agonistic or antagonistic effects on hERα or hAR-induced transcriptional activation	Suzuki (2012)

CYP2B: cytochrome P450 2B; DNA: deoxyribonucleic acid; hAR: human androgen receptor; hERα: human estrogen receptor alpha; T₄: thyroxine; TSH: thyroid stimulating hormone; UGT: uridine diphosphate glucuronosyltransferase

In repeated-dose toxicity studies, the primary target organ of mandestrobin was the liver in all species examined. The thyroid was also a target organ in the rat, but not in the mouse or dog. The primary liver finding in the rat was hypertrophy (increased liver weight and/or hepatocellular hypertrophy), and the main thyroid finding was follicular cell hypertrophy. However, no tumorigenicity was observed in rat or mouse carcinogenicity studies.

In conjunction with evidence from the literature, two in vivo studies were performed (one in rats, one in mice) to gain insight into the mechanistic basis of the liver and thyroid effects observed in the main studies in rats.

Two in vitro studies were performed to address possible hormonal effects of mandestrobin and its metabolites: 5-COOH-S-2200, 4-OH-S-2200, 5-CH₂OH-S-2200 and 5-CA-S-2200-NHM.

Short-term study in rats

In a non-GLP-compliant mechanistic study, groups of 10 male and 10 female Wistar rats were fed diets containing mandestrobin (purity 93.4%) at a concentration of 0 (control), 400, 2000, 7000 or 15 000 ppm (equal to 0, 23.3, 115.7, 378.9 and 744.4 mg/kg bw per day for males and 0, 25.7, 131.2, 420.2 and 811.8 mg/kg bw per day for females, respectively) for 7 days. The following parameters were measured at necropsy: serum thyroxine (T₄), triiodothyronine (T₃) and thyroid stimulating hormone (TSH) concentrations, CYP4A activity, CYP2B activity, T₄-UGT, replicative DNA synthesis of hepatocytes (determined as 5-bromo-2'-deoxyuridine [BrdU] incorporation), microscopy of liver and thyroid and ultrastructural analysis of hepatocytes using electron microscopy. To evaluate the time course of alterations at 15 000 ppm, 10 rats of each sex per group were also fed diets containing 0 or 15 000 ppm (equal to 796.3 mg/kg bw per day for males and 952.4 mg/kg bw per day for females) mandestrobin for 14 days. Data from both the 7- and 14-day treatment groups were compared to determine whether enhancement or attenuation of alterations was observed. To evaluate the reversibility of any findings, 10 rats of each sex per group were also fed diets containing 0 or 15 000 ppm (equal to 804.9 mg/kg bw per day for males and 896.1 mg/kg bw per day for females) mandestrobin for 7 days followed by a 7-day recovery period. As a positive control for CAR activation, groups of rats were treated with 1000 ppm phenobarbital (PB).

Treatment with mandestrobin caused no deaths, severe toxicity or changes in clinical signs.

Body weight gain during the first 4 days of treatment was statistically significantly lower in both sexes administered 15 000 ppm in both the 7- and 14-day treatment groups, and this resulted in lower body weights. After the first 4 days, however, body weight gain was generally equivalent to or higher than the control level, but total body weight gain remained lower (8% [not statistically significant] in males and 50% in females) at 15 000 ppm in both the 7- and 14-day treatment groups. In the recovery group, no remarkable findings for evaluation of reversibility were observed, because no findings were observed after the 7-day treatment with mandestrobin. Males at 7000 ppm also had decreased body weight and body weight gain after the 7-day treatment.

Although suppression of body weight gains was observed in females administered PB on day 8 in both the 7- and 14-day treatment groups, the recovery group demonstrated no difference from controls at day 8. Statistically significantly suppressed or a tendency towards suppression of feed consumption was observed in both sexes administered 15 000 ppm, especially in the early phase of the treatment period; however, these alterations were not observed after the 14-day treatment. Less severe changes were observed in both sexes at 7000 ppm. In the recovery group, feed consumption was increased after cessation of treatment with mandestrobin.

No remarkable consistent changes were observed in the PB group in any phase.

Liver: After 7 days of treatment with mandestrobin, statistically significantly increased absolute liver weights for both sexes at 15 000 ppm and relative liver weights for males at 7000 ppm and above and for females at 2000 ppm and above were observed. After the 14-day treatment, statistically significant increases were observed in the absolute and relative liver weights in both sexes at 15 000 ppm and in the PB group. In the recovery group, statistically significant increases were observed in

several parameters: absolute and relative liver weights in males administered 15 000 ppm, and relative liver weights in males administered PB. However, all findings were less severe than those after the 7-day treatment (Table 35).

Table 35. Key findings of the short-term mechanistic study in rats

Finding	Males						Females					
	0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	PB	0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	PB
Organ weights												
Relative liver weight (% of control value)												
7 days	100	99	103	110**	124**	124**	100	99	106*	110**	121**	118**
14 days	100	ND	ND	ND	130**	135**	100	ND	ND	ND	128**	127**
7 days + 7 days of recovery	100	ND	ND	ND	111	109*	100	ND	ND	ND	108	105
Relative thyroid weight (% of control value)												
7 days	100	108	112	104	119	119	100	106	103	132**	131**	122*
14 days	100	ND	ND	ND	117*	127**	100	ND	ND	ND	133**	121**
7 days + 7 days of recovery	100	ND	ND	ND	102	124**	100	ND	ND	ND	112	105
Hepatic enzymatic activity												
CYP2B activity (% of control value)												
7 days	100	148	289*	872*	1 358*	2 510*	100	112	305*	2 581*	7 987*	26 948*
7 days + 7 days of recovery	100	ND	ND	ND	135	235**	100	ND	ND	ND	108	334**
CYP4A activity (% of control value)												
7 days	100	98	99	120	124	167**	100	94	86	89	85	129*
7 days + 7 days of recovery	100	ND	ND	ND	141**	121	100	ND	ND	ND	76*	77*
UGT activity (% of control value)												
7 days	100	123*	130	150*	148*	191*	100	95	100	117	136**	123*
7 days + 7 days of recovery	100	ND	ND	ND	113	136**	100	ND	ND	ND	109	110
Serum hormone levels												
Serum [T ₄] (% of control value)												
7 days	100	106	106	90	81*	88	100	100	96	92	84	67**
14 days	100	ND	ND	ND	72**	71**	100	ND	ND	ND	75*	54**
7 days + 7 days of recovery	100	ND	ND	ND	90*	102	100	ND	ND	ND	109	108

Finding	Males						Females					
	0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	PB	0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	PB
Serum [TSH] (% of control value)												
7 days	100	85	87	78	87	123	100	103	110	149	248**	167**
14 days	100	ND	ND	ND	146	159	100	ND	ND	ND	202	174
7 days + 7 days of recovery	100	ND	ND	ND	92	111	100	ND	ND	ND	122	98
Serum [T ₃] (% of control value)												
7 days	100	100	100	80	100	100	100	100	100	100	100	100
14 days	100	ND	ND	ND	80	100	100	ND	ND	ND	71**	71**
7 days + 7 days of recovery	100	ND	ND	ND	100	117*	100	ND	ND	ND	114	114
Histopathology												
Hepatocyte centrilobular hypertrophy												
7 days	0/10	0/10	0/10	0/10	0/10	10/10**	0/10	0/10	0/10	0/10	0/10	10/10**
14 days	0/10	ND	ND	ND	0/10	10/10**	0/10	ND	ND	ND	0/10	10/10**
7 days + 7 days of recovery	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	1/10
Hepatocyte diffuse hypertrophy												
7 days	0/10	0/10	1/10	8/10**	9/10**	0/10	1/10	1/10	5/10	6/10*	8/10**	0/10
14 days	2/10	ND	ND	ND	10/10**	0/10	0/10	ND	ND	ND	5/10*	0/10
7 days + 7 days of recovery	2/10	ND	ND	ND	3/10	1/10	0/10	ND	ND	ND	0/10	0/10
SER proliferation												
7 days	0/2	ND	ND	ND	1/2	ND	0/2	ND	ND	ND	1/2	ND
Hepatocyte intracellular lipid droplet												
7 days	0/2	ND	ND	ND	0/2	ND	0/2	ND	ND	ND	1/2	ND
BrdU labelling indices (% of control value)												
7 days	100	98	182	233**	389**	388**	100	192	197	225*	241**	308**
14 days	100	ND	ND	ND	138*	110	100	ND	ND	ND	111	174*
Thyroid diffuse follicular hypertrophy												
7 days	1/10	1/10	2/10	2/10	3/10	5/10	0/10	1/10	1/10	4/10*	6/10**	3/10
14 days	2/10	ND	ND	ND	5/10	6/10	0/10	ND	ND	ND	5/10*	3/10
7 days + 7 days of recovery	1/10	ND	ND	ND	2/10	4/10	0/10	ND	ND	ND	0/10	0/10

BrdU: 5-bromo-2'-deoxyuridine; CYP: cytochrome P450; ND: not determined; PB: phenobarbital; ppm: parts per million; SER: smooth endoplasmic reticulum; T₃: triiodothyronine; T₄: thyroxine; TSH: thyroid stimulating hormone; *: $P < 0.05$; **: $P < 0.01$

Source: Asano (2012e)

After the 7-day treatment, an increased incidence of enlarged liver was found in males receiving mandestrobin at 15 000 ppm and in females receiving PB at 1000 ppm. After the 14-day treatment, the incidences of enlarged liver were increased in males and females receiving mandestrobin at 15 000 ppm and in males and females receiving PB at 1000 ppm.

After the 7-day treatment, the incidences of diffuse hypertrophy of hepatocytes were increased in females at 2000 ppm and in both sexes at 7000 and 15 000 ppm. Centrilobular hypertrophy of hepatocytes was observed in all animals of the PB group. After the 14-day treatment, the incidences of diffuse hypertrophy of hepatocytes were increased in both sexes at 15 000 ppm. Centrilobular hypertrophy of hepatocytes was observed in all animals of the PB groups (Table 35).

In the recovery group, the incidences of diffuse hypertrophy of hepatocytes were not disturbed among males administered mandestrobin at 15 000 ppm or PB at 1000 ppm. Centrilobular hypertrophy of hepatocytes was observed in one female administered PB at 1000 ppm (Table 35). There was no evidence of necrosis or increased apoptosis in any of the groups at any time point. In addition, the incidences of brown pigment in bile duct were 4/10 for males and 1/10 for females at 15 000 ppm after the 7-day treatment and 2/10 for males at 15 000 ppm after the 14-day treatment. No pigment was observed in the recovery animals. The morphology of hepatocytes was assessed in controls and the highest-dose group using electron microscopy. After the 7-day treatment, proliferation of smooth endoplasmic reticulum (SER) was observed in the hepatocytes from both sexes in the 15 000 ppm mandestrobin-treated groups (Table 35). Peroxisome size and number were not changed in the treatment groups. Enlarged lipid droplets in the hepatocytes were observed in one female administered mandestrobin at 15 000 ppm.

After 7 days of treatment, statistically significant increases in the BrdU labelling indices were noted in animals at 7000 ppm and above and in the PB group. After 14 days of treatment, statistically significant increases were still observed in males administered 15 000 ppm and in females administered PB, but were attenuated compared with the 7-day treatment (Table 35).

After 7 days of treatment, statistically significant increases in CYP2B activity were observed in both sexes at 2000 ppm and above and in the PB group. In the recovery group, a statistically significant increase in CYP2B activity was still observed relative to controls in both sexes of the PB group; however, it was clear that some recovery had taken place, with a decrease in activity compared with the peak at 7 days of treatment (Table 35).

After 7 days of treatment, a statistically significant increase in CYP4A activity was observed in both sexes of the PB group; however, no statistically significant alterations were observed in the mandestrobin groups. In the recovery group, a statistically significant increase in CYP4A activity was observed in males that had been administered 15 000 ppm, and a statistically significant decrease in CYP4A activity was observed in females that had been administered 15 000 ppm or PB (Table 35). However, these alterations are marginal and likely to be due to individual variation. Therefore, they are considered not to be of toxicological relevance.

After 7 days of treatment, statistically significant increases in T₄-UGT activity were observed in males administered mandestrobin at 400 ppm and higher (except for 2000 ppm) and females administered 15 000 ppm. (Although the 2000 ppm group did not show statistical significance, the value was deemed to represent an increase over the controls.) A statistically significant increase in T₄-UGT activity was observed in both sexes of the PB group. In the recovery group, a clear decrease in T₄-UGT activity was detected (Table 35).

All changes, including liver weight, enzyme induction and hepatocellular hypertrophy, showed partial recovery 7 days after cessation of mandestrobin treatment, indicating that the alterations of rat liver by mandestrobin are reversible, similar to the situation with PB.

Thyroid: After the 7-day treatment, statistically significant increases were observed in the absolute and relative thyroid weights in females at 7000 ppm and above. After the 14-day treatment, statistically significant increases were observed in the absolute and relative thyroid weights in both sexes at 15 000 ppm and the PB group (Table 35).

After the 7-day treatment, the incidences of diffuse follicular cell hypertrophy were statistically significantly increased in females at 7000 and 15 000 ppm mandestrobin. This finding was also observed in the PB group. After 14 days, the incidences of diffuse follicular cell hypertrophy were increased for males and females at 15 000 ppm. This finding was also observed in the PB group. After the 7-day recovery period, the incidences of diffuse follicular cell hypertrophy remained minimally elevated for males at 1000 ppm PB (Table 35).

After 7 days of treatment with mandestrobin, a decrease in the serum concentration of T_4 was observed in both sexes administered 15 000 ppm (statistically significant only in males), and a statistically significant increase in TSH was observed in females administered 15 000 ppm. Statistically significant decreases in the serum concentration of T_4 and an increase in TSH were observed in females administered PB (Table 35).

After 14 days of treatment, statistically significant decreases in the serum concentration of T_4 and an increase in the serum concentration of TSH were observed in both sexes of the 15 000 ppm mandestrobin and PB groups. Statistically significant decreases in the serum concentrations of T_3 were observed in females of the same groups (Table 35).

In the recovery group, statistically significant decreases in the serum concentration of T_4 were observed in males administered 15 000 ppm mandestrobin, and statistically significant increases in T_3 were observed in males administered PB (Table 35).

Overall, in females treated with mandestrobin for 7 days, a slight, dose-related decrease in T_4 concentration, a slight, dose-related increase in TSH and a dose-related increase in the incidence of diffuse follicular cell hypertrophy were observed in females, but not all were statistically significant. These effects were not seen in males after the 7-day treatment, but were observed in both sexes after the 14-day treatment. Upon cessation of treatment, T_4 , TSH and thyroid follicular cell hypertrophy findings had partially reversed by 7 days after the last dose. The serum concentration reductions of T_4 and TSH and changes in thyroid morphology were associated with the induction of hepatic T_4 -UGT, suggesting that mandestrobin affected rat thyroid due to perturbation of the hypothalamus–pituitary–thyroid axis by induction of hepatic UGT activity, which depletes T_4 . These findings were consistent with those caused by PB.

In conclusion, mandestrobin administration in the rat resulted in increased liver weight, diffuse hepatocellular hypertrophy with proliferation of SER, increased CYP2B activity and a transient increase in the rate of replicative DNA synthesis, with a dose–response relationship and reversibility. Mandestrobin increased T_4 -UGT activity and secondarily perturbed the hypothalamus–pituitary–thyroid hormone axis. These effects are similar to those of PB, a known CAR activator. Therefore, it is reasonable to conclude that mandestrobin is a hepatic enzyme inducer via CAR activation in the rat, in a manner similar to PB (Asano, 2012e).

Short-term study in mice

In a second mechanistic non-GLP-compliant study, mandestrobin (purity 93.4%) was administered to 10 male CD-1 mice at a dietary concentration of 0 (control) or 7000 ppm (equal to 814 mg/kg bw per day) for 7 days. Hepatic CYP2B activity (determined by 7-pentoxoresorufin *O*-depentylase [PROD] activity) was then examined. In addition, replicative DNA synthesis of hepatocytes (determined by BrdU labelling index) was examined, because CAR activators often increase the BrdU labelling index of hepatocytes during the early phase of treatment.

No mortality or clinical signs were noted. No changes in body weight or feed consumption were recorded that could confound the evaluation of hepatic CYP2B induction or BrdU labelling.

Absolute liver weight tended to increase, but not statistically significantly (1.06-fold relative to control value), whereas relative liver weight was statistically significantly increased by mandestrobin treatment (1.07-fold relative to control value) (Table 36). No clear alterations in gross pathology of the liver were observed.

Table 36. Key findings of the mechanistic study in mice

Finding	0 ppm	7 000 ppm
Absolute liver weight (% of control value)	100	106
Relative liver weight (% of control value)	100	107*
S9 protein content of liver (% of control value)	100	115*
Hepatic CYP2B activity (% of control value)	100	171**
BrdU labelling index of hepatocytes (% of control value)	100	108
Liver gross pathology (incidence with no remarkable findings)	10/10	10/10
Liver histopathology (incidence)		
Eosinophilic change/hepatocyte hypertrophy	0/10	3/10
Focal hepatocyte brownish pigment	0/10	2/10
Focal mononuclear cell infiltration	2/10	4/10
Focal necrosis	2/10	3/10

BrdU: 5-bromo-2'-deoxyuridine; CYP2B: cytochrome P450; ppm: parts per million; S9: 9000 × g supernatant fraction of rat liver homogenate; *: $P < 0.05$; **: $P < 0.01$

Source: Yamada (2012b)

Slight eosinophilic change/hypertrophy of hepatocytes was observed in 3/10 animals treated with mandestrobin at 7000 ppm. Hepatic CYP2B activity was also statistically significantly increased by mandestrobin treatment (1.71-fold relative to control value). No clear alterations were observed in replicative DNA synthesis of hepatocytes.

Overall, treatment of the mouse with mandestrobin resulted in increased liver weight with slight eosinophilic change/hypertrophy of hepatocytes and increased CYP2B activity. Therefore, it is concluded that mandestrobin is a weak hepatic enzyme inducer via at least CAR activation in the mouse. In contrast to PB, however, mandestrobin did not enhance replicative DNA synthesis of hepatocytes during the early phase of treatment (Yamada, 2012b).

In conclusion, the effects observed in the two in vivo studies were compared with CAR-mediated induction of liver enzymes and subsequent perturbations of thyroid hormones. The proposed phenobarbital-like mode of action for mandestrobin tested in the in vivo rat mechanistic study is considered to satisfy the Bradford Hill criteria of dose and temporal concordance, biological plausibility, coherence, strength, consistency and specificity for thyroid follicular cell hypertrophy. Although the phenobarbital-like mode of action could theoretically operate in humans, the markedly different susceptibility for thyroid abnormality renders it non-relevant for humans. Furthermore, no increased tumour rates were observed up to the highest doses tested in the long-term toxicity and carcinogenicity studies.

In vitro hormonal activity studies

Mandestrobin (purity 93.4%) was evaluated for its effects on androgen and estrogen production in a non-GLP-compliant steroidogenesis assay. H295R cells cultured in 24-well plates were incubated with mandestrobin in triplicate for 48 hours. For the evaluation of testosterone and estradiol production, four (runs 1, 2, 3 and 4) and three (runs 1, 2 and 3) independent experiments were performed, respectively. DMSO was used as the vehicle at a final concentration of 0.1%. Positive controls (forskolin, a known inducer, and prochloraz, a known inhibitor) were evaluated concurrently with each run to confirm the changes in testosterone and estradiol levels in the assay. Ten micromoles of forskolin per litre increased the levels of testosterone and estradiol by more than 1.5-fold and 7.5-fold,

respectively, compared with the solvent control. One micromole of prochloraz per litre decreased the levels of both testosterone and estradiol by less than 0.5-fold compared with the solvent control. These results indicated that present assay systems were validated by the positive control experiments. Testosterone and estradiol levels were measured using enzyme-linked immunosorbent assay systems. In run 1, mandestrobin showed cytotoxicity to H295R cells at 100 $\mu\text{mol/L}$. Therefore, the maximum mandestrobin concentration was set to 30 $\mu\text{mol/L}$ in subsequent runs. For testosterone, through runs 1–4, a significant change in testosterone production relative to the controls was seen only at 30 $\mu\text{mol/L}$ in run 2, but not in runs 3 and 4. For estradiol, no significant change was observed in runs 1 and 2. In run 3, the production of estradiol was statistically significantly increased at 10 $\mu\text{mol/L}$, but not at either 3 or 30 $\mu\text{mol/L}$. Consequently, a consistent, dose-related increase in estradiol was not observed.

It is concluded that mandestrobin does not influence testosterone or estradiol production in H295R cells at concentrations up to 30 $\mu\text{mol/L}$ (Kubo, 2012).

In a second in vitro study, mammalian cell-based luciferase reporter gene assays were performed for detecting estrogenic, anti-estrogenic, androgenic and anti-androgenic effects of mandestrobin (purity 93.4%) and its metabolites: 5-COOH-S-2200 (purity 99.7%), 4-OH-S-2200 (purity 99.9%), 5-CH₂OH-S-2200 (purity 99.9%) and 5-CA-S-2200-NHM (purity 94.9%) on human estrogen receptor alpha (hER α)– and human androgen receptor (hAR)–mediated mechanisms. In order to determine the appropriate concentration ranges of substances to be tested, the test substances were evaluated by cytotoxicity assays (100 pmol/L to 100 $\mu\text{mol/L}$), the concentrations at or above the cytotoxic concentration being excluded from the evaluation. Based on the results of the cytotoxicity assays, the maximum concentrations of mandestrobin, 5-COOH-S-2200, 4-OH-S-2200, 5-CH₂OH-S-2200 and 5-CA-S-2200-NHM were 1, 100, 10, 10 and 100 $\mu\text{mol/L}$ in the hER α assays, respectively, and 10, 100, 10, 100 and 100 $\mu\text{mol/L}$ in the hAR assays, respectively.

In the reporter gene assay for hER α , positive controls for hER α (agonist: 17 β -estradiol; antagonist: 4-hydroxytamoxifen) showed marked agonistic and antagonistic activities, respectively. However, mandestrobin and its metabolites showed neither agonistic nor antagonistic activity in the assays. In the reporter gene assay for hAR, positive controls for hAR (agonist: dihydrotestosterone; antagonist: hydroxyflutamide) showed marked agonistic and antagonistic activities, respectively. However, neither agonistic nor antagonistic activity was observed for mandestrobin and its metabolites.

Overall, mandestrobin and its metabolites did not show agonistic or antagonistic effects on hER α or hAR, indicating that mandestrobin and its metabolites did not have any impact on hER α - and hAR-mediated transcriptional activities in vitro (Suzuki, 2012).

It is concluded that mandestrobin had no influence on testosterone or estradiol production and that mandestrobin and its metabolites 5-COOH-S-2200, 4-OH-S-2200, 5-CH₂OH-S-2200 and 5-CA-S-2200-NHM did not have any effects on estrogen- or androgen-mediated reporter gene activity.

(d) *Toxicity studies on metabolites and impurities*

An assessment of the representativeness (i.e. impurity profile) of the batches used in the toxicity studies was performed. According to the impurity profile, the batch used in the toxicity studies represents a worst-case scenario, as it contains impurities that are not present in the commercial specification. Only four impurities are present in both the batch used in the toxicity studies and the commercial specification. All of them are at comparable levels; therefore, their toxicological properties can be considered to be covered by the toxicity studies.

2-COOH-S-2200

2-COOH-S-2200 (2-({2-[(1*RS*)-1-methoxy-2-(methylamino)-2-oxoethyl]benzyl}oxy)-4-methylbenzoic acid) is a rat, hen and goat metabolite and is also present in groundwater. Results of the toxicity studies with this metabolite are summarized in Table 37.

Table 37. Results of toxicity studies with the metabolite 2-COOH-S-2200

Study	Species	Purity (%)	Results	Reference
Acute oral toxicity study	Rat (Slc:WistarHannover/Rcc)	99.0	LD ₅₀ > 2 000 mg/kg bw	Asano (2012a)
Ames test	<i>Salmonella typhimurium</i> , <i>Escherichia coli</i>	99.0	Not mutagenic	Kitamoto (2012a)
In vitro chromosomal aberration assay	Chinese hamster lung (CHL/IU) cells	99.0	Weak potential to induce chromosomal aberrations only at highly toxic dose of 2 200 µg/mL	Kitamoto (2012b)
In vitro mammalian gene mutation assay	Chinese hamster V79 cells	99.0	Not mutagenic	Wollny (2011a)
In vivo micronucleus assay	Mouse	99.0	Not genotoxic	Tanaka (2012)

bw: body weight; LD₅₀: median lethal dose

In an acute oral toxicity study, a single oral dose of 2-COOH-S-2200 (purity 99.0%), as a suspension in 0.5% aqueous methylcellulose solution, of 2000 mg/kg bw was administered by gavage to six female rats. Clinical and mortality observations were made at 10 and 30 minutes and 1, 2 and 4 hours after treatment and once daily for 14 days thereafter. Body weights were determined on the day of dosing (day 0), day 7 and day 14. On day 14, animals were euthanized, and all organs and tissues were examined macroscopically. No mortality or clinical signs were noted during the study. Body weights were not affected, and no gross abnormalities at necropsy were found. Therefore, the oral LD₅₀ of 2-COOH-S-2200 in female rats was greater than 2000 mg/kg bw (Asano, 2012a).

In a reverse gene mutation assay in bacteria (Ames test), 2-COOH-S-2200 (purity 99.0%) was tested on four strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and one strain of *Escherichia coli* (WP2uvrA) in the presence and absence of S9 mix. As neither cytotoxicity nor precipitation was noted in a dose range-finding assay, doses ranged from 156 to 5000 µg/plate with and without S9 mix in the main assays. There was no statistically significant dose-related increase in the number of revertant colonies with or without S9 mix. Positive controls showed the appropriate increase in the number of revertant colonies. 2-COOH-S-2200 was not mutagenic under these test conditions (Kitamoto, 2012a).

The clastogenic potential of 2-COOH-S-2200 (purity 99.0%) was examined in an in vitro chromosomal aberration test using Chinese hamster lung (CHL/IU) cells with and without metabolic activation (S9 mix). In a preliminary cytotoxicity assay, marked growth inhibitions were noted in both the presence and absence of S9 mix at 3500 µg/mL after 6 and 24 hours of exposure. Precipitation was noted after 6 hours of treatment at the concentration of 3500 µg/mL with and without S9 mix. In the chromosomal aberration assay, doses ranged from 550 to 3000 µg/mL without S9 mix and from 700 to 2800 µg/mL with S9 mix. When the CHL/IU cells were exposed for 6 hours to 2-COOH-S-2200 with and without S9 mix, no increase in chromosomally aberrant cells (structural or numerical) was observed. When the CHL/IU cells were exposed for 24 hours without S9 mix, a small increase in structural aberrant cells (but not numerical) was observed only at the high concentration of 2200 µg/mL (equivalent to 6.4 mmol/L). All negative and positive control cultures gave values of chromosomal aberrations within the expected range. 2-COOH-S-2200 has a weak potential to induce chromosomal

aberrations only at the high concentration of 2200 µg/mL (equivalent to 6.4 mmol/L) in CHL/IU cells under the test conditions (Kitamoto, 2012b). It should also be noted that this high concentration exceeds the current Organisation for Economic Co-operation and Development (OECD)–recommended top concentration of 2000 µg/mL (2 mg/mL) (OECD, 2014).

If the relative increase in cell count (RICC) is used to estimate cytotoxicity, as is highly recommended in the latest OECD test guideline, the clastogenic effects occur only at a concentration at which significant cytotoxicity is observed and are considered an artefact of cytotoxicity, as outlined in Kirkland (1998). The result is a classic example of a “false positive”, as outlined in Kirkland (1998), for what he terms “high toxicity [in vitro] clastogens”. Relative cell count (RCC) was used in the study with 2-COOH-S-2200 as the indicator for cytotoxicity. The RCC index is now well known to underestimate cytotoxicity and often leads to overdosing. The use of the RICC index is therefore more appropriate, as discussed by Galloway et al. (2011). As shown in Table 38, the RICC for 2200 µg/mL (where the response was seen) was just 21.2% of the control value, which is considerably in excess of the top-dose toxicity required by OECD Test Guideline 473. 2-COOH-S-2200 is therefore a “high toxicity [in vitro] clastogen” (i.e. the positive result occurred only at >60% toxicity). The other in vitro assays were negative, which is also typical of this type of false-positive result discussed by Kirkland (1998). There was also no response at lower concentrations. The response seen at 2200 µg/mL (with precipitate: maximum soluble concentrate estimated at approximately 1600 µg/mL) was practically as intense as for the positive control, but reduced to zero at 1100 µg/mL. This would be extremely unusual for a classic non-threshold genotoxic carcinogen. It is considered, therefore, that the result in this assay is an artefact of the high-dose “high toxicity” and is of little biological relevance. In addition, no in vivo clastogenic activity was identified in the mouse micronucleus assay on 2-COOH-S-2200, as described below.

Table 38. Growth rates using RCC and RICC in the in vitro chromosomal aberration test with 2-COOH-S-2200 (24-hour exposure, without S9 mix)

Group	Dose (µg/mL)	Cell number	RCC ^a (%)	RICC ^b (%)
At the start of treatment ^c	–	3.35 × 10 ⁵	–	–
Control	0	9.54 × 10 ⁵	100	100
2-COOH-S-2200	550	8.17 × 10 ⁵	85.7	77.9
	1 100	7.58 × 10 ⁵	79.5	68.3
	2 200	4.66 × 10 ⁵	48.8	21.2

RCC: relative cell count; RICC: relative increase in cell count

^a RCC = (Number of cells in treated cultures / number of cells in control cultures) × 100.

^b RICC = (Increase in number of cells in treated cultures [final – starting] / increase in number of cells in control cultures [final – starting]) × 100.

^c The cell number is available in raw data.

Source: Adapted from Kitamoto (2012b)

Metabolite 2-COOH-S-2200 (purity 99.0%) was evaluated in a gene mutation assay at the *HPRT* locus in V79 cells of the Chinese hamster. The study was performed in two independent experiments using identical experimental procedures. In the first experiment, the treatment period was 4 hours with and without metabolic activation. The second experiment was performed with a treatment time of 4 hours with metabolic activation and 24 hours without metabolic activation. As no relevant cytotoxic effect occurred up to the 3500 µg/mL concentration with or without S9 mix, the main assay was conducted at concentrations ranging from 109.4 to 3500 µg/mL with and without S9 mix. Again, the high concentration substantially exceeds the current OECD-recommended top concentration of 2000 µg/mL (2 mg/mL) (OECD, 2014).

Precipitation of the test item at the end of treatment was observed by the unaided eye at 3500 µg/mL in both main experiments with and without metabolic activation. Negative and positive controls

were within the expected historical range. Neither a relevant nor reproducible increase in mutant colony cells (≥ 3 times control values) was observed in the main experiments up to the maximum concentration with and without metabolic activation. In the 24-hour experiment in the absence of S9, a modest 3-fold increase in mutation frequency was seen in one cell culture, but was not seen in the second culture from the same experiment. No increases were seen in two separate experiments that employed 4-hour exposures with or without S9. Overall, it was concluded that 2-COOH-S-2200 did not induce gene mutations at the *HPRT* locus in V79 cells under the experimental conditions and is considered non-mutagenic in this assay (Wollny, 2011a).

In an *in vivo* bone marrow micronucleus test, 2-COOH-S-2200 (purity 99.0%) was administered by gavage to groups of six male CD-1 mice at a dose of 0, 500, 1000 or 2000 mg/kg bw in the 24-hour treatment groups and to six male CD-1 mice at 2000 mg/kg bw in the 48-hour treatment group. Bone marrow smears were prepared 24 and 48 hours (five animals per group) after dosing and analysed for the presence of micronuclei by microscopy. There were an additional mitomycin C positive control group (smears prepared at 24 hours) and two solvent negative control groups (smears prepared at 24 and 48 hours). The incidence of micronuclei in 2000 polychromatic erythrocytes (PCEs) and the incidence of PCEs in 500 erythrocytes (including normochromatic erythrocytes [NCEs] and PCEs) were recorded for each animal.

There were no signs of toxicity as a result of the administration of 2-COOH-S-2200 in treated animals. The frequencies of micronucleated PCEs (MNPCEs) after administration of 2-COOH-S-2200 were 0.31%, 0.36% and 0.46% at 500, 1000 and 2000 mg/kg bw, respectively. This apparent increase was not statistically significant. From individual animal data, only two animals had the %MNPCE exceeding the upper limit of the negative historical controls (0.56%): one at 500 mg/kg bw (0.7%) and one at 2000 mg/kg bw (0.75%). In the 1000 mg/kg bw group, all animals had the %MNPCE falling within the negative historical controls. There was no change in the PCE/(PCE+NCE) ratio.

The positive control induced marked statistically significant increases in micronuclei. 2-COOH-S-2200 showed no potential to induce micronuclei in mouse bone marrow cells (Tanaka, 2012).

The following evidence was presented that the target organ is reached at sufficient levels: in the rat ADME study performed with the parent molecule (Kendrick & Farrell, 2012), mandestrobin-derived radioactivity in bone marrow peaked at 234 ng equivalents/g (male) and 217 ng equivalents/g (female) at 0.5 hour post-dosing ($T_{\max}$) in the 5 mg/kg bw dose group and 7455 ng equivalents/g (male) and 3828 ng equivalents/g (female) at 8 hours post-dosing ($T_{\max}$) in the 1000 mg/kg bw dose group. It was still measureable in the bone marrow of male rats at 36 hours after treatment at the high dose, indicating reasonable exposure over the whole 24-hour period. These results indicated that mandestrobin-derived radioactivity reached the bone marrow at significant and sufficient concentration at the low and high doses, respectively; therefore, the negative outcome of the study can be considered to be reliable.

There is no estimated change to the physicochemical properties of the parent compound (octanol–water partition coefficient, molecular weight, water solubility) by the addition of the carboxyl group that would significantly reduce exposure of the bone marrow to 2-COOH-S-2200 when compared with the parent (Table 39). The ADME study with the parent compound suggests that 2-COOH-S-2200 is formed in the gut and liver and absorbed and excreted in urine, ostensibly unchanged, indicating that systemic exposure is not obstructed by the liver. This, coupled with the increased water solubility but unchanged lipophilicity of the metabolite, would potentially increase exposure of the bone marrow: higher concentrations compared with parent compound could be carried in the plasma.

As such, there is no reason to expect that exposure of the bone marrow to 2-COOH-S-2200 would be less than that of the parent compound or at least would not be reduced to an extent that would render the *in vivo* micronucleus study with 2-COOH-S-2200 invalid.

Table 39. Comparison of the physicochemical properties of 2-COOH-S-2200 with those of mandestrobin

Properties	Mandestrobin	2-COOH-S-2200
Molecular weight (g/mol)	313.39	343.38
Aqueous solubility at 20 °C (mg/L)	15.8	607 ^a
Log octanol–water partition coefficient	3.51/3.44	2.53 ^{a,b}

^a Estimated at 25 °C using EPISUITE.^b New estimate not in dossier, effectively the un-ionized form.**5-COOH-S-2200**

5-COOH-S-2200 (3-({2-[(1*RS*)-1-methoxy-2-(methylamino)-2-oxoethyl]benzyl}oxy)-4-methylbenzoic acid) is a rat, hen and goat metabolite. It is also present in soil, groundwater, surface water and crops (including rotational). Results of toxicity studies with this metabolite are summarized in Table 40.

Table 40. Results of toxicity studies with the metabolite 5-COOH-S-2200

Study	Species	Purity (%)	Results	Reference
Acute oral toxicity study	Rat (Slc:WistarHannover/Rcc)	97.6	300 mg/kg bw < LD ₅₀ < 2 000 mg/kg bw	Asano (2012b)
Ames test	<i>Salmonella typhimurium</i> , <i>Escherichia coli</i>	97.6	Not mutagenic	Kitamoto (2012c)
In vitro chromosomal aberration assay	Chinese hamster lung (CHL/IU) cells	97.6	Not clastogenic	Kitamoto (2012d)
In vitro mammalian gene mutation assay	Chinese hamster V79 cells	97.6	Not mutagenic	Wollny (2011b)

bw: body weight; LD₅₀: median lethal dose

In an acute oral toxicity study in rats, 5-COOH-S-2200 (purity 97.6%) was administered, as a suspension in 0.5% aqueous methylcellulose solution, by gavage to three or six female rats in a single oral dose of 300 or 2000 mg/kg bw. Clinical and mortality observations were made at 10 and 30 minutes and 1, 2 and 4 hours after treatment and once daily for 14 days thereafter. Body weights were determined on the day of dosing (day 0), day 7 and day 14. On day 14, animals were euthanized, and all organs and tissues were examined macroscopically. All three animals treated at 2000 mg/kg bw died, and clinical signs included lateral position and bradypnoea. All six animals treated at 300 mg/kg bw survived the observation period with no clinical signs. All animals gained weight. Retention of foamy fluid in the trachea was observed in two animals at 2000 mg/kg bw, and “uncollapse” and pale in all lobes of the lung were observed in one animal at 300 mg/kg bw during the gross pathological examination. The oral LD₅₀ of 5-COOH-S-2200 in rats was therefore greater than 300 mg/kg bw but less than 2000 mg/kg bw (Asano, 2012b).

In a reverse gene mutation assay in bacteria (Ames test), 5-COOH-S-2200 (purity 97.6%) was tested on four strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and one strain of *Escherichia coli* (WP2uvrA) in the presence and absence of S9. As neither cytotoxicity nor precipitation was noted up to 5000 µg/plate in a dose range-finding assay, concentrations ranged from 156 to 5000 µg/plate with and without S9 mix. There was no statistically significant dose-related increase in the number of revertant colonies, with or without S9 mix. Positive controls showed the

appropriate increase in the number of revertant colonies. 5-COOH-S-2200 was not mutagenic under these test conditions (Kitamoto, 2012c).

The clastogenic potential of 5-COOH-S-2200 (purity 97.6%) was examined in an in vitro chromosomal aberration test using Chinese hamster lung (CHL/IU) cells, with and without metabolic activation (S9 mix). In a preliminary cytotoxicity assay, relevant cytotoxicity was noted after 6 hours of treatment at 1750 µg/mL with S9 mix and after 24 hours of treatment at and above 875 µg/mL without S9 mix. Precipitation was noted in the medium at the end of treatment at 3500 µg/mL. Main assays were conducted with concentrations ranging from 219 to 3500 µg/mL without S9 mix and from 438 to 1750 µg/mL with S9 mix. The 3500 µg/mL concentration substantially exceeds the current OECD-recommended top concentration of 2000 µg/mL (2 mg/mL) (OECD, 2014). When the CHL/IU cells were exposed for 6 or 24 hours to 5-COOH-S-2200 with and without S9 mix, no increase in chromosomally aberrant cells (structural or numerical) was observed. All negative and positive control cultures gave values of chromosomal aberrations within the expected range. 5-COOH-S-2200 has no potential to induce chromosomal aberrations in CHL/IU cells under the test conditions (Kitamoto, 2012d).

Metabolite 5-COOH-S-2200 (purity 97.6%) was evaluated in a gene mutation assay at the *HPRT* locus in V79 cells of the Chinese hamster, with and without metabolic activation (S9 mix). The study was performed in two independent experiments using identical experimental procedures. In the first experiment, the treatment period was 4 hours with and without metabolic activation. The second experiment was performed with a treatment time of 4 hours with metabolic activation and 24 hours without metabolic activation. As no relevant cytotoxic effect occurred up to the maximum concentration in the preliminary cytotoxicity assay, the main assay was conducted at concentrations ranging from 109.4 to 3500 µg/mL with and without S9 mix. Precipitation of the test item at the end of treatment was observed by the unaided eye at 3500 µg/mL in both main experiments with and without metabolic activation. In experiment I, turbidity of the medium was noted at 875 and 1750 µg/mL with metabolic activation.

No relevant cytotoxic effect occurred up to the maximum concentration with metabolic activation. A moderate cytotoxic effect, indicated by a relative cloning efficiency I below 50%, was observed in the first experiment without metabolic activation at the highest soluble concentration of 1750 µg/mL. The relative cloning efficiency I was back above 50% at the next higher precipitating concentration of 3500 µg/mL. Following 24 hours of treatment in the second experiment without metabolic activation, relevant cytotoxic effects were observed at 1750 and 3500 µg/mL. Again, the 3500 µg/mL concentration exceeds the current OECD-recommended top concentration of 2000 µg/mL (OECD, 2014). No substantial or reproducible dose-dependent increase of the mutant frequencies exceeding the threshold of 3 times over the solvent control was observed at any concentration either with or without S9 mix or after 4- or 24-hour treatment. Appropriate reference mutagens were used as positive controls and showed a distinct increase in induced mutant colonies. 5-COOH-S-2200 did not induce gene mutations at the *HPRT* locus in V79 cells under the experimental conditions and is considered non-mutagenic in this assay (Wollny, 2011b).

2-CH₂OH-S-2200

2-CH₂OH-S-2200 ((2*RS*)-2-[2-(5-hydroxymethyl-2-methylphenoxy)methyl]phenyl)-2-methoxy-*N*-methylacetamide) is a rat, hen and goat metabolite and is also present in crops (including rotational). Results of toxicity studies with this metabolite are summarized in Table 41.

In an acute oral toxicity study in rats, 2-CH₂OH-S-2200 (purity 100%) was administered, as a suspension in 0.5% aqueous methylcellulose solution, by gavage to two groups of three female rats in a single oral dose of 2000 mg/kg bw. Clinical and mortality observations were made at 10 and 30 minutes and 1, 2 and 4 hours after treatment and once daily for 14 days thereafter. Body weights were determined on the day of dosing (day 0), day 7 and day 14. On day 14, animals were euthanized, and all organs and tissues were examined macroscopically. All animals survived the observation period with

no clinical signs. All animals gained weight, and no pathological findings were noted during necropsy. Therefore, the oral LD₅₀ of 2-CH₂OH-S-2200 in rats was greater than 2000 mg/kg bw (Asano, 2012c).

Table 41. Results of toxicity studies with the metabolite 2-CH₂OH-S-2200

Study	Species	Purity (%)	Results	Reference
Acute oral toxicity study	Rat (Slc:WistarHannover/Rcc)	100	LD ₅₀ > 2 000 mg/kg bw	Asano (2012c)
Ames test	<i>Salmonella typhimurium</i> , <i>Escherichia coli</i>	100	Not mutagenic	Kitamoto (2012e)

bw: body weight; LD₅₀: median lethal dose

In a reverse gene mutation assay in bacteria (Ames test), 2-CH₂OH-S-2200 (purity 100%) was tested on four strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and one strain of *Escherichia coli* (WP2uvrA) in the presence and absence of S9 mix. In a dose range-finding assay, precipitation was observed at and above a concentration of 1250 µg/plate. No cytotoxicity was observed. In the main assay, concentrations ranged from 156 to 5000 µg/plate with and without S9 mix. There was no statistically significant, dose-related increase in the number of revertant colonies, with or without S9 mix. Positive controls showed the appropriate increase in the number of revertant colonies. 2-CH₂OH-S-2200 was not mutagenic under these test conditions (Kitamoto, 2012e).

4-OH-S-2200

4-OH-S-2200 ((2*RS*)-2-[2-(4-hydroxy-2,5-dimethylphenoxy)methyl]phenyl]-2-methoxy-*N*-methylacetamide) is a rat, hen and goat metabolite and is also present in crops (including rotational). Results of toxicity studies with this metabolite are summarized in Table 42.

Table 42. Results of toxicity studies with the metabolite 4-OH-S-2200

Study	Species	Purity (%)	Results	Reference
Acute oral toxicity study	Rat (Slc:WistarHannover/Rcc)	99.9	LD ₅₀ > 2 000 mg/kg bw	Asano (2012d)
Ames test	<i>Salmonella typhimurium</i> , <i>Escherichia coli</i>	99.9	Not mutagenic	Kitamoto (2012f)

bw: body weight; LD₅₀: median lethal dose

In an acute oral toxicity study in rats, 4-OH-S-2200 (purity 99.9%) was administered, as a suspension in 0.5% aqueous methylcellulose solution, by gavage to two groups of three female rats in a single oral dose of 2000 mg/kg bw. Clinical and mortality observations were made at 10 and 30 minutes and 1, 2 and 4 hours after treatment and once daily for 14 days thereafter. Body weights were determined on the day of dosing (day 0), day 7 and day 14. On day 14, animals were euthanized, and all organs and tissues were examined macroscopically. All animals survived the observation period with no clinical signs. All animals gained weight, and no pathological findings were noted during necropsy. Therefore, the oral LD₅₀ of 4-OH-S-2200 in rats was greater than 2000 mg/kg bw (Asano, 2012d).

In a reverse gene mutation assay in bacteria (Ames test), 4-OH-S-2200 (purity 99.9%) was tested on four strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and one strain of *Escherichia coli* (WP2uvrA) in the presence and absence of S9. As no cytotoxicity was noted in the dose range-finding assay, concentrations ranged from 156 to 5000 µg/plate with and without S9 mix.

Precipitation was observed at and above 1250 µg/plate with and without S9 mix in both the dose range-finding and main assays. There was no statistically significant, dose-related increase in the number of revertant colonies, with or without S9 mix. Positive controls showed the appropriate increase in the number of revertant colonies. 4-OH-S-2200 was not mutagenic under these test conditions (Kitamoto, 2012f).

De-Xy-S-2200

De-Xy-S-2200 ((2*RS*)-2-(2-hydroxymethylphenyl)-2-methoxy-*N*-methylacetamide) is a rat, hen and goat metabolite and is also present in crops (including rotational). Results of toxicity studies with this metabolite are summarized in Table 43.

Table 43. Results of toxicity studies with the metabolite De-Xy-S-2200

Study	Species	Purity (%)	Results	Reference
Acute oral toxicity study	Rat (Slc:WistarHannover/Rcc)	100	LD ₅₀ > 2 000 mg/kg bw	Asano (2011)
Ames test	<i>Salmonella typhimurium</i> , <i>Escherichia coli</i>	100	Not mutagenic	Kitamoto (2011)

bw: body weight; LD₅₀: median lethal dose

In an acute oral toxicity study in rats, De-Xy-S-2200 (purity 100%) was administered, as a suspension in 0.5% aqueous methylcellulose solution, by gavage to two groups of three female rats in a single oral dose of 2000 mg/kg bw. Clinical and mortality observations were made at 10 and 30 minutes and 1, 2 and 4 hours after treatment and once daily for 14 days thereafter. Body weights were determined on the day of dosing (day 0), day 7 and day 14. On day 14, animals were euthanized, and all organs and tissues were examined macroscopically. All animals survived the observation period. No mortality was observed. All animals showed a decrease of spontaneous activity from 30 minutes after administration. This finding disappeared on day 3. In four animals, ataxic gait from 1 to 4 hours after administration and prone position from 2 to 4 hours after administration were observed. In addition, abdominal stains and lateral position were observed in three and two animals, respectively. Both findings were noted from 4 hours after administration and disappeared on day 2. Furthermore, one animal showed lacrimation at 4 hours after administration and irregular respiration on day 2. All animals gained weight, and no pathological findings were noted during necropsy. Therefore, the oral LD₅₀ of De-Xy-S-2200 in rats was greater than 2000 mg/kg bw (Asano, 2011).

In a reverse gene mutation assay in bacteria (Ames test), De-Xy-S-2200 (purity 100%) was tested on four strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and one strain of *Escherichia coli* (WP2uvrA) in the presence and absence of S9. As no cytotoxicity and no precipitation were observed in a dose range-finding assay, concentrations ranged from 156 to 5000 µg/plate with and without S9 mix. There was no statistically significant, dose-related increase in the number of revertant colonies, with or without S9 mix. Positive controls showed the appropriate increase in the number of revertant colonies. De-Xy-S-2200 was not mutagenic under these test conditions (Kitamoto, 2011).

(e) Microbiological effects

The fungicide mandestrobin was evaluated at the 2018 JMPR to determine its impact on microbiota in the gastrointestinal tract. As no data were submitted by the sponsors, a literature search was performed using a number of search engines. These included BioOne (<http://www.bioone.org/>), Google (<https://www.google.com/>), Google Scholar (<http://scholar.google.com/>), PubMed

(<http://www.ncbi.nlm.nih.gov/pubmed>), ScienceDirect (<http://www.sciencedirect.com/>) and Web of Science (<https://apps.webofknowledge.com>).

The search strategy used the input keywords of the fungicide chemical name (mandestrobin), chemical structure, antimicrobial mode of action, antimicrobial spectrum of activity, antimicrobial resistance, resistance mechanisms and genetics, microbiome, microbiota, gut microbiota, gut microbiome, gastrointestinal microbiota, gastrointestinal microbiome, etc., as well as the Boolean operators AND, OR and NOT.

The extensive search and review of the scientific literature did not find any reports on the effects of mandestrobin on the intestinal microbiome to include in the toxicological risk assessment.

3. Observations in humans

A formal statement from the manufacturer noted that members of staff involved in the synthesis and development of mandestrobin are routinely monitored and that no indication of mandestrobin-related ill-health have been detected by, or reported to, medical staff (Nishioka, 2012).

In a recent report (Nishimoto, 2017), the health of 27 factory workers involved in the manufacturing of mandestrobin technical material was monitored for 2 years in accordance with Japanese law. At least once a year, every employee underwent a medical examination, which included body measurements, eyesight and audibility, blood pressure measurement, haematological examination, liver function test, blood lipid examination, blood sugar and haemoglobin A1c, urine analysis and renal function test. Since the production of mandestrobin started, there have been no findings to suggest adverse effects caused by mandestrobin. This is consistent with the fact that there have been no claims or reports of health problems, nor any incident of intoxication related to the manufacturing/packing operations.

Comments

Biochemical aspects

In metabolism studies in which rats were administered racemic [^{14}C]mandestrobin labelled at either the benzyl or phenoxy ring as a single dose of 5 or 1000 mg/kg bw, excretion was approximately 90% (>70% within 48 hours). The major routes of excretion were through the faeces (67–75%) and urine (16–22%). There were no significant sex-, dose- or radiolabel-related differences in either the rates or routes of excretion. In bile duct-cannulated rats, elimination of radioactivity after a single low dose of 5 mg/kg bw was rapid, with more than 95% of the dose eliminated within 24 hours. About 80% of the excreted radioactivity was eliminated via the bile, and the amount recovered in faeces of the cannulated rats accounted for less than 2% of the total radioactivity. Elimination via urine was comparable to that of intact animals. For both low and high doses, there were no significant sex differences in the pharmacokinetic parameters. Mean plasma elimination half-lives were approximately 20 and 27 hours at the low and high doses, respectively. C_{max} was observed within 3 and 10 hours post-dosing for the low and high doses, respectively. At the high dose, additional plasma concentration peaks were observed, which were particularly prominent during the first 12 hours post-dosing. The multiple peaks could possibly reflect enterohepatic recirculation, which is supported by the enhanced rapidity of elimination in bile duct-cannulated animals. At the high dose, systemic exposure (AUC) was sub-dose-proportional, indicating saturation of absorption processes following administration.

Following repeated administration of a low dose of radiolabelled compound at 5 mg/kg bw for up to 14 consecutive days, radioactivity was primarily excreted in the faeces (50–57%), with urine containing 13–15% of the administered dose. There were no significant sex differences. In a metabolism study conducted with the *R*- or *S*-isomer, after a single low dose of 5 mg/kg bw, the excretion of *R*-isomer (>95% at 72 hours) was more rapid than excretion of the *S*-isomer (>94% at 120 hours), likely due to enterohepatic recirculation. The majority of radioactivity was excreted in faeces (up to 73% *R*-

isomer; up to 77% *S*-isomer). Urinary excretion was higher in female rats (32% *R*-isomer; 25% *S*-isomer) than in male rats (22% *R*-isomer; 15% *S*-isomer).

Mandestrobin was widely distributed throughout the body. No major differences in distribution were observed, regardless of dose, sex or label position. The major tissue residues were seen in the gastrointestinal tract, liver, kidney, uterus and ovaries at 168 hours after dosing. A similar distribution of radioactivity into tissues was observed following repeated dosing. There was evidence of accumulation in tissues, but no persistence.

Mandestrobin was extensively metabolized to numerous metabolites. Unchanged parent was found in faeces at less than 0.2% and less than 6% of the administered dose after a single low and single high dose, respectively. The primary routes of metabolism were by oxidation and subsequent conjugation with glucuronic acid, demethylation with subsequent oxidation, or oxidation with subsequent demethylation. The metabolic profile was generally independent of sex, dose, label position or whether the animals were bile duct cannulated or not. Metabolite fractions in plasma, liver and kidney were identified. There was generally no discernible shift in metabolism at high or repeated doses except in the kidney. Metabolic profiles in the kidney exhibited differences in the type and number of metabolites observed depending on both sex and dosing regimen (single versus repeated dosing). In an investigation of the ADME of the isomers, 12 metabolites were identified and quantified; 5-CA-*S*-2200-NHM was the predominant metabolite of the *R*-isomer, and 4-OH-*S*-2200 followed by 5-COOH-*S*-2200 were the most abundant metabolites of the *S*-isomer. However, the same metabolites were identified for both the *R*-isomer and the *S*-isomer. 4-OH-*S*-2200-GlucA is likely to be subject to enterohepatic circulation. This metabolic pathway is likely to occur more commonly with the *S*-isomer, because the rate of radiolabel excretion was slower (delayed presumably by enterohepatic recirculation) than that for the *R*-isomer (Mikata, 2011; Kendrick & Farrell, 2012; Kendrick, Farrell & Murphy, 2012).

Toxicological data

In rats, the acute oral and dermal LD₅₀ values were greater than 2000 mg/kg bw (Asano, 2010a,b), and the acute inhalation LC₅₀ was greater than 4.96 mg/L (Deguchi, 2010). Mandestrobin was not irritating to the skin of rabbits (Ota, 2010a), but was mildly irritating to the eyes of rabbits (Ota, 2010b). Mandestrobin was not sensitizing to the skin of guinea-pigs (Ota, 2010c).

The short-term toxicity of mandestrobin was tested in mice, rats and dogs, and the long-term toxicity and carcinogenicity were tested in mice and rats. The target organ in all species was the liver, with the dog being the most sensitive species. In the rat, thyroid follicular hypertrophy was also observed, concurrently with or at higher doses than effects on the liver.

In a 90-day study in mice in which mandestrobin was administered at a dietary concentration of 0, 1750, 3500 or 7000 ppm (equal to 0, 204, 405 and 807 mg/kg bw per day for males and 0, 252, 529 and 1111 mg/kg bw per day for females, respectively), the NOAEL was 3500 ppm (equal to 529 mg/kg bw per day), based on reduced body weight gain in females at 7000 ppm (equal to 1111 mg/kg bw per day) (Beck, 2011a).

In a 90-day study in rats in which mandestrobin was administered at a dietary concentration of 0, 800, 4000, 10 000 or 20 000 ppm (equal to 0, 54, 283, 743 and 1545 mg/kg bw per day for males and 0, 62, 320, 788 and 1886 mg/kg bw per day for females, respectively), the NOAEL was 4000 ppm (equal to 283 mg/kg bw per day), based on increased liver weights accompanied by hepatocyte hypertrophy, increased total cholesterol and thyroid follicular hypertrophy in both sexes at 10 000 ppm (equal to 743 mg/kg bw per day) (Beck, 2011b).

In a 90-day study in dogs in which mandestrobin was administered at a dietary concentration of 0, 4000, 12 000 or 40 000 ppm (equal to 0, 90.9, 267.8 and 933.1 mg/kg bw per day for males and 0, 102.7, 304.4 and 820.4 mg/kg bw per day for females, respectively), the NOAEL was 4000 ppm (equal to 90.9 mg/kg bw per day), based on increased liver weights, histopathological changes in the liver and increased alkaline phosphatase activity in blood in both sexes at 12 000 ppm (equal to 267.8 mg/kg bw per day) (Beck, 2012a).

In a 1-year study in dogs in which mandestrobin was administered at a dietary concentration of 0, 200, 800, 4000 or 8000 ppm (equal to 0, 4.3, 19.2, 92.0 and 180.7 mg/kg bw per day for males and 0, 4.5, 20.4, 92.0 and 225.7 mg/kg bw per day for females, respectively), the NOAEL was 800 ppm (equal to 19.2 mg/kg bw per day), based on increased relative liver weights, hepatocyte hypertrophy and hepatocyte pigment and disturbances to clinical biochemistry parameters (increased ALP, GGT and triglycerides) in males at 4000 ppm (equal to 92.0 mg/kg bw per day) (Beck, 2012b).

In an 18-month toxicity and carcinogenicity study in mice, mandestrobin was administered at a dietary concentration of 0, 700, 2000 or 7000 ppm (equal to 0, 82.5, 238.8 and 823.9 mg/kg bw per day for males and 0, 99.2, 280.3 and 994.0 mg/kg bw per day for females, respectively). The NOAEL for toxicity was 7000 ppm (equal to 823.9 mg/kg bw per day), the highest dose tested. No treatment-related increases in tumour incidence were observed in this study (Beck, 2012c).

In a 2-year toxicity and carcinogenicity study in rats, mandestrobin was administered at a dietary concentration of 0, 400, 2000, 7000 or 15 000 ppm (equal to 0, 21.0, 105.1, 375.6 and 804.3 mg/kg bw per day for males and 0, 26.7, 135.2, 475.0 and 1016.2 mg/kg bw per day for females, respectively). The NOAEL for toxicity was 400 ppm (equal to 26.7 mg/kg bw per day), based on effects on body weight and liver (histopathological changes and increased liver weights) in females at 2000 ppm (equal to 135.2 mg/kg bw per day). The NOAEL for carcinogenicity was 7000 ppm (equal to 375.6 mg/kg bw per day), based on an equivocal increase in the incidence of tumours (ovarian sex cord stromal adenomas and testicular interstitial cell adenomas) at the highest dose (Beck, 2012d).

Mandestrobin was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found (Kitamoto 2010a,b,c; Wollny, 2010).

The Meeting concluded that mandestrobin is unlikely to be genotoxic.

In view of the lack of genotoxicity, the absence of carcinogenicity in mice and the fact that only an equivocal increase in testicular interstitial cell and ovarian sex cord stromal adenomas was seen in rats at the highest dose tested, the Meeting concluded that mandestrobin is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation reproductive toxicity study in which rats were given mandestrobin at a dietary concentration of 0, 1000, 3000 or 10 000 ppm (equal to 0, 47.77, 145.7 and 511.7 mg/kg bw per day for males and 0, 65.68, 200.3 and 672.0 mg/kg bw per day for females, respectively), the NOAEL for parental toxicity was 1000 ppm (equal to 47.77 mg/kg bw per day), based on increased liver weights, diffuse liver hypertrophy, brown pigment in the bile duct/periportal area in both sexes in the F₁ generation and periductular inflammatory cell infiltration in F₁ females at 3000 ppm (equal to 145.7 mg/kg bw per day). The NOAEL for offspring toxicity was 1000 ppm (equal to 47.77 mg/kg bw per day), based on decreased spleen weights in F₁ male and F₂ female pups. The NOAEL for reproductive toxicity was 10 000 ppm (equal to 511.7 mg/kg bw per day), the highest dose tested (Matsuura, 2012).

In a developmental toxicity study in rats given mandestrobin by gavage at 0, 100, 300 or 1000 mg/kg bw per day from GDs 6 to 19, the NOAEL for maternal toxicity was 300 mg/kg bw per day, based on reduced feed consumption at 1000 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 300 mg/kg bw per day, based on an increase in a limited number of visceral and skeletal malformations at 1000 mg/kg bw per day (Rhodes, 2012a).

In a developmental toxicity study in rabbits given mandestrobin by gavage at 0, 100, 300 or 1000 mg/kg bw per day from GDs 7 to 28, the NOAELs for both maternal and embryo/fetal toxicity were 1000 mg/kg bw per day, the highest dose tested (Rhodes, 2012b).

The Meeting concluded that mandestrobin is teratogenic in rats, but not in rabbits.

In an acute neurotoxicity study, mandestrobin was given to rats by gavage at a dose of 0, 500, 1000 or 2000 mg/kg bw. The NOAEL for neurotoxicity was 1000 mg/kg bw, based on decreased overall locomotor activity (total and/or ambulatory counts) at 2000 mg/kg bw. The NOAEL for systemic toxicity was 2000 mg/kg bw, the highest dose tested (Herberth, 2011b).

In a 90-day neurotoxicity study in rats given mandestrobin at a dietary concentration of 0, 1500, 5000 or 15 000 ppm (equal to 0, 99, 338 and 1024 mg/kg bw per day for males and 0, 122, 415 and 1223 mg/kg bw per day for females, respectively), the NOAEL for systemic toxicity was 5000 ppm (equal to 338 mg/kg bw per day), based on transient decreases in body weight, body weight gain and feed consumption in males at 15 000 ppm (equal to 1024 mg/kg bw per day). The NOAEL for neurotoxicity was 15 000 ppm (equal to 1024 mg/kg bw per day), the highest dose tested (Herberth, 2012).

Although there were no indications of neuropathological effects of mandestrobin, the Meeting concluded that mandestrobin may cause transient, acute neurobehavioural effects at high doses.

In a 28-day immunotoxicity study in female rats given mandestrobin at a dietary concentration of 0, 1500, 5000 or 15 000 ppm (equal to 0, 147, 471 and 1419 mg/kg bw per day, respectively), the NOAEL for immunotoxicity (splenic AFC response) was 15 000 ppm (equal to 1419 mg/kg bw per day), the highest dose tested (Hosako, 2011b).

Two in vivo studies (one in rats, one in mice) to gain insight into the mechanistic basis of the liver and thyroid effects observed in the main studies in rats were provided (Asano, 2012e; Yamada, 2012b). The effects observed indicated a constitutive androstane receptor (CAR)-mediated induction of liver enzymes and subsequent perturbations of thyroid hormones, similar to a phenobarbital-like mode of action (Capen et al., 1999; Meek et al., 2003; Cohen et al., 2004).

In an in vitro non-GLP-compliant human estrogen and androgen receptor transactivation assay, mandestrobin and its metabolites (5-COOH-S-2200, 4-OH-S-2200, 5-CH₂OH-S-2200 and 5-CA-S-2200-NHM) did not show agonistic or antagonistic effects on human estrogen receptor alpha or human androgen receptor (Suzuki, 2012). In a second in vitro non-GLP-compliant study, a steroidogenesis assay in NCI-H295R cells, mandestrobin did not influence testosterone or estradiol production (Kubo, 2012).

No information on the potential effects of mandestrobin on the microbiome of the human gastrointestinal tract is available.

Toxicological data on metabolites

Metabolite 2-CH₂OH-S-2200 (free and conjugated)

The free form of 2-CH₂OH-S-2200 ((2RS)-2-[2-(5-hydroxymethyl-2-methylphenoxy)methyl]-phenyl]-2-methoxy-N-methylacetamide) is a rat (<1% of the applied dose in urine), hen, goat and plant metabolite. The conjugated form is also a plant and animal metabolite. The submitted studies were performed with the free form of the metabolite only.

The acute oral LD₅₀ of metabolite 2-CH₂OH-S-2200 in rats was greater than 2000 mg/kg bw (Asano, 2012c).

Metabolite 2-CH₂OH-S-2200 was tested in a gene mutation assay in bacteria. There was no evidence of mutagenicity (Kitamoto, 2012e).

The acute toxicity of the free and conjugated 2-CH₂OH-S-2200 metabolite is considered to be similar to that of the parent compound. For chronic toxicity, the threshold of toxicological concern (TTC) approach could be applied using Cramer class III.

Metabolite 4-OH-S-2200 (free and conjugated)

Both the free and conjugated forms of 4-OH-S-2200 ((2RS)-2-[2-(4-hydroxy-2,5-dimethylphenoxy)methyl]phenyl]-2-methoxy-N-methylacetamide) are rat (free form <1% of the applied dose in urine; conjugated form >30% of the applied dose in bile; present in plasma), hen, goat and plant metabolites. Submitted studies were performed with the free form of the metabolite only.

The acute oral LD₅₀ of metabolite 4-OH-S-2200 in rats was greater than 2000 mg/kg bw (Asano, 2012d).

Metabolite 4-OH-S-2200 was tested in a gene mutation assay in bacteria. There was no evidence of mutagenicity (Kitamoto, 2012f).

Based on the high levels in bile, the toxicity of the free and conjugated 4-OH-S-2200 metabolite is considered to be covered by the parent compound.

Metabolite De-Xy-S-2200

De-Xy-S-2200 ((2*RS*)-2-(2-hydroxymethylphenyl)-2-methoxy-*N*-methylacetamide) is a rat (<1% of the applied dose in urine; present in liver and kidney), hen, goat and plant metabolite.

The acute oral LD₅₀ of metabolite De-Xy-S-2200 in rats was greater than 2000 mg/kg bw (Asano, 2011).

Metabolite De-Xy-S-2200 was tested in a gene mutation assay in bacteria. There was no evidence of mutagenicity (Kitamoto, 2011).

The acute toxicity of metabolite De-Xy-S-2200 is considered to be similar to that of the parent compound. For chronic toxicity, the TTC approach could be applied using Cramer class III.

Metabolite 2-COOH-S-2200

2-COOH-S-2200 (2-({2-[(1*RS*)-1-methoxy-2-(methylamino)-2-oxoethyl]benzyl}oxy)-4-methylbenzoic acid) is a rat (<1% of the applied dose in urine and bile; present in plasma and kidney), hen and goat metabolite.

The acute oral LD₅₀ of metabolite 2-COOH-S-2200 in rats was greater than 2000 mg/kg bw (Asano, 2012a).

Metabolite 2-COOH-S-2200 was tested in an adequate range of in vitro and in vivo genotoxicity assays. Negative results were obtained in gene mutation assays (Wollny, 2011a; Kitamoto, 2012a) and an in vivo micronucleus assay (Tanaka, 2012). A weak positive response was seen in an in vitro chromosomal aberration assay (Kitamoto, 2012b).

The acute toxicity of metabolite 2-COOH-S-2200 is considered to be similar to that of the parent compound. For chronic toxicity, the TTC approach could be applied using Cramer class III.

Metabolite 5-COOH-S-2200

5-COOH-S-2200 (3-({2-[(1*RS*)-1-methoxy-2-(methylamino)-2-oxoethyl]benzyl}oxy)-4-methylbenzoic acid) is a rat (1.3% of the applied dose in urine and faeces; present in plasma, liver and kidney), plant, hen and goat metabolite.

The acute oral LD₅₀ of metabolite 5-COOH-S-2200 in rats was greater than 300 mg/kg bw and less than 2000 mg/kg bw (Asano, 2012b).

Metabolite 5-COOH-S-2200 was tested in an adequate range of in vitro genotoxicity assays. There was no evidence of genotoxicity (Wollny, 2011b; Kitamoto, 2012c,d).

The acute toxicity of metabolite 5-COOH-S-2200 is considered to be similar to that of the parent compound. For chronic toxicity, the TTC approach could be applied using Cramer class III.

Human data

In reports on manufacturing plant personnel, no adverse effects were noted (Nishioka, 2012; Nishimoto, 2017). No information on accidental or intentional poisoning in humans is available.

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

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) for mandestrobin of 0–0.2 mg/kg bw, based on a NOAEL of 19.2 mg/kg bw per day for increased liver weights, histopathological changes in the liver and associated disturbance of blood biochemistry parameters in a 1-year dog study and using a safety factor of 100. This ADI provides a margin of about 4000 relative to the lowest-observed-adverse-effect level (LOAEL) for equivocal carcinogenic effects in rats.

The Meeting established an acute reference dose (ARfD) of 3 mg/kg bw, based on a NOAEL of 300 mg/kg bw per day for malformations observed in a developmental toxicity study in rats and using a safety factor of 100. This ARfD is applicable to women of childbearing age only.

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

Levels relevant to risk assessment of mandestrobin

Species	Study	Effect	NOAEL	LOAEL
Mouse	Seventy-eight-week study of toxicity and carcinogenicity ^a	Toxicity	7 000 ppm, equal to 823.9 mg/kg bw per day ^b	–
		Carcinogenicity	7 000 ppm, equal to 823.9 mg/kg bw per day ^b	–
Rat	Two-year study of toxicity and carcinogenicity ^a	Toxicity	400 ppm, equal to 26.7 mg/kg bw per day	2 000 ppm, equal to 135.2 mg/kg bw per day
		Carcinogenicity	7 000 ppm, equal to 375.6 mg/kg bw per day	15 000 ppm, equal to 804.3 mg/kg bw per day ^c
	Two-generation study of reproductive toxicity ^a	Reproductive toxicity	10 000 ppm, equal to 511.7 mg/kg bw per day ^b	–
		Parental toxicity	1 000 ppm, equal to 47.77 mg/kg bw per day	3 000 ppm, equal to 145.7 mg/kg bw per day
		Offspring toxicity	1 000 ppm, equal to 47.77 mg/kg bw per day	3 000 ppm, equal to 145.7 mg/kg bw per day
	Developmental toxicity study ^d	Maternal toxicity	300 mg/kg bw per day	1 000 mg/kg bw per day
		Embryo and fetal toxicity	300 mg/kg bw per day	1 000 mg/kg bw per day
	Acute neurotoxicity study ^d	Neurotoxicity	1 000 mg/kg bw	2 000 mg/kg bw
Rabbit	Developmental toxicity study ^d	Maternal toxicity	1 000 mg/kg bw per day ^b	–
		Embryo and fetal toxicity	1 000 mg/kg bw per day ^b	–
Dog	Ninety-day toxicity study ^a	Toxicity	4 000 ppm, equal to 90.9 mg/kg bw per day	12 000 ppm, equal to 267.8 mg/kg bw per day
	One-year toxicity study ^a	Toxicity	800 ppm, equal to 19.2 mg/kg bw per day	4 000 ppm, equal to 92.0 mg/kg bw per day

^a Dietary administration.

^b Highest dose tested.

^c Based on an equivocal increase in the incidence of tumours.

^d Gavage administration.

Acceptable daily intake (ADI)

0–0.2 mg/kg bw

Acute reference dose (ARfD)

3 mg/kg bw (applies to women of childbearing age only)

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

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

Critical end-points for setting guidance values for exposure to mandestrobin*Absorption, distribution, excretion and metabolism in mammals*

Rate and extent of oral absorption	>95% based on urinary and biliary excretion within 24 hours
Dermal absorption	No information provided
Distribution	Extensively distributed throughout the body (mainly in gastrointestinal tract, liver and kidneys)
Potential for accumulation	Evidence of accumulation, but not persistence
Rate and extent of excretion	Faecal elimination, via the bile, was the primary route of elimination (~80%); urinary excretion up to ~20%
Metabolism in animals	Extensively metabolized to numerous metabolites; primary routes of metabolism are oxidation and subsequent conjugation with glucuronic acid, demethylation with subsequent oxidation, oxidation with subsequent demethylation
Toxicologically significant compounds in animals and plants	Mandestrobin

Acute toxicity

Rat, LD ₅₀ , oral	>2 000 mg/kg bw
Rat, LD ₅₀ , dermal	>2 000 mg/kg bw
Rat, LC ₅₀ , inhalation	>4.96 mg/L air (maximal attainable concentration)
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Mildly irritating
Guinea-pig, dermal sensitization	Not sensitizing (Magnusson & Kligman)

Short-term studies of toxicity

Target/critical effect	Liver and clinical chemistry
Lowest relevant oral NOAEL	19.2 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	1 000 mg/kg bw per day, highest dose tested (rat)
Lowest relevant inhalation NOAEC	Not available

Long-term studies of toxicity and carcinogenicity

Target/critical effect	Liver
Lowest relevant NOAEL	26.7 mg/kg bw per day (rat)

Carcinogenicity	Not carcinogenic in mice; equivocal increases in testicular interstitial cell and ovarian sex cord stromal adenomas in rats ^a
<i>Genotoxicity</i>	
	No evidence of genotoxicity ^a
<i>Reproductive toxicity</i>	
Target/critical effect	Parental: increased liver weights, diffuse liver hypertrophy, pigment in the bile duct and periductal inflammatory cell infiltration Offspring: lower spleen weights
Lowest relevant parental NOAEL	47.77 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	47.77 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	511.7 mg/kg bw per day, highest dose tested (rat)
<i>Developmental toxicity</i>	
Target/critical effect	Maternal effects: decreased feed consumption Developmental effects: visceral and skeletal malformations
Lowest relevant maternal NOAEL	300 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	300 mg/kg bw per day (rat)
<i>Neurotoxicity</i>	
Acute neurotoxicity NOAEL	1 000 mg/kg bw (rat)
Subchronic neurotoxicity NOAEL	1 024 mg/kg bw per day, highest dose tested (rat)
Developmental neurotoxicity NOAEL	Not available
<i>Other toxicological studies</i>	
Immunotoxicity NOAEL	1 419 mg/kg bw per day, highest dose tested (rat)
<i>Studies on toxicologically relevant metabolites</i>	
2-COOH-S-2200	Oral LD ₅₀ > 2 000 mg/kg bw Not genotoxic in vivo
5-COOH-S-2200	300 < LD ₅₀ < 2 000 mg/kg bw Not genotoxic in vitro
2-CH ₂ OH-S-2200	LD ₅₀ > 2 000 mg/kg bw Not genotoxic in vitro
4-OH-S-2200	LD ₅₀ > 2 000 mg/kg bw Not genotoxic in vitro
De-Xy-S-2200	LD ₅₀ > 2 000 mg/kg bw Not genotoxic in vitro
<i>Human data</i>	
	No detrimental health effects in manufacturing personnel

^a Unlikely to pose a carcinogenic risk to humans via exposure from the diet.

Summary

	Value	Study	Safety factor
ADI	0–0.2 mg/kg bw	One-year toxicity study in dogs	100
ARfD	3 mg/kg bw ^a	Developmental toxicity study in rats	100

^a Applies to women of childbearing age only.

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