

Volume 3

Annex B

Mandestrobin

B.6

Toxicology and metabolism

Table of contents

B.6.1.	Absorption, distribution, excretion and metabolism (toxicokinetics) (Annex IIA 5.1)	5
B.6.1.1	Oral ADME studies – single dose, oral route in rats	5
B.6.1.2	Oral ADME studies – second single dose, oral route in rats	17
B.6.1.3	Oral ADME studies – repeated dose, oral route in rats	23
B.6.1.4	Summary of ADME studies	33
B.6.2	Acute toxicity including irritancy and skin sensitisation (Annex IIA 5.2)	38
B.6.2.1	Acute oral toxicity	38
B.6.2.2	Acute percutaneous toxicity	39
B.6.2.3	Acute inhalation toxicity	40
B.6.2.4	Skin irritation	41
B.6.2.5	Eye irritation	41
B.6.2.6	Skin sensitisation	43
B.6.2.7	Potential/interactions of multiple active substances or products	45
B.6.2.8	Summary of acute toxicity	45
B.6.3	Short term toxicity (Annex IIA 5.3)	45
B.6.3.1	Oral 28-day toxicity (rodents)	45
B.6.3.2	Oral 90-day toxicity (rodents)	46
B.6.3.3	Oral 90-day toxicity (dog)	61
B.6.3.4	Oral 1-year toxicity (dog)	73
B.6.3.5	Repeated dose (28-day) inhalation toxicity (rodents)	82
B.6.3.6	Repeated dose (90-day) inhalation toxicity (rodents)	82
B.6.3.7	Repeated dose (28-day) percutaneous toxicity (rodents)	82
B.6.3.8	Repeated dose (90-day) percutaneous toxicity (rodents)	86
B.6.3.9	Immunotoxicity	86
B.6.3.10	Summary of short-term toxicity studies	95
B.6.4	Genotoxicity (Annex IIA 5.4)	97
B.6.4.1	<i>In vitro</i> assays	97
B.6.4.1.1	Bacterial assay for gene mutation	97
B.6.4.1.2	Test for clastogenicity in mammalian cells	99
B.6.4.1.3	Test for gene mutation in mammalian cells	102
B.6.4.2	<i>In vivo</i> assays	106
B.6.4.2.1	<i>In vivo</i> studies in somatic cells	106
B.6.4.2.2	<i>In vivo</i> studies in germ cells	108
B.6.4.3	Summary and overall conclusions of genotoxicity studies	108
B.6.5	Long term toxicity and carcinogenicity (Annex IIA 5.5)	109
B.6.5.1	Long term oral toxicity and carcinogenicity study in the rat	109
B.6.5.2	Carcinogenicity study in the mouse	124
B.6.5.3	Summary of long term toxicity/carcinogenicity studies	130
B.6.6	Reproductive toxicity (Annex IIA 5.6)	132
B.6.6.1	Multi-generation reproductive toxicity in the rat	132
B.6.6.2	Developmental toxicity studies	160
B.6.6.2.1	Teratogenicity test by the oral route in the rat	160
B.6.6.2.2	Teratogenicity test by the oral route in the rabbit	168
B.6.6.3	Summary of reproductive toxicity	177
B.6.7	Neurotoxicity (Annex IIA 5.7)	180
B.6.7.1	Acute neurotoxicity – rat	180
B.6.7.2	Delayed neurotoxicity following acute exposure	187
B.6.7.3	Subchronic neurotoxicity – rat 90-day	188
B.6.7.4	Postnatal developmental neurotoxicity	195

WARNING: This document forms part of the Confidentiality data package and should not be made available outside the package. Registration must not be granted on the basis of this document.

B.6.7.5	Summary on neurotoxicity	195
B.6.8	Further toxicological studies (Annex IIA 5.10)	196
B.6.8.1	Mechanistic studies.....	196
B.6.8.1.1	Liver and thyroid changes.....	196
B.6.8.1.2	Ovary issues	210
B.6.8.2	Summary of mechanistic studies	220
B.6.8.3	Toxicity studies on metabolites	221
B.6.8.4	Summary of toxicological studies on metabolites	252
B.6.8.5	Assessment of the relevance of metabolites in groundwater	253
B.6.9	Medical data and information (Annex IIA 5.9)	255
B.6.9.1	Report on medical surveillance on manufacturing plant personnel	255
B.6.9.2	Report on clinical cases and poisoning incidents	255
B.6.9.3	Observations on exposure of the general population and epidemiological studies	255
B.6.9.4	Clinical signs and symptoms of poisoning and details of clinical tests	255
B.6.9.5	First aid measures	255
B.6.9.6	Therapeutic regimes	255
B.6.9.7	Expected effects and duration of poisoning as a function of the type, level and duration of exposure or ingestion	255
B.6.9.8	Expected effects and duration of poisoning as a function of varying time periods between exposure or ingestion and commencement of treatment.....	256
B.6.10	Summary of mammalian toxicity and overall evaluation (Annex IIA 5.11)	256
B.6.10.1	Proposal for ADI.....	262
B.6.10.2	Proposal for ARfD	264
B.6.10.3	Proposal for drinking water limit.....	267
B.6.10.4	Proposal for AOEL	268
B.6.11	Acute toxicity of the preparations (Annex IIIA 7.1)	271
B.6.11.1	Acute oral toxicity	271
B.6.11.2	Acute percutaneous (dermal) toxicity	272
B.6.11.3	Acute inhalation toxicity to rats	273
B.6.11.4	Skin irritation	276
B.6.11.5	Eye irritation	277
B.6.11.6	Skin sensitisation	279
B.6.11.7	Summary of toxicity studies with the formulation.....	280
B.6.12	Dermal absorption (Annex IIIA 7.6)	281
B.6.12.1	Dermal absorption, <i>in vivo</i> in the rat	281
B.6.12.2	Comparative dermal absorption, <i>in vitro</i> using rat and human skin	281
B.6.12.3	Summary on dermal absorption.....	287
B.6.13	Toxicological data on non-active substances (Annex IIIA 7.9).....	288
B.6.13.1	Material safety data sheet for each formulant	288
B.6.13.2	Available toxicological data for each formulant	288
B.6.14	Exposure data (Annex IIIA 7.3, 7.4 and 7.5)	288
B.6.14.1	Operator exposure (Annex IIIA 7.3).....	288
B.6.14.1.1	Estimation of operator exposure assuming personal protective equipment is used	289
B.6.14.1.2	Measurement of operator exposure – (Mixer/Loader/Applicator)	289
B.6.14.1.3	Summary on operator exposure	289
B.6.14.2	Bystander exposure (Annex IIIA 7.4).....	289
B.6.14.2.1	Estimation of bystander exposure	289
B.6.14.2.2	Measurement of bystander exposure	290
B.6.14.2.3	Summary on bystander exposure.....	290
B.6.14.3	Worker exposure (Annex IIIA 7.5)	290
B.6.14.3.1	Estimation of worker exposure assuming personal protective equipment is not used	290

WARNING: This document forms part of an EU evaluation package and should not be read in isolation. Registration must not be granted on the basis of this document.

B.6.14.3.2	Estimation of worker exposure assuming personal protective equipment is used	291
B.6.14.3.3	Estimation of operator exposure assuming personal protective equipment is used and using data generated on dislodgeable residues under the proposed conditions of use	291
B.6.14.3.4	Measurement of worker exposure	291
B.6.14.3.5	Summary on worker exposure	291
B.6.15	Epidemiology (Annex IIIA 7.8)	292
Appendix 1:	Exposure models	293
Appendix 2:	Position papers	302
B.6.16	References relied on	355

WARNING: This document forms part of an EC evaluation data package and should not be read in isolation. Registration must not be granted on the basis of this document.

B.6.1. Absorption, distribution, excretion and metabolism (toxicokinetics) (Annex IIA 5.1)

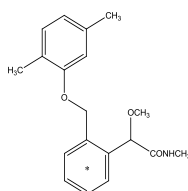
B.6.1.1 Oral ADME studies – single dose, oral route in rats

Reference:	Amended Final Report 1: [¹⁴ C]S-2200: Absorption, Distribution, Metabolism and Excretion following Single Oral Administration to the Rat
Author(s), year:	2012
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROM-0033
Guideline(s):	US EPA OPPTS 870.7485 (August 1998) Japanese MAFF (12-Nousan-No. 8147, Part 2-3-1) OECD 417 (April 1984).
GLP:	Yes (lab certified by National Authority)
Deviations:	Minor deviations not affecting the integrity of the study: humidity in animal room rose to 77% for one day; no microhaematocrit samples taken at study termination for dose groups D and E
Validity:	Yes

Material and methods:

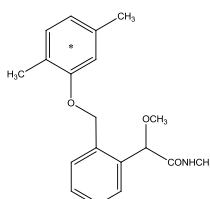
Test Material

Non-radiolabelled:	S-2200
Batch:	081103G
Purity:	100.0% (analytical standard)
Radiolabelled:	[benzyl- ¹⁴ C]S-2200
Batch:	CFQ40467 Batch B1
Specific activity:	4.55 GBq/mmol
Radiopurity:	98.9%
Vehicle:	0.5% aqueous methylcellulose



* = position of radiolabel

Radiolabelled:	[phenoxy- ¹⁴ C]S-2200
Batch:	CFQ40466 Batch B1
Specific activity:	4.44 GBq/mmol
Radiopurity:	99.8%
Vehicle:	0.5% aqueous methylcellulose



* = position of radiolabel

Test animals

Species:	Rat
Strain:	Hannover Wistar (CrI)
Source:	UK Home Office approved source
Number and sex:	59 males and 59 females
Age:	7-8 weeks at dosing
Body weight:	187-266 g (males), 146-234 g (females) at dosing

Eight groups (A-H) of male and female Wistar rats were administered a single high (1000 mg/kg) or low (5 mg/kg) oral dose of [benzyl-¹⁴C]S-2200 or [phenoxy-¹⁴C]S-2200 in 0.5% aqueous methylcellulose. The pharmacokinetic parameters of blood and plasma total radioactivity and the rates and routes of elimination were determined at both dose levels. The rates and routes of elimination were also determined in bile duct-cannulated animals after a dose of 5 mg/kg of [benzyl-¹⁴C]S-2200. The tissue distribution of radioactivity was examined over a time course of up to 168 hours post-dose.

Table B.6.1.1-1: Dose group assignment

Dose Group	Label position	Parameter(s) investigated	Dose level		Number of animals	
			(mg/kg)	(MBq/kg)	Males	Females
A	[benzyl- ¹⁴ C]	Excretion balance	5	5	4	4
B	[phenoxy- ¹⁴ C]		5	5	4	4
C	[benzyl- ¹⁴ C]		1000	5	4	4
D	[benzyl- ¹⁴ C]	Pharmacokinetics	5	10	4	4
E	[benzyl- ¹⁴ C]		1000	10	4	4
F	[benzyl- ¹⁴ C]	Biliary excretion	5	5	7*	7*
G	[benzyl- ¹⁴ C]	Tissue distribution	5	5	16	16
H	[benzyl- ¹⁴ C]		1000	5	16	16

*Additional animals underwent surgery to ensure that 4 animals per gender completed the experimental regime

Animal treatment, housing, and group assignment corresponded to the conditions specified in OECD-guideline 417. The animals were fed SQC Rat and Mouse Maintenance Diet No 1 *ad libitum* and had unlimited access to tap water.

All animals were examined twice daily. No signs of pharmacological or toxicological effects attributable to the test substance have been recorded throughout the study. Body weights were recorded the day after arrival, before dose administration (prior to surgery for biliary excretion analysis), and at necropsy where applicable.

Preparation of the dose solutions:

[benzyl-¹⁴C]- and [phenoxy-¹⁴C]S-2200 were re-purified prior to dosing by HPLC followed by Solid Phase Extraction cartridge chromatography.

To prepare dose suspensions, radiolabelled and non-radiolabelled S-2200 were weighed into dose vessels and the appropriate volume of acetonitrile added. For the higher concentration formulations, ethyl acetate was also added to aid dissolution. Once dissolution was achieved, solvents were evaporated under a gentle stream of nitrogen gas, and dose vehicle (0.5% w/v aqueous methylcellulose) was added to the dose vessel. The formulation was shaken for at least 1 hour, before being stirred continuously for approximately 88 hours. The stability of the formulations was assessed by determining the radiochemical purity of the formulated material before and after dosing using HPLC and was >99% pre and post dose in all groups. The ratios of the isomers within the formulations were also checked pre and post-dose. The homogeneity of the dose formulation and radioactivity concentrations of the formulated test compounds were determined by taking triplicate aliquots from each solution from the top, middle and bottom of the formulations vessels, where volume permitted, and subjected to Liquid Scintillation Counting (LSC) Variations were below 5% and the formulations were considered homogenous.

Sample analysis:

Samples were stored at <-10°C, with the exception of whole blood, which was stored at 2-8°C. Volumes and weights of all samples were measured where appropriate. Test compound solutions and formulations, plasma, bile, urine and cage washings were added directly to liquid scintillant prior to LSC. Tissues were homogenised in deionised water, solubilised (Solvable™) or digested (KOH/methanol solution under reflux) prior to LSC. Faeces, liver, kidney and cage debris were homogenised in deionised water, and solubilised, digested, or combusted as appropriate prior to LSC.

Findings:

Excretion balance investigation (Groups A, B, and C):

Following the administration of [¹⁴C]S-2200, four rats of each sex from each dose group were placed in individual glass metabolism cages suitable for the separate collection of urine and faeces. Urine and faecal collection vessels were cooled by solid carbon dioxide.

Urine was collected at the following time points following dose administration: 6, 12 and 24 hours and then at 24 hour intervals until 168 hours. Faeces were collected at 24 hour intervals until 168 hours following dose administration.

At each collection of excreta, cage debris was removed and the cages rinsed with water. Cage debris was pooled separately for each animal over the 168 hour collection period. Cage washings were retained separately per animal and time point. After the final water wash, the cages were washed with methanol which was added to the final water wash. Following the final sample collection, animals were exsanguinated by cardiac puncture under Isoflurane anaesthesia and 31 tissues including carcass, plasma, red blood cells and GIT were analysed for radioactivity. GIT contents were also analysed.

Table B.6.1.1-2: Cumulative recovery of radioactivity in excreta following a single oral administration of [benzyl-¹⁴C]- or [phenoxy-¹⁴C]S-2200 to rats (% administered)

Hours after dosing	5 mg/kg [benzyl- ¹⁴ C]		5 mg/kg [phenoxy- ¹⁴ C]		1000 mg/kg [benzyl- ¹⁴ C]	
	Males	Females	Males	Females	Males	Females
Urine						
0-6	5.767	5.320	6.645	5.270	1.642	1.611
0-12	9.541	8.516	8.728	10.54	4.491	4.501
0-24	12.97	14.55	11.77	16.60	10.41	11.14
0-48	15.20	18.57	13.95	19.25	15.68	15.36
0-72	16.48	19.70	14.79	20.48	16.64	16.44
0-96	17.13	20.23	15.21	20.97	16.97	16.85
0-120	17.57	20.40	15.48	21.25	17.08	17.01
0-144	17.83	20.53	15.65	21.40	17.15	17.11
0-168	17.97	20.61	15.77	21.47	17.19	17.18
Subtotal	17.97	20.61	15.77	21.47	17.19	17.18
Faeces						
0-24	34.40	33.03	33.57	32.56	27.15	32.99
0-48	55.28	58.17	59.23	53.82	62.63	65.43
0-72	61.78	64.46	66.74	59.75	70.38	69.22
0-96	67.19	68.49	70.29	62.83	73.33	71.08
0-120	70.09	69.99	72.02	64.47	74.16	71.60
0-144	71.69	70.76	73.21	65.80	74.57	71.83
0-168	72.88	71.26	73.90	66.45	74.77	71.96
Subtotal	72.88	71.26	73.90	66.45	74.77	71.96

Cage wash						
0-24	1.997	2.955	1.734	3.535	1.430	3.427
0-48	3.008	4.342	2.677	4.899	2.681	5.265
0-72	3.869	5.124	3.214	5.558	3.202	6.040
0-96	4.321	5.405	3.513	5.966	3.343	6.318
0-120	4.636	5.586	3.733	6.179	3.433	6.471
0-144	4.755	5.693	3.849	6.309	3.500	6.585
0-168	4.879	5.994	3.981	6.513	3.647	6.739
Subtotal	4.879	5.994	3.981	6.513	3.647	6.739
Cage debris						
0-168	0.282	0.069	0.172	0.038	0.048	0.117
Final cage wash						
168	0.150	0.108	0.071	0.087	0.082	0.357
Tissues and carcass						
168	2.129	0.974	1.687	1.311	1.222	0.702
Total	98.28	99.02	95.58	95.87	96.96	97.05

Recoveries of radioactivity were complete at 168 hours (95.6 - 99%) in all dose groups.

After 168 hours, only 0.7 - 2.1% radioactivity remained in carcass and tissues.

The majority of radioactivity was eliminated 48 hours post dose (>75%), with the faeces accounting for approximately three quarters of the radioactivity administered.

There were no gender related differences in rates and routes of excretion in either the high or low dose groups, and no significant differences between the radiolabels were observed.

Biliary excretion (Group F):

Surgery was performed at least 48 hours prior to dose administration.

The animals were placed into individual all glass metabolism cages suitable for the separate collection of bile, urine and faeces cooled by solid carbon dioxide. Bile was collected separately for each animal, and the bile flow calculated. Only animals deemed to have recovered from the effects of surgery, and whose behaviour and bile flow were normal (typically 0.5-1.0 mL/hour for a 250 g rat) were dosed.

Following administration of [¹⁴C]S-2200, animals were returned to their metabolism cages.

The following samples were collected at the stated times:

bile: pre-dose and 0-6, 6-12, 12-24, 24-48 and 48-72 hours following dose administration

urine and faeces: 0-24, 24-48 and 48-72 hours following dose administration

At each collection of faeces, cage debris was removed and the cages were rinsed with water. The aqueous cage washings were retained separately for each animal and time point. Cage debris was pooled for each animal over the entire collection period. After the final water wash, the cages were washed with methanol which was added to the final water wash.

Table B.6.1.1-3: Cumulative recovery of radioactivity in excreta of bile duct cannulated rats following a single oral administration of [benzyl-¹⁴C] S-2200 (% administered)

Hours after dosing	5 mg/kg [benzyl- ¹⁴ C]	
	Males	Females
Urine		
0-24	17.42	13.30
0-48	17.84	13.57
0-72	17.97	13.65
Subtotal	17.97	13.65
Faeces		

0-24	1.087	1.539
0-48	1.357	1.887
0-72	1.425	1.924
Subtotal	1.425	1.924
Cage wash		
0-24	0.790	0.993
0-48	1.108	1.232
0-72	1.392	1.354
Subtotal	1.392	1.354
Bile		
Pre-dose	--	--
0-6	76.75	76.42
0-12	79.24	80.34
0-24	79.58	81.41
0-48	79.71	81.91
0-72	79.73	81.93
Subtotal	79.73	81.93
Cage debris		
0-168	0.036	0.068
Final cage wash		
168	0.157	0.264
Carcass		
168	0.262	0.388
Total	101.0	99.58

Recoveries of radioactivity were complete at 168 hours, and only minimal amounts of radioactivity were detected in the carcass at terminal sacrifice.

Absorption calculated from urine and bile was >95% of the administered dose.

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 total radioactivity.

Elimination via urine was comparable to non-bile duct cannulated animals.

Elimination was substantially more rapid than in non-surgically altered animals, where it was apparently slowed by enterohepatic recirculation.

Pharmacokinetic investigation (Groups D and E):

Following the administration of 5 or 1000 mg/kg bw [benzyl-¹⁴C]S-2200, four rats of each sex from each dose group were returned to their holding cages. Two samples of approximately 75 µL of blood were taken from a lateral tail vein into heparinised microhaematocrit tubes.

Samples were taken 0.5, 1, 2, 4, 6, 9, 12, 24, 48, 72, 96 and 120 hours after administration.

One sample was analysed to determine the radioactivity content in the blood, the remaining sample was centrifuged to prepare plasma for analysis. At the last blood sampling time (120 hours after administration), animals were exsanguinated by cardiac puncture under Isoflurane anaesthesia followed by cervical dislocation. The carcasses were discarded.

Table B.6.1.1-4: Concentrations of radioactivity (mean ng equivalents/g) in blood and plasma

Time (hr)	[benzyl- ¹⁴ C]-S2200							
	5 mg/kg bw				1000 mg/kg bw			
	Males		Females		Males		Females	
	Blood	Plasma	Blood	Plasma	Blood	Plasma	Blood	Plasma
0.5	307	501	393	667	33040	49820	23340	36090
1	369	589	340	553	26950	37970	20000	28240
2	451	721	402	687	19490	25630	9916	15180
4	441	723	252	395	38710	60550	18210	27970
6	238	537	312	362	47410	61840	23320	33320
9	171	249	244	371	39110	53080	21180	31800
12	164	261	239	342	42420	59340	31400	45700
24	139	206	177	238	17230	23280	17430	24750
48	92	138	63	81	4581	5852	5373	6361
72	29	49	29	31	2653	2616	2749	2345
96	33	25	55	15	1785	1462	1996	1342
120	13	12	9	6	1430	1009	1525	951

Table B.6.1.1-5: Mean pharmacokinetic parameters (radioactivity in plasma)

Pharmacokinetic parameters	5 mg/kg bw [benzyl- ¹⁴ C]-S2200		1000 mg/kg bw [benzyl- ¹⁴ C]-S2200	
	Males	Females	Males	Females
t _{1/2} (hours)	22.52	18.32	24.51	29.40
T _{max} (hours)	2.625	1.250	7.000	9.125
C _{max} (ng eq/g)	841.7	829.3	68990	49220
AUC ₀₋₁₂₀ (h.ng eq/g)	15640	13920	1541000	1262000
AUC _{0-∞} (h.ng eq/g)	16020	14080	1577000	1303000
AUC ₀₋₁₂₀ as a % of AUC _{0-∞}	98	99	98	97

t_(1/2) = Kinetic half-life, calculated from the linear terminal portion of the individual time curves when plotted semi-logarithmically

T_{max} = Time to maximum concentration

C_{max} = Maximum concentration in blood or plasma

AUC = Area under the Curve, calculated by the log/linear trapezoid Rule

For both doses, there were no discernible gender differences in the pharmacokinetic parameters.

Mean plasma terminal elimination half-lives were approximately 20 hours (low dose) and 27 hours (high dose).

At the low dose, C_{max} was observed within 3 hours of dosing (individual animal T_{max} values ranged from 0.5 to 4 hours). At the high dose, C_{max} observed within 10 hours post-dose (individual animal T_{max} values ranged from 0.5 to 12 hours).

At the high dose, the pharmacokinetic profiles contained a region of multiple maxima up to 12 hours post-dose. The multiple maxima could possibly reflect enterohepatic recirculation, which is supported by the enhanced rapidity of elimination in biliary-cannulated animals.

Systemic exposure (AUC) to S-2200 derived radioactivity was ca. 90- to 100-fold higher in male and female animals receiving the 1000 mg/kg dose level, compared to animals receiving the 5 mg/kg dose group. As such, exposure was sub-proportional, indicating that saturation of absorption processes may also have occurred.

At the terminal time point, plasma radioactivity concentrations were ≤ 2% of the concentrations observed at C_{max}, indicating that at these later time points the vast majority of radioactivity had been cleared from the circulation.

Tissue Distribution (Groups G and H, and terminal timepoint Groups A, B, and C):

Following the administration of [¹⁴C]S-2200, sixteen rats of each sex from each dose group were returned to their holding cages.

At 0.5, 2, 24 and 72 hours post-dose (dose Group G) and at 2, 8, 36 and 72 hours post-dose (dose Group H) four animals per sex from each dose group were exsanguinated by cardiac puncture under Isoflurane anaesthesia) and 31 tissues including carcass, plasma, red blood cells and GIT were analysed for radioactivity. GIT contents were also analysed.

Table B.6.1.1-6: Tissue distribution of radioactivity (mean ng equivalents/g), low dose groups

Dose	5 mg/kg bw [benzyl- ¹⁴ C]-S2200										5 mg/kg bw [phenoxy- ¹⁴ C]-S2200	
Timepoint (hours)	0.5		2		24		72		168		168	
Tissue	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Adrenals	551	391	432	384	38	33	8	ND	21	7	8	ND
Blood	534	501	461	264	53	47	13	3	10	3	9	3
Bone	55	44	46	28	8	5	ND	ND	ND	3	ND	ND
Bone marrow	234	217	151	52	ND	ND	ND	ND	ND	ND	ND	ND
Brain	68	45	36	19	12	9	ND	ND	ND	ND	ND	ND
Eyes	78	54	66	36	14	12	7	3	3	1	1	10
Fat	211	153	1255	982	198	196	35	23	50	19	21	31
Hair & Skin	211	209	162	108	29	25	7	ND	8	3	14	8
Heart	329	246	221	108	31	25	2	ND	2	ND	2	2
Kidney	2954	2441	1138	772	142	130	43	16	34	14	35	19
Liver	9859	10730	4927	3527	733	718	217	132	147	98	130	92
Lung	400	335	336	157	41	37	10	ND	9	ND	6	4
Mandibular gland	237	205	157	89	25	20	3	ND	2	ND	3	2
Muscle (quad.)	137	99	175	92	23	21	2	ND	ND	ND	2	ND
Ovaries	NA	432	NA	1626	NA	231	NA	39	NA	32	NA	46
Pancreas	521	633	1103	1324	500	469	164	53	61	20	7	30
Pituitary	415	318	169	26	ND	ND	ND	ND	ND	ND	ND	ND
Plasma	793	801	720	434	76	69	16	3	11	2	9	4
Red Blood Cells	246	178	190	63	19	14	7	2	9	6	8	2
Sciatic nerve	244	130	218	118	49	36	7	ND	ND	ND	ND	ND
Spinal cord	76	137	81	26	9	ND	ND	ND	ND	ND	ND	ND
Spleen	286	394	299	364	89	93	18	8	19	5	7	44
Testes	91	NA	109	NA	23	NA	2	NA	ND	NA	ND	NA
Thymus	192	136	149	66	33	18	2	ND	ND	ND	2	6
Thyroid	321	407	277	150	47	15	25	ND	23	ND	ND	ND
Uterus	NA	616	NA	3347	NA	463	NA	72	NA	62	NA	26
Gastrointestinal tract												
Caecum	716	1298	9661	39150	9665	8757	980	380	247	103	300	177

Caecum contents	130	146	5408	35440	15190	15110	1856	601	358	132	456	146
Large intestine contents	152	364	1429	8670	8807	13350	1225	371	295	105	312	190
Large intestine	845	1300	6239	9721	3170	5467	473	174	198	62	162	110
Small intestine contents	44510	51630	49940	65170	9806	12140	2977	1193	2050	12	1241	1134
Small intestine	22510	29270	41230	33160	4062	4412	1404	392	775	624	638	526
Stomach	30700	44680	23190	11950	588	430	106	53	55	41	55	58
Stomach contents	100600	149100	31180	17650	1296	438	18	51	118	98	41	114

NA: not available/not applicable

ND: not detected

Table B.6.1.1-7: Tissue distribution of radioactivity (mean ng equivalents/g), high dose groups

Dose	1000 mg/kg bw [benzyl- ¹⁴ C]-S2200									
Timepoint (hours)	2		8		36		72		168	
Tissue	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Adrenals	22510	23160	37430	53470	5596	12400	ND	1396	ND	ND
Blood	21720	10620	37680	28950	7097	6610	1349	2558	732	1352
Bone	ND	ND	24110	19680	ND	ND	ND	ND	ND	ND
Bone marrow	2561	1534	7455	3828	343	ND	ND	ND	ND	ND
Brain	4047	4769	7319	12990	421	ND	ND	ND	ND	ND
Eyes	4273	2924	8223	7625	2221	1606	ND	ND	ND	ND
Fat	48000	45660	54320	94910	15470	14120	2024	ND	593	ND
Hair & Skin	9172	11220	23810	45140	5363	5649	2475	3539	522	ND
Heart	15400	9759	26090	24930	3689	3114	ND	ND	762	ND
Kidney	54460	31810	72380	71160	19320	17560	4064	4050	2627	1458
Liver	106300	100000	164600	173100	74790	70290	20620	22570	9033	7144
Lung	23840	21370	164600	25990	4901	4768	ND	528	644	ND
Mandibular gland	11220	9132	164600	26830	2941	2453	ND	ND	ND	ND
Muscle (quad.)	6995	4365	164600	13670	1431	1517	ND	ND	ND	ND
Ovaries	NA	82630	164600	64090	NA	14570	NA	2068	NA	874
Pancreas	50010	46670	164600	54020	22910	46910	3540	4098	1561	1225
Pituitary	ND	ND	164600	15150	ND	ND	ND	ND	ND	ND
Plasma	30740	17100	164600	44810	9682	9183	1627	2164	170	ND
Red Blood Cells	15910	6616	164600	11550	4030	4116	2009	3360	1910	2712

Sciatic nerve	15080	18600	164600	27410	3698	722	ND	ND	ND	ND
Spinal cord	4886	5491	164600	17800	ND	ND	ND	ND	ND	ND
Spleen	13940	16170	164600	21510	6370	6511	ND	734	607	ND
Testes	6711	NA	164600	NA	1764	NA	ND	NA	ND	NA
Thymus	7764	5756	164600	15600	2058	1352	ND	ND	ND	ND
Thyroid	14550	6778	164600	29830	ND	1984	ND	ND	ND	NA
Uterus	NA	57660	164600	61190	NA	26630	NA	3447	NA	982
Gastrointestinal tract										
Caecum	536300	445500	2500000	1819000	869200	773000	52480	108400	5547	5494
Caecum contents	2017000	4211000	6516000	7903000	2172000	1928000	124200	145700	8387	8775
Large intestine contents	84290	918900	6719000	13460000	1868000	1981000	73190	112100	7380	6664
Large intestine	160700	1252000	1523000	1365000	541200	492500	28260	48960	4179	2994
Stomach	3637000	1606000	3011000	3273000	21100	75260	3131	15510	1093	1780
Stomach contents	14740000	14880000	12020000	12770000	13060	153200	2376	67410	3194	655
Small intestine	820600	773200	1556000	926300	281100	306100	84880	69170	17840	8373
Small intestine contents	5969000	1693000	4073000	4884000	920000	959800	196900	180900	39380	23880

NA: not available/ not applicable

ND: not detected

Radioactivity distribution was similar at high and low doses.

At the first two timepoints (0.5 and two hours post-dose for the low dose and 2 and 8 hours post-dose for the high dose groups), the majority of radioactivity was detected in the upper half of the GIT. Transit through the GIT was evident over time, and at the terminal timepoint, only about 1% of total radioactivity was associated with tissue and carcass.

Excluding the components of the GIT, the greatest concentrations of radioactivity were measured in liver and kidneys (up to 9 and 0.4% of administered radioactivity). Notable amounts of radioactivity were also detected in fat, muscle, pancreas, ovaries and uterus.

Plasma:tissue radioactivity ratios were generally below one, excepting liver, kidney, fat, spleen, pancreas, and ovaries and uterus in female animals.

Metabolite profiling:

Samples from dose groups A-C and F-H were pooled per time point, sex, and dose group by taking equal percentage sample weights (typically about 10%).

Metabolites were determined in urine, faeces, liver, kidney, plasma and bile.

Urine and bile samples were centrifuged, and aliquots of the supernatant taken to check recoveries. The supernatants were analysed by radio HPLC.

Faeces, liver, kidney, and plasma samples were extracted three times with acetonitrile or acetonitrile/water, with sequential vortex mixing and centrifugation. Extracts were analysed by radio HPLC.

Retention times of radiolabelled metabolites were compared with those of authentic standards. LC-MS was also used to confirm metabolite identity.

Urine (Group C) and bile (Group F) from female animals indicated the presence of potential hydroxylated glucuronide metabolites. Enzyme deconjugation using β -glucuronidase (*Helix pomatia*) was conducted to identify conjugated metabolites. This enzyme was chosen since it has both glucuronidase and sulphatase activity. Inhibition of *H. pomatia* β -glucuronidase (inhibited by D-saccharo-1,4-lactone, providing sulphatase activity only) was also investigated to examine the presence of any sulphate conjugates within the samples. Following enzyme deconjugation, samples were examined for evidence of metabolism using HPLC with radiodetection.

S-2200 was shown to undergo extensive metabolism. Unchanged parent was found in faeces only at <0.2% of administered dose at low dose and <6% at high dose. No major gender differences were observed, and no major differences in the profiles from intact and bile duct-cannulated animals were apparent. Comparison between the profiles generated following administration with the benzyl or phenoxy labels also indicated that little cleavage occurred between the two rings in the molecules. The majority of ADME work was therefore appropriately conducted using only [benzyl-¹⁴C]S-2200. The primary routes of metabolism of S-2200 were by oxidation and subsequent conjugation with glucuronic acid, demethylation with subsequent oxidation, or oxidation with subsequent demethylation.

The metabolite profiles in urine, faeces, and bile are summarised in table B.6.1.1-8:

In the liver, three metabolites dominated the profiles at both dose levels: 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-S2200-NDM were detected.

Table B.6.1.1-8: Quantification of metabolites (%dose administered) in pooled excreta

Label	[benzyl- ¹⁴ C]												[phenoxy- ¹⁴ C]			
Dose level	5 mg/kg								1000 mg/kg				5 mg/kg			
Matrix	Urine (Group A, non-cannulated)		Urine (Group F, cannulated)		Bile (Group F, cannulated)		Faeces (Group A, non-cannulated)		Urine (Group C)		Faeces (Group C)		Urine (Group B)		Faeces (Group B)	
Sex	Male 48 hr	Female 48 hr	Male 24 hr	Female 24 hr	Male 12 hr	Female 24 hr	Male 96 hr	Female 72 hr	Male 48 hr	Female 48 hr	Male 72 hr	Female 48 hr	Male 48 hr	Female 48 hr	Male 72 hr	Female 72 hr
Metabolite Identity¹																
S-2200							0.093	0.139			3.915	5.489			0.081	
5-CA-S-2200-NHM	3.578	5.695	4.756	4.929	6.857	3.432	19.866	16.566	4.154	4.353	11.843	8.559	2.944	5.478	18.114	15.275
4-OH-S-2200		0.026					9.474	18.213			10.449	12.592			7.092	16.474
4-OH-S-2200-GlucA	0.084	0.010			33.784	36.670				0.105			0.016	0.032		
COOH-S-2200-Glucuronides					20.616	23.757										
5-COOH-S-2200	1.259	3.254			1.627	3.380	7.417	8.315	1.779	4.428	10.735	8.712	0.636	2.632	8.054	6.792
5-CA-2-HM-S-2200	0.849	0.653	0.923	0.730	0.102	1.398	4.864	3.974	1.582	0.601	4.739	5.043	0.897	0.681	4.743	4.443
5-CA-2-HM-S-2200-NHM	0.885	0.550	1.590	0.839	1.667	0.489	6.485	0.683	0.607	0.181	2.121	1.510	0.878	0.595	6.372	2.877
De-Xy-S-2200	0.122	0.188					1.731	2.754	0.181	0.149	3.290	4.435				0.011
2-COOH-S-2200	0.330	0.641	0.540	0.222			0.777	0.230	0.580	0.286		0.775	0.356	0.856	0.062	0.597
5-CH ₂ OH-S-2200	0.003		0.275	0.126			0.212	0.168		0.033		1.138			0.920	0.364
2-CH ₂ OH-S-2200	0.024	0.012					0.153					0.367			0.141	
5-CA-MCBX-NDM	1.791	1.769	3.055	1.577			0.086	0.064	0.265	0.230	0.957	0.438	1.616	2.186	2.088	0.115
5-CA-S-2200-NDM	0.377	0.595	0.688	0.636	0.037	0.947		0.092	0.188	0.363			0.462	0.741	0.265	
5-CA-2-HM-MCBX	0.873	0.328	0.629	0.141			0.039		0.870	0.281	0.527		1.164	0.338	0.287	0.140
DX-CA-S-2200									0.127							
5-CA-2-HM-MCBX(-2H)	0.070	0.091							0.785	0.088			0.040			
MCBX											0.040					
% Dose identified	10.245	13.812	12.456	9.200	64.690	70.073	51.197	51.198	11.118	11.098	48.616	49.058	8.969	13.579	48.219	47.088

WARNING: This document forms part of an EC evaluation data package and should not be read in isolation. Registrants must not be granted the basis of this document.

% Dose detected	15.198	18.565	17.420	13.300	79.240	81.409	67.186	64.457	15.680	15.359	70.387	65.430	13.944	19.253	66.741	59.747
-----------------	--------	--------	--------	--------	--------	--------	--------	--------	--------	--------	--------	--------	--------	--------	--------	--------

Blank: not detected or <LOQ

WARNING: This document forms part of an EC evaluation data package and should not be read in isolation. Registration must not be granted on the basis of this document.

Conclusion:

Following a single oral high or low dose administration, [¹⁴C]S-2200 was rapidly absorbed and widely distributed throughout the body. Absorption of S-2200, calculated from the radioactivity recovered in urine and bile, was greater than 90% of the administered dose at 5 mg/kg bw. At the high dose systemic exposure to S-2200 derived radioactivity was sub-proportional, indicating saturation of absorption processes following administration. Secondary absorption phases following administration of 1000 mg/kg doses of S-2200 indicated that systemic exposure at high concentrations may have resulted from one or more of the following: (a) enterohepatic recirculation, (b) differential absorption during transit through the gastro-intestinal tract, or (c) transient solubility of the S-2200 at 200 mg/mL. There did not appear to be any major gender differences in the pharmacokinetics of S-2200 at 5 or 1000 mg/kg bodyweight. There was no major difference in distribution between high and low doses or between sexes. The major tissue residues were seen in the GI tract, and in liver and kidney. Tissue distribution of radioactivity was similar in animals administered [benzyl-¹⁴C]S-2200 or [phenoxy-¹⁴C]S-2200 indicating that the core of the molecule was stable during systemic exposure. Clearance from the plasma was almost complete by 120 hours post-administration after 5 and 1000 mg/kg bodyweight doses. Faecal elimination was the primary route of elimination of radioactivity, primarily via the bile. However, renal elimination was also important for the excretion of metabolites. There did not appear to be any gender-, dose- or radiolabel related differences in either the rates or routes of excretion. S-2200 was extensively metabolised. The primary routes of metabolism were by oxidation and subsequent conjugation with glucuronic acid, demethylation with subsequent oxidation, or oxidation with subsequent demethylation. Metabolite fractions in plasma, liver and kidney were identified. There was no discernible shift in radiolabel distribution between low and high doses.

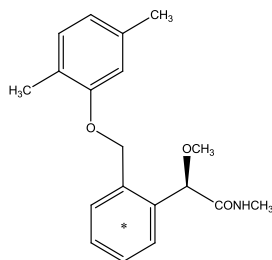
B.6.1.2 Oral ADME studies – second single dose, oral route in rats

Reference:	Metabolism of S-2200 R-isomer (S-2167) and S-2200 S-isomer (S-2354) in Rats.
Author(s), year:	██████████ 2011
Report/Doc. number:	Sumitomo Chemical Co. Ltd. Report No. ROM-0021
Guideline(s):	US EPA OPPTS 870.7485 (August 1998), Japanese MAFF (12-Nousan-No. 8147, Part 2-3-1), and OECD 417 (April 1984).
GLP:	Yes (lab certified by National Authority)
Deviations:	Minor deviations not expected to compromise the reliability of the study were listed in the study report
Validity:	Yes

Material and methods:

Test Material

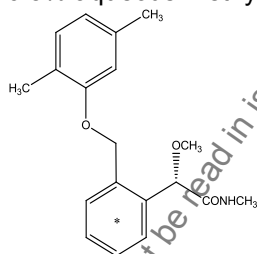
Non-radiolabelled:	S-2200 (<i>R</i> -isomer), also known as S-2167
Batch:	060020652 (Lot No.)
Purity:	100.0% (analytical standard)
Radiolabelled:	[benzyl- ¹⁴ C]S-2200 (<i>R</i> -isomer)
Batch:	RIS2008-010 (Lot No.)
Specific activity:	4.22 GBq/mmol
Radio purity:	99.2%
Vehicle:	0.5% aqueous methylcellulose



* = position of radiolabel

Non-radiolabelled: S-2200 (S-isomer), also known as S-2354
Batch: 060020653 (Lot No.)
Purity: 99.7% (analytical standard)

Radiolabelled: [benzyl-¹⁴C]S-2200 (S-isomer)
Batch: RIS2008-009 (Lot No.)
Specific activity: 4.22 GBq/mmol
Radiopurity: 99.4%
Vehicle: 0.5% aqueous methylcellulose



* = position of radiolabel

Test animals

Species: Rat
Strain: Slc Wistar Hannover/RCC
Source: [REDACTED]
Number and sex: 16 animals (4 males and 4 females per dose and time-point)
Age: 7 weeks old
Body weight: 223-253g (males), 159-188g (females)

The study was designed to determine the rates and routes of excretion and the metabolic reactions of the [benzyl-¹⁴C]-labelled S and R isomer of S-2200 after a single application.

Table B.6.1.2-1: Study design for the excretion, tissue distribution, and metabolism study of the S-2200 R- and S- isomer in rats

Group	Study Item	Dose	Number of rats		Sampling times (hr)
		(mg/kg)	Males	Females	
A (S-2200 R-isomer)	Excretion, Tissue distribution, Metabolite profiling	5	4	4	Faeces and urine collected at 6, 12, 24, 48, 72, 96, 120 and 168 hours. At 168 hours, rats were sacrificed and tissues and organs were collected for radioactivity analysis.
B (S-2200 S-isomer)		5	4	4	

Animals were housed three per cage in polypropylene cages with saw dust bedding. After test article administration, rats were housed individually in glass metabolism cages. There was a 12 hour light/dark photoperiod, temperature was 23±2°C, humidity was 55±15%; and there were at least 10 air changes per hour. Animals were fed with pelleted CRF-1 diet (Oriental Yeast Co., Ltd, Japan) and tap water *ad libitum*.

Dose suspensions: Both isomers were mixed with non-radioactive material to a specific activity of 1.00 MBq/mg in acetonitrile. After evaporation under a gentle stream of nitrogen, the residue was suspended in 0.5% aqueous methyl cellulose at a concentration of 1 mg/mL. Dosing suspensions were analysed (three aliquots) measured in duplicate by Liquid Scintillation counting for concentration, specific activity and homogeneity, and were then stored frozen. Radiochemical purity was confirmed after dosing by RI HPLC.

Urine and cage wash samples were radioassayed by liquid scintillation counting. Faecal samples were homogenised and then combusted for radioassay. Trapped CO₂ was radioassayed by liquid scintillation counting. Whole blood, blood cells, plasma and tissues were analysed by combustion radioanalysis. Carcasses were dissolved in 3M potassium hydroxide. The mass balance was determined. Identification of metabolites was performed by co-HPLC with known standards (UV absorption retention time vs that from the RI chromatogram) and confirmed using LC-MS.

Findings:

No behavioural or toxicological abnormalities were observed in any rat.

Absorption/ Elimination:

Both isomers were absorbed immediately after administration and metabolised almost completely (parent materials detected by LC-MS but below LOQ).

Total recoveries after seven days were > 95% with both isomers and both sexes.

The *R*-isomer (Group A) was almost completely excreted after three days. Elimination of the *S*-isomer (Group B) was slower and reached around 95% after five days.

Both isomers were eliminated mainly via faeces, about 15 to 30% of radioactivity was eliminated via urine.

¹⁴C-Excretion into expired air was negligible with both isomers and both sexes.

Table B.6.1.2-2: Cumulative elimination of radioactivity in rats after a single low dose of radiolabelled isomers of S-2200

Time (hr)	¹⁴ C]S-2200 <i>R</i> -isomer						¹⁴ C]S-2200 <i>S</i> -isomer					
	Males			Females			Males			Females		
	Urine	Faeces	Total	Urine	Faeces	Total	Urine	Faeces	Total	Urine	Faeces	Total
0-6	11.2		11.2	18.9		18.9	4.7		4.7	9.1		9.1
0-12	14.4		14.4	23.6		23.6	8.2		8.2	14.2		14.2
0-24	19.1	54.8	73.9	29.0	42.8	71.8	11.8	41.8	53.6	19.2	16.9	36.1
0-48	21.0	70.2	91.2	31.4	59.2	90.6	14.0	62.8	76.7	22.7	50.5	73.2
0-72	21.7	73.4	95.1	32.3	62.8	95.1	14.9	71.9	86.8	24.2	64.1	88.2
0-96	22.0	74.7	96.7	32.7	63.9	96.5	15.4	76.2	91.6	24.8	69.7	94.6
0-120	22.2	75.3	97.4	32.8	64.2	97.0	15.6	78.6	94.2	25.1	71.5	96.6
0-168	22.3	75.7	97.9	32.9	64.5	97.4	15.8	80.6	96.4	25.4	73.3	98.7

Tissue distribution:

Seven days after administration, tissue radioactivity was generally low (total radioactivity < 1%). Residuals were slightly higher in group B (*S*-isomer), reflecting the slower elimination. Otherwise, no isomer-specific distribution patterns were observed.

Table B.6.1.2-3: Tissue distribution of radioactivity (µg S-2200 equivalents/g tissue) 7 days after administration

	¹⁴ C]S-2200 <i>R</i> -isomer		¹⁴ C]S-2200 <i>S</i> -isomer	
	Males	Females	Males	Females
Adrenals	<0.015	<0.014	<0.013	<0.013
Whole blood	0.004	<0.003	<0.003	0.006

Blood cells	0.008	0.005	0.005	0.008
Plasma	<0.003	<0.003	<0.003	<0.003
Bone	<0.003	<0.003	<0.003	<0.003
Bone marrow	<0.007	<0.009	<0.007	<0.008
Brain	<0.003	<0.005	<0.003	<0.003
Caecum	0.016	0.019	0.189	0.168
Eye	<0.007	<0.011	<0.007	<0.006
Fat	<0.006	<0.009	<0.006	<0.007
Hair and skin	<0.003	<0.005	<0.003	<0.003
Heart	<0.003	<0.005	<0.003	<0.003
Kidney	0.008	0.011	0.010	0.007
Large intestine	0.008	0.009	0.132	0.072
Liver	0.058	0.084	0.060	0.153
Lung	<0.003	<0.005	<0.003	<0.003
Mandibular gland	<0.003	<0.005	<0.003	<0.004
Muscle	<0.003	<0.005	<0.003	<0.003
Ovary		<0.010		<0.009
Pancreas	<0.003	<0.005	0.004	<0.003
Pituitary gland	<0.074	<0.160	<0.119	<0.083
Sciatic nerve	<0.030	<0.043	<0.026	<0.022
Small intestine	0.006	<0.005	0.153	0.081
Spinal cord	<0.005	<0.006	<0.005	<0.005
Spleen	<0.003	<0.005	<0.003	<0.003
Stomach	0.005	<0.005	0.042	0.020
Testis	<0.003		<0.003	
Thymus	<0.003	<0.005	<0.003	<0.003
Thyroid	<0.055	<0.097	<0.056	<0.046
Uterus		<0.005		<0.003
Caecum contents	0.067	0.054	0.657	0.479
Large Intestine contents	0.057	0.042	0.452	0.356
Small intestine contents	0.062	0.061	0.718	0.646
Stomach content	<0.003	<0.005	0.006	0.019

Metabolism:

Twelve metabolites were identified and quantified.

In group A (*R*-isomer), 5-CA-S-2200-NHM was the predominant metabolite, accounting for 38.6% and 41.2% 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 between 4 and 12% each.

In group B (*S*-isomer), 4-OH-S-2200 was the predominant metabolite, accounting for 23.1% and 28.4% 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 at between 3 and 19.2% of the administered dose each.

S-2200 *R*-isomer was absorbed into the body and metabolised 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 into urine and faeces. No potential for accumulation in tissues was demonstrated.

The rate of excretion of S-2200 *S*-isomer was slower than that of S-2200 *R*-isomer. The major metabolite in the S-2200 *S*-isomer dose group was 4-OH-S-2200, and its glucuronide conjugate (4-OH-S-2200-GlucA) which was detected after enzyme hydrolysis. It is likely that 4-OH-S-2200-GlucA is hydrolysed in the small

intestine, is subject to enterohepatic circulation, and finally excreted as 4-OH-S-2200 in faeces. The enterohepatic recirculation slowed excretion of S-2200 S-isomer derived radioactivity.

Table B.6.1.2-4: Metabolites identified in rat excreta 3 (R-isomer) or 4 days (S-isomer) after administration of radiolabeled S-2200 (% dose administered)

Metabolite	R-isomer of S-2200		S-isomer of S-2200	
	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
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*	6.9	6.1	6.6	8.2
Total of 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**	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 of faeces	73.4	62.8	76.5	69.8

Data were obtained from the pooled sample of four rats. ND: Not detected.

*: Sum of the 16 unidentified metabolites which are below 3.6% of the dose.

**: Sum of the 9 unidentified metabolites which are below 2.7% of the dose.

Conclusion:

Both-isomers were absorbed immediately after administration and metabolised completely.

S-2200 R-isomer derived radioactivity was rapidly excreted after a single oral administration, excretion of the S-2200 S-isomer was less rapid.

Twelve metabolites were identified and quantified: 5-CA-S-2200-NHM was the predominant metabolite the

R-isomer. 4-OH-S-2200, followed by 5-COOH-S-2200, were the major metabolites of the *S*-isomer.

Because 4-OH-S-2200 and its glucuronide were detected in faeces, and urine and faeces, respectively, it is likely that 4-OH-S-2200-GlucA is subjected to enterohepatic circulation. This metabolic pathway is likely to occur more commonly with S-2200 *S*-isomer, because the rate of radiolabel excretion was slower (delayed by enterohepatic recirculation).

On the basis of the identified metabolites in this study, the following metabolic pathways of S-2200 *R*-isomer/S-2200 *S*-isomer in rats are proposed.

The main metabolic pathways of S-2200 *R*-isomer are 1) carboxylation of the methyl group at the 5-position of the phenoxy group, 2) hydroxylation of *N*-methyl group, 3) further hydroxylation of the methyl group at 2-position of the phenoxy group, or 4) *N*-demethylation, followed by 5) *O*-demethylation.

In the case of S-2200 *S*-isomer, the main metabolic reactions are 1) hydroxylation at 4-position of the phenoxy group, followed by glucuronide conjugation, or 2) carboxylation of the methyl group at 5-position of the phenoxy group, followed by 3) hydroxylation of *N*-methyl group, or 4) hydroxylation of the methyl group at 2-position of phenoxy group.

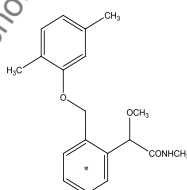
B.6.1.3 Oral ADME studies – repeated dose, oral route in rats

Reference:	Amended Final Report 1: [¹⁴ C]S-2200: Absorption, Distribution, Metabolism and Excretion following Repeat Oral Administration to the Rat. [REDACTED]
Author(s), year:	[REDACTED] 2011
Report/Doc. number:	Sumitomo Chemical Co. Ltd. Report No. ROM-0036
Guideline(s):	US EPA OPPTS 870.7485 (August 1998), Japanese MAFF (12-Nousan-No. 8147, Part 2-3-1), and OECD 417 (April 1984).
GLP:	Yes (lab certified by National Authority)
Deviations:	Minor deviations not expected to compromise the reliability of the study were listed in the study report
Validity:	Yes

Material and methods:

Test Material

Non-radiolabelled:	S-2200
Batch:	081103G
Purity:	100.0% (analytical standard)
Radiolabelled:	[benzyl- ¹⁴ C]S-2200
Batch:	CFQ40467 Batch B1
Specific activity:	4.55 GBq/mmol
Radiopurity:	98.9%
Vehicle:	0.5% aqueous methylcellulose



* = position of radiolabel

Test animals

Species:	Rat
Strain:	Hannover Wistar (CrI)
Source:	UK Home Office approved source
Number and sex:	52 animals (26 males and 26 females)
Age:	7 weeks
Body weight:	197-234 g (males) and 143-182g (females) at dosing

Low doses (5 mg/kg bw) of [benzyl-¹⁴C]S-2200 were repeatedly administered to 6 groups of male and female rats to study excretion, tissue distribution, and metabolism of S-2200 after repeated exposure (two animals were dosed additionally to ensure equal group size).

Table B.6.1.3-1: Study design for the repeat dose ADME study in rats

Dose Group	Days treated	Sacrifice (hours after last dose)	Study type	Dose level		Number of animals	
				(mg/kg)	(MBq/kg/day)	Males	Females

A	1	2	TD, MT	5	1.5	4	4
B	6	2	TD	5	1.5	4	4
C	10	2	TD	5	1.5	4	4
D	14	2	TD, MT	5	1.5	4	4
E	14	168	TD, Ex, ME	5	1.5	4	4
F	14	336	TD, Ex, ME	5	1.5	4	4

Ex Excretion balance
TD Tissue distribution
MT Metabolism study in tissues
ME Metabolism study in excreta

Animal treatment, housing, and group assignment corresponded to the conditions specified in OECD-guideline 417. The animals were fed SQC Rat and Mouse Maintenance Diet No 1 *ad libitum* and had unlimited access to tap water.

All animals were examined twice daily. No signs of pharmacological or toxicological effects attributable to the test substance have been recorded throughout the study. Body weights were recorded the day after arrival, before dose administration, and at necropsy except for group F where the animals stayed in metabolism cages for 2 weeks.

Preparation of the dose solutions:

Appropriate amounts of radiolabelled and non-radiolabeled S-2200 were weighed into dose vessels and the appropriate volume of acetonitrile added. The solvent was evaporated under a gentle stream of nitrogen gas, and the dose vehicle was added to the dose vessel. Appropriate amounts of [benzyl-¹⁴C]S-2200 and S-2200 were suspended in 0.5% w/v aqueous methylcellulose at a nominal concentration of 1 mg/mL. The formulation was shaken for 2 hours, before being stirred continuously for approximately 88 hours prior to removal of aliquots for dose formulation checks.

The stability of the formulations was assessed by determining the radiochemical purity of the formulated material on days 1, 7 and 14; using HPLC. The homogeneity and radioactivity concentrations of the formulated test compounds were determined by taking triplicate aliquots on days 1, 7 and 8-14, and subjected to Liquid Scintillation Counting (LSC). The radioactive concentration of the replicates on each day varied by less than 5% and the formulation was considered homogeneous on a daily basis.

Dosing and sampling regimens:

Four male and four female rats (per dose group) were administered daily oral doses of [¹⁴C]S-2200 for 14 days.

After each dose, rats were returned to holding cages (with the exception of animals in group E).

Urine and faeces were collected at 24 hour intervals until removal to holding cages or termination. At each collection of excreta, cage debris was removed and the cages rinsed with water. Cage washings were retained separately per animal and time point. After the final water wash, the cages were washed with methanol which was added to the final water wash. Following the final sample collection, animals were exsanguinated by cardiac puncture under Isoflurane anaesthesia, and 31 tissues were then sampled including whole blood, blood plasma, red blood cells and carcass.

Animals in group E were housed in metabolism cages, following the first dose administration. Twenty four hours after the fourteenth dose administration, the animals were transferred to holding cages until termination on day 21.

Animals in group F were returned to holding cages following dose administration on days 1-13. Following dose administration on day 14, animals were placed in individual glass metabolism cages suitable for the separate collection of urine and faeces.

Findings:

No signs of pharmacological or toxicological effects attributable to the test substance have been recorded throughout the study.

Elimination

Table B.6.1.3-2: Cumulative recovery of radioactivity in excreta after repeated administration (% of dose administered, calculated from the sum of 14 administered doses)

Hours after dosing	Days 1-14 (Group E)		Days 14-28 (Group F)	
Sex	Males	Females	Males	Females
Urine				
0-24	0.360	0.389	1.352	1.361
0-48	0.893	0.882	1.607	1.751
0-72	1.368	1.404	1.710	1.865
0-96	1.931	2.038	1.784	1.946
0-120	2.818	3.290	1.822	1.991
0-240	8.071	9.021	1.887	2.064
0-336	13.25	14.81	1.894	2.074
Faeces				
0-24	0.930	0.493	5.784	5.529
0-48	3.429	2.498	8.140	8.233
0-72	5.460	4.194	8.972	9.194
0-96	7.540	5.590	9.459	9.774
0-120	10.45	8.152	9.617	10.04
0-240	35.22	28.89	9.929	10.51
0-336	57.25	49.45	9.965	10.56
Cage wash				
0-24	0.077	0.113	0.201	0.444
0-48	0.226	0.548	0.296	0.578
0-72	0.384	0.800	0.362	0.637
0-96	0.633	1.257	0.391	0.674
0-120	0.898	1.718	0.404	0.693
0-240	3.153	4.720	0.454	0.754
0-336*	6.112	7.998	0.566	0.837
Total ¹	76.61	72.25	12.42	13.47

* Includes cage debris, final cage wash and tissues

¹: Recovery calculated from sum of the fourteen daily doses administered

Overall total recoveries of radioactivity were approximately 80% for both male and female animals over the entire sample collection.

During the first 14 days, elimination of the administered radioactivity was consistent within each 24 hour collection period, with approximately 5% of the total administered dose eliminated during each day.

Following completion of the daily dosing regime, the elimination of radioactivity was approximately 0.1-0.5% of the total administered dose during each 24 hour period.

Radioactivity was eliminated primarily in the faeces, with about two thirds to three quarters of the administered radioactivity recovered in the faeces of both sexes. Renal elimination (including cage washings) was also prominent and accounted for a recovery of up to one quarter of the administered dose in male

animals. Radioactivity remaining associated with the residual carcass (including those tissues harvested at termination) accounted for about 0.2% of the administered dose in both males and females 168 hours after the 14th daily dose.

Tissue distribution

Radioactivity was widely distributed at all time points examined (2 hours after 1, 6, 10 and 14 daily doses, and at 168 and 336 hours after the 14th daily dose). Radioactivity was detected in all of the tissues examined on at least one occasion. At 648 h post-dose (336 h after the final dose), radioactivity was detected in around half of the tissues examined.

Excluding the components of the gastrointestinal tract, the greatest mean concentrations of radioactivity were measured in the tissues involved in the metabolism and excretion of xenobiotics (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 the accumulation of radioactivity with repeated daily administration of [¹⁴C]S-2200 at a dose level of 5 mg/kg/day.

Table B.6.1.3-3: Concentrations of radioactivity (ng equivalents/g) in organs and tissues following repeated oral administration of 5 mg/kg bw [¹⁴C] S-2200 to rats

Hours after 1 st dose	2 (group A)		122 (group B)		218 (Group C)		314 (Group D)		480 (Group E)		648 (Group F)	
Hours after 14 th daily dose	-		-		-		2		168		336	
Tissue ^a	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Liver	4724	2025	7762	4687	11760	6618	10150	8868	415.3	686.3	115.2	150.0
Carcass	215.1	238.8	620.5	602.9	995.7	1041	932.6	1165	59.20	61.79	85.69	51.70
Skin+ hair	167.0	67.3	300.6	144.8	452.1	301.6	449.6	271.9	92.50	43.99	81.04	40.03
Bone	65.56	21.89	93.59	55.46	151.2	92.14	133.8	114.6	19.69	9.502	ND	32.15
Kidney	1157	446.0	1730	923.2	3057	1094	2459	1574	108.9	91.11	39.83	26.59
Red Blood Cells	274.9	67.21	318.6	140.6	430.6	148.1	382.1	208.1	104.6	67.60	38.23	21.48
Ovaries		874.4		1813		2360		1123		104.5		11.50
Spleen	169.1	167.7	504.2	724.3	475.5	1319	707.3	839.5	28.68	36.27	20.56	11.46
Uterus		1310		3189		4379		4810		89.54		7.677
Pancreas	571.4	635.4	1019	3239	1622	2878	11590	4370	40.04	67.14	ND	ND
Plasma	772.8	227.3	1052	513.0	1582	582.2	1197	867.7	13.74	8.352	ND	ND
Fat	339.2	270.4	602.6	1124	996.6	1151	1956	803.0	6.861	36.29	ND	ND
Thyroid	322.0	245.6	492.4	249.1	907.6	1201	1122	685.5	27.81	ND	ND	ND
Blood	495.1	145.1	685.8	322.3	1031	375.9	781.3	570.6	63.74	44.18	6.613	ND
Adrenals	315.2	90.20	462.0	298.1	733.9	426.5	673.1	522.1	13.31	17.40	ND	ND
Lung	287.8	136.5	472.8	214.6	736.7	416.7	631.7	425.9	32.42	33.21	ND	ND
Sciatic nerve	220.9	ND	301.2	94.73	475.9	222.5	432.7	336.4	38.16	ND	ND	ND
Pituitary	67.64	ND	94.25	90.94	751.1	ND	428.4	261.2	ND	ND	ND	ND
Muscle(quad)	91.53	57.56	144.7	99.44	253.1	167.2	237.2	247.5	ND	6.384	ND	ND
Heart	208.8	80.54	301.4	127.8	484.1	184.9	336.4	244.4	7.898	ND	ND	ND
Mandibular gland	147.4	50.57	231.4	111.8	366.2	192.0	301.5	227.2	ND	ND	ND	ND
Testes	130.9		178.6		272.7		228.5		ND		ND	
Thymus	93.91	50.03	197.3	98.01	280.3	148.8	249.5	170.7	6.783	ND	ND	ND
Eyes	75.91	41.24	130.8	58.76	227.3	125.0	212.4	118.6	7.752	ND	ND	ND
Bone Marrow	57.26	ND	195.3	ND	145.6	ND	128.5	91.73	ND	ND	ND	ND
Spinal Cord	33.28	27.44	79.90	35.41	159.0	96.76	101.8	75.02	ND	ND	ND	ND
Brain	39.11	11.97	70.91	39.25	107.6	44.92	77.96	61.15	ND	ND	ND	ND
Gastro-Intestinal Tract												
Caecum	2648	8859	20270	30420	33170	56130	42420	71510	351.1	486.1	31.54	46.68
Caecum contents	3234	12290	34520	41870	55550	78190	53210	51580	464.6	551.2	47.00	65.43
Large intestine Contents	160.0	497.8	15550	14710	34300	44160	27340	36900	315.8	621.8	39.52	33.06
Large Intestine	643.6	1466	6706	10630	16680	14570	20320	16980	167.6	270.5	9.270	13.80
Stomach	7592	5772	19310	8914	33770	17400	26010	27010	30.15	119.1	ND	5.886
Stomach Contents	20250	22320	54780	16440	83540	31430	94490	49320	43.88	103.0	15.71	35.26
Small Intestine	21980	17450	43020	37680	62130	57840	95130	67350	380.7	814.4	60.87	65.42

Small Intestine Contents	44260	45220	132700	132000	176400	167000	228300	100600	1240	1508	100.2	97.53
--------------------------	-------	-------	--------	--------	--------	--------	--------	--------	------	------	-------	-------

^a In descending order of magnitude of residues in group F female tissues excluding GIT.

Metabolism

S-2200 underwent extensive metabolism. In the urine, faeces, plasma and liver profiles, no major gender-dependent differences were observed following single or repeated daily administration of [benzyl-¹⁴C]S-2200 at 5 mg/kg/day. However, the kidney profiles did exhibit both gender dependent, and differences between repeated and single dosing, in the type and number of metabolites observed.

Table B.6.1.3-4: Quantification of metabolites (% administered dose) in pooled excreta following single or repeated oral administration of 5 mg/kg bw [¹⁴C] S-2200 to rats

Metabolite Identity ¹	Urine				Faeces			
	0-24 hr (group E, single adm.)		312-336 hr (Group F, repeated adm.)		0-24 hr (group E, single adm.)		312-336 hr (Group F, repeated adm.)	
	Males	Females	Males	Females	Males	Females	Males	Females
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
%DOSE	6.881	7.741	1.352	1.361	17.64	9.594	5.784	5.529

ND
1 not detected
confirmed by LC-MS

WARNING: This document forms part of an EC evaluation data package and should not be read in isolation. Registration must not be granted on the basis of this document.

In **urine**, the major metabolites following 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 metabolites 2-COOH-S-2200, 5-COOH-S-2200 and the 4-OH-S-2200-GlucA were especially prominent.

In **faeces** the metabolites following both single and repeated daily doses 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. Although quantitative differences exist in the proportions of some metabolites between male and female profiles, there were no major qualitative differences. Repeated administration did not significantly affect the profile of the metabolites observed.

In **plasma** from animals receiving a single dose, nearly twice as many metabolites were identified in males. In animals receiving 14 daily doses, a greater number of metabolites could be identified.

Although quantitative differences exist in the proportions of some metabolites, there are no major qualitative differences between the sexes or between single and repeated administration.

Table B.6.1.3-5: Quantification of metabolites (ng equivalents/g) in plasma following single or repeated oral administration of 5 mg/kg bw [¹⁴C] S-2200 to rats

Metabolite Identity ¹	2 hr (Group A)		314 hr (Group D)	
	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 31.3-31.3)	ND	ND	66.58	ND
UK (Rt 33.9-33.9)	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
Conc. (ng eq./g)	778.2	229.7	1215	869.8

ND not detected

< LOQ below the limit of quantification

¹ confirmed by LC-MS

UK unknown

Rt retention time

In the **liver**, following a single administration, both aqueous and organic extracts from male and female animals were dominated by the presence of 5-CA-S-2200-NDM and 5-COOH-S-2200. The major metabolites in the male and female profiles were similar, and included De-Xy-S-2200, 5-CA-MCBX-NDM, 5-CA-S-2200-NHM, 5-CA-S-2200-NDM, 5-COOH-S-2200, 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 male and female organic liver extract profile De-Xy-S-2200, 5-CA-S-2200-NHM, 5-CA-S-2200-NDM and 5-COOH-S-2200 were the dominant metabolites identified. An additional region of interest in the female profile was assigned the identity of 5-CA-MCBX-NDM. Therefore, although quantitative differences exist in the proportions of some metabolites, in the liver there are no major qualitative differences between the sexes. Repeated administration did not significantly alter the metabolic profile.

Table B.6.1.3-6: Quantification of metabolites (ng equivalents/g) in liver following single or repeated oral administration of 5 mg/kg bw [¹⁴C] S-2200 to rats

Metabolite Identity ¹	2 hr (Group A)				314 hr (Group D)			
	Aqueous phase		Organic phase		Aqueous phase		Organic phase	
	Male	Female	Male	Female	Male	Female	Male	Female
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	1275	812.0	1500	580.4
5-CA-S-2200-NDM	1393	651.2	2710	1016	1331	779.1	3885	1787
5-CA-S-2200-NHM	303.3	163.9	548.0	ND	412.9	826.0	1135	1790
5-COOH-S-2200	1127	745.4	1221	1238	1686	1644	2114	2912
De-Xy-S-2200	441.7	332.5	534.5	ND	1286	2081	1073	1174
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 24.5-24.5)	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 27.9-27.9)	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
Concentration (ng eq./g)	4814	2223	5014	2254	10070	8244	10070	8244

ND not detected

<LOQ below the limit of quantification confirmed by LC-MS

¹ confirmed by LC-MS

UK unknown

Rt retention time

In the **kidney** following the single oral administration of [benzyl-¹⁴C]S-2200, the profiles of male animals contained a greater number of metabolites compared to female animals. The major metabolites in the male profiles corresponded to 5-CA-2-HM-S-2200-NHM, an unknown region of interest, 5-CA-S-2200-NHM and 5-COOH-S-2200. In the profile from the female animals, 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 the female profiles an unknown region of interest and 5-CA-MCBX-NDM were considered major regions in addition to those observed following single dosing (5-CA-S-2200-NHM and 5-COOH-S-2200).

Chromatographic resolution of the kidney profiles from male animals was much greater. The four most prominent metabolites observed in the female kidney profile were also prominent in the male profile. However, seven additional metabolites were also observed in the male profile. 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 of > 100 ng equivalents/g. Therefore, in the kidney, there were both quantitative and qualitative differences in the metabolites observed between male and female animals, and following both single and repeated administration of S-2200.

Table B.6.1.3-7: Quantification of metabolites (ng equivalents/g) in kidney following single or repeated oral administration of 5 mg/kg bw [¹⁴C] S-2200 to rats

Metabolite Identity ¹	2 hr (Group A)		314 hr (Group D)	
	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 24.9-24.9)	ND	ND	134.9	ND
UK (Rt 27.1-27.1)	ND	ND	157.4	ND
UK (Rt 28.1-28.1)	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
Conc. (ng eq./g)	1101	402.5	2371	1597

ND not detected
 <LOQ below the limit of quantification
¹ confirmed by LC-MS
 UK unknown
 Rt retention time

Conclusion:

There were no gender-dependent differences in either the rates or routes of excretion of radioactivity following repeated daily administration of [benzyl-¹⁴C]S-2200 at 5 mg/kg/day for 14 days. Faecal elimination was the primary route of elimination of radioactivity, but renal elimination was also important for the excretion of metabolites.

There were no gender-dependent differences in the distribution of radioactivity into tissues following repeated daily administration. A similar distribution of radioactivity into tissues was observed following 1, 6, 10 and 14 daily doses indicating that association with tissues was transient. This is supported by the very low recoveries of radioactivity in tissues 168 and 336 hours after the 14th dose. Clearance of radioactivity from the plasma was complete by 336 hours after the 14th daily dose.

Besides the main excretory organs liver and kidney, spleen, pancreas, fat, thyroid, and uterus and ovaries contained notable amounts of radioactivity.

S-2200 was extensively metabolised. The primary routes of metabolism of S-2200 were by oxidation and subsequent conjugation with glucuronic acid, demethylation with subsequent oxidation, or oxidation with subsequent demethylation.

In the urine, faeces, plasma and liver metabolism profiles, no major gender-dependent differences were observed following a single or repeated daily administration of [benzyl-¹⁴C]S-2200 at 5 mg/kg/day. However, the kidney profiles exhibited both gender and repeat dose differences, in the type and number of metabolites observed.

B.6.1.4 Summary of ADME studies

Absorption and Excretion

Following a single oral high (1000 mg/kg bw) or low (5 mg/kg bw) dose administration of racemic [^{14}C]S-2200, labelled at either the benzyl or phenoxy rings, radioactivity was rapidly absorbed (more rapid at low than high doses). At the low dose, absorption of S-2200, calculated from the radioactivity recovered in urine and bile, was greater than 90% of the administered dose. At the high dose, systemic exposure (AUC) to S-2200 derived radioactivity was sub-proportional, indicating saturation of absorption processes following administration. Secondary absorption phases following administration of 1000 mg/kg doses of S-2200 indicated that systemic exposure at high concentrations may have resulted from one or more of the following: (a) enterohepatic recirculation, (b) differential absorption during transit through the gastro-intestinal tract, or (c) transient solubility of the S-2200. There were no significant gender differences in the pharmacokinetics of S-2200 at 5 or 1000 mg/kg bodyweight.

Clearance from the plasma was almost complete by 120 hours post-administration after a single oral dose of 5 or 1000 mg/kg bodyweight. Faecal elimination, via the bile, was the primary route of elimination of radioactivity. However, renal elimination was also important for the excretion of metabolites. More than 70% of radioactivity was eliminated within 48 hours after single oral administration. There did not appear to be any gender-, dose- or radiolabel- related differences in either the rates or routes of excretion.

In an investigation of ADME of the two stereo isomers of the active substance, coded S-2200 *R*-isomer and S-2200 *S*-isomer, both were absorbed immediately after administration. S-2200 *R*-isomer derived radioactivity was rapidly excreted after single oral administration, whereas excretion of radiolabel from S-2200 *S*-isomer was less rapid (likely due to enterohepatic recirculation). In this study, urinary excretion was greater in female than male rats for both isomers. Excretion into expired air was negligible.

Following the repeated daily administration of [benzyl- ^{14}C]S-2200 at 5 mg/kg bw for 14 days, there were no differences compared to single dose of 5 mg/kg bw. Faeces was again the primary route of excretion.

Distribution

Following oral exposure, S-2200 is widely distributed throughout the body. After single oral administration there was no major difference in distribution between high and low doses (1000 and 5 mg/kg bw, respectively), or between sexes. The major tissue residues were seen in the gastrointestinal tract, and in liver and kidney, as well as in uterus and ovaries at 168 hours after dosing. Tissue distribution was similar in animals administered [benzyl- ^{14}C]S-2200 or [phenoxy- ^{14}C]S-2200 indicating that the core of the molecule was stable during systemic exposure. Furthermore, a similar distribution of radioactivity into tissues was observed following 1, 6, 10 and 14 daily doses of 5 mg/kg bw. There was no evidence of accumulation into tissues.

Metabolism

S-2200 was extensively metabolised to numerous metabolites. Unchanged parent was found in faeces only at < 0.2% of administered dose after a single low dose of 5 mg/kg bw and at < 6% after single high dose of 1000 mg/kg bw. The primary routes of metabolism were by (i) oxidation and subsequent conjugation with glucuronic acid, (ii) demethylation with subsequent oxidation, or (iii) oxidation with subsequent demethylation. Metabolite fractions in plasma, liver and kidney were identified. Residues were in general readily extractable from tissue matrix. There was no discernible shift in radiolabel distribution between low and high dose.

In an investigation of ADME of the two stereo isomers of the active substance, coded S-2200 *R*-isomer and

S-2200 S-isomer, twelve metabolites were identified and quantified: 5-CA-S-2200-NHM was the predominant metabolite of the S-2200 *R*-isomer. 4-OH-S-2200, followed by 5-COOH-S-2200, were the major metabolites of S-2200 *S*-isomer. However, the same metabolites were identified both in S-2200 *R*-isomer and *S*-isomer. The “A” glucuronide of 4-OH-S-2200 (4-OH-S-2200-Glucuronide A) is likely to be subject to enterohepatic circulation. This metabolic pathway is likely to occur more commonly with S-2200 *S*-isomer, because the rate of radiolabel excretion was slower (delayed by enterohepatic recirculation) than that for S-2200 *R*-isomer.

Following repeated administration of radiolabelled S-2200 at 5 mg/kg bw for 14 days, metabolism was similar to that after single dose, however, kidney profiles exhibited both gender and repeat dose differences, in the type and number of metabolites observed.

B.6.1.4-1 Proposed metabolic pathway in rats

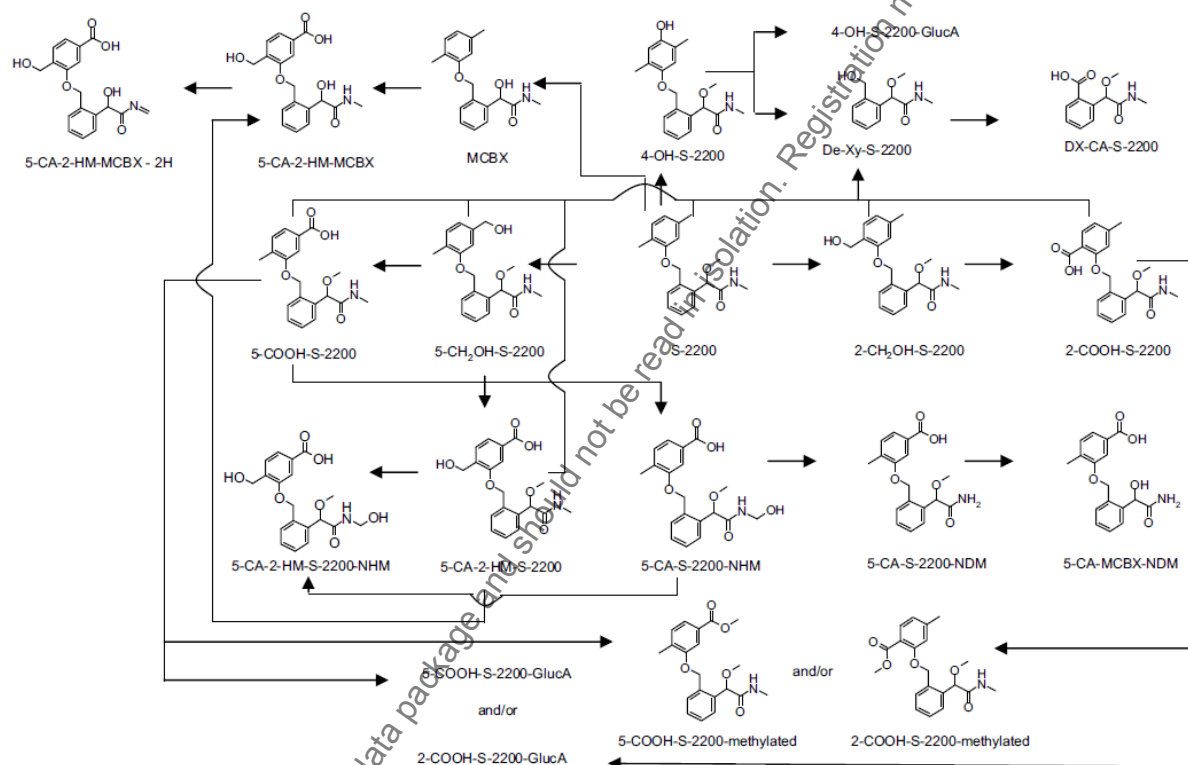
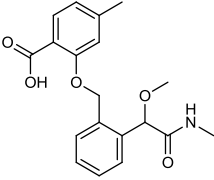
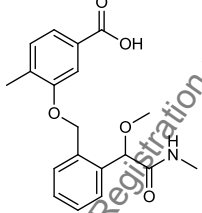
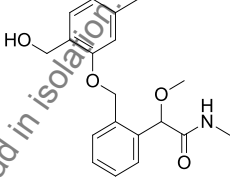
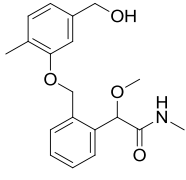
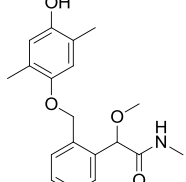
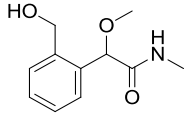
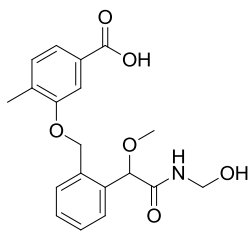
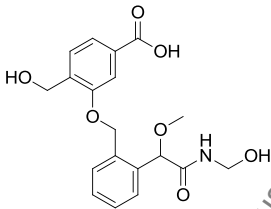
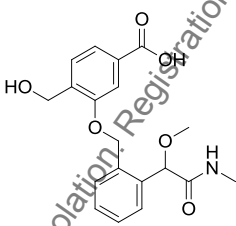
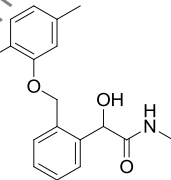
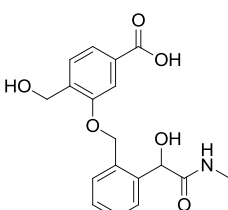
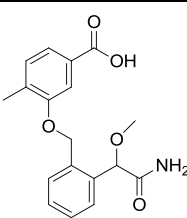
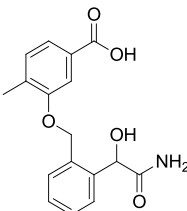


Table B.6.1.4-1: Metabolites identified in excreta of rats (after single and repeated dose)

Substance	IUPAC Name	Molecular weight	Structure	% of applied radioactivity (max. values)	
				Single dose	Repeated dose
2-COOH-S-2200	(<i>RS</i>)-2-[2-[1-methoxy-1-(<i>N</i> -methylcarbamoyl)methyl]benzyloxy]-4-methylbenzoic acid	343.38		0.856 ¹ R: 0.8 S: 2 ² 0.338 ³	0.153 ⁴
5-COOH-S-2200	(<i>RS</i>)-3-[2-[1-methoxy-1-(<i>N</i> -methylcarbamoyl)methyl]benzyloxy]-4-methylbenzoic acid	343.38		10.765 ¹ R: 3.5, S: 19.2 ² 2.186 ³	0.91 ⁴
2-CH ₂ OH-S-2200	(<i>RS</i>)-2-[2-(2-hydroxymethyl-5-methylphenoxy)methyl]phenyl]-2-methoxy- <i>N</i> -methylacetamide	329.39		0.367 ¹	ND
5-CH ₂ OH-S-2200	(<i>RS</i>)-2-[2-(5-hydroxymethyl-2-methylphenoxy)methyl]phenyl]-2-methoxy- <i>N</i> -methylacetamide	329.39		1.138 ¹	ND
4-OH-S-2200	(<i>RS</i>)-2-[2-(4-hydroxy-2,5-dimethylphenoxy)methyl]phenyl]-2-methoxy- <i>N</i> -methylacetamide	329.39		18.213 ¹ R: 5.5, S: 28.4 ² 0.896 ³	1.164 ⁴
4-OH-S-2200-Glucuronide				36.670 ¹ R: 4.5, S: 3.9 ² 1.048 ³	0.147 ⁴
De-Xy-S-2200	(<i>RS</i>)-2-(2-hydroxymethylphenyl)-2-methoxy- <i>N</i> -methylacetamide	209.24		4.435 ¹ R: 1.8, S: 4.3 ² 0.446 ³	1.053 ⁴

5-CA-S-2200-NHM	(<i>RS</i>)-3-[2-[1-(<i>N</i> -hydroxymethylcarbamoyl)-1-methoxymethyl]benzyloxy]-4-methylbenzoic acid	359.37		19.866 ¹ R: 41.2, S: 8.3 ² 8.46 ³	1.68 ⁴
5-CA-2-HM-S-2200-NHM	(<i>RS</i>)-4-(hydroxymethyl)-3-[2-[1-(<i>N</i> -hydroxymethylcarbamoyl)-1-methoxymethyl]benzyloxy]benzoic acid	375.37		6.485 ¹ R: 6.4, S: 2.4 ² 2.768 ³	0.518 ⁴
5-CA-2-HM-S-2200	(<i>RS</i>)-4-(hydroxymethyl)-3-[2-[1-methoxy-1-(<i>N</i> -methylcarbamoyl)methyl]benzyloxy]benzoic acid	359.37		5.043 ¹ R: 2.3, S: 6.5 ² 1.834 ³	0.471 ⁴
MCBX	(<i>RS</i>)-2-hydroxy- <i>N</i> -methyl-2-[α-(2,5-xylyloxy)- <i>o</i> -tolyl]acetamide	299.36		0.040 ¹	ND
5-CA-2-HM-MCBX	(<i>RS</i>)-4-(hydroxymethyl)-3-[2-[1-hydroxy-1-(<i>N</i> -methylcarbamoyl)methyl]benzyloxy]benzoic acid	345.35		1.164 ¹ R: 2.1, S: 3.6 ² 0.774 ³	0.214 ⁴
5-CA-S-2200-NDM	(<i>RS</i>)-3-[2-(1-carbamoyl-1-methoxymethyl)benzyloxy]-4-methylbenzoic acid	329.35		0.947 ¹ R: 5.8, S: 2 ² 0.638 ³	0.222 ⁴
5-CA-MCBX-NDM	(<i>RS</i>)-3-[2-(1-carbamoyl-1-hydroxymethyl)benzyloxy]-4-methylbenzoic acid	315.32		2.186 ¹ R: 11.8, S: 3.4 ² 1.823 ³	0.529 ⁴

R
S

R-isomer
S-isomer

Maximal Values from Table B.6.1.1-8: Quantification of metabolites (%dose administered) in pooled excreta;

Study: ██████████ (2012), Single oral administration

Maximal values detected in pooled faeces and urine from Table B.6.1.2-4: Metabolites identified in rat excreta 3 (R-isomer) or 4 days (S-isomer) after administration of radiolabelled S-2200 (% dose administered);

Study: ██████████ (2011), Second single dose, oral route

- ³ Maximal values detected in pooled faeces and urine from Table B.6.1.4-4: Quantification of metabolites (% administered dose) in pooled excreta following single or repeated oral administration of 5 mg/kg bw [¹⁴C] S-2200 to rats after single exposure
Study: [REDACTED] 2011, repeated dose, oral route
- ⁴ Maximal values detected in pooled faeces and urine from Table B.6.1.4-4: Quantification of metabolites (% administered dose) in pooled excreta following single or repeated oral administration of 5 mg/kg bw [¹⁴C] S-2200 to rats after repeated exposure
Study: [REDACTED] 2011, repeated dose, oral route

WARNING: This document forms part of an EC evaluation data package and should not be read in isolation. Registration must not be granted on the basis of this document.

B.6.2 Acute toxicity including irritancy and skin sensitisation (Annex IIA 5.2)

B.6.2.1 Acute oral toxicity

Reference:	Acute Oral Toxicity Study of S-2200 TG in Rats
Author(s), year:	██████████ 2010a
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0010
Guideline(s):	OECD 423; EC Directive 2004/73/EC, Acute toxic (oral); Japanese MAFF (12-Nousan No. 8147)
GLP:	Yes (Lab certified by National Authority)
Deviations:	None
Validity:	Yes

Material and methods:

<i>Test Material:</i>	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	Lot No.: ST-0811G (Batch specification 01)
Purity:	93.4%
Stability of test compound:	Expiry date: 21 November 2011 (after completion of treatment) (see certificate p179, ROT-0022)

Test animals:

Species:	Rat (females only)
Strain:	Slc:WistarHanover/Rcc
Age:	8 weeks (at start of study)
Weight at dosing:	First group: 155 – 165 g Second group: 149 – 160 g
Source:	██
Diet:	CRF-1 Pellet diet from Oriental Yeast Co, Ltd. Tokyo, Japan; <i>ad libitum</i>

Animals in the first group of three were each given a single gavage dose of 2000 mg/kg bw S-2200 TG in 0.5% aqueous methylcellulose. When no response was observed in the first group, a second group of three animals was dosed at 2000 mg/kg bw. Accuracy of dose formulation was confirmed by analysis. Clinical and mortality observations were made at 10 and 30 minutes, 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/tissues examined macroscopically (no further description).

Findings:

No mortality was observed.

Clinical signs observed during the observation period included stains around the anus and liquid stools containing a white compound. Clinical signs appeared from 4 hours after administration and had disappeared on day 1.

The body weights were not affected by the administration of the test compound throughout the study period. There were no findings at the gross pathological examination.

Conclusion:

No mortality occurred after administration of 2000 mg/kg bw (limit test). According to Regulation (EC) No. 1272/2008, classification of mandestrobin for acute toxicity is not required.

B.6.2.2 Acute percutaneous toxicity

Reference:	Acute Dermal Toxicity Study of S-2200 TG in Rats
Author(s), year:	██████████ 2010b
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0011
Guideline(s):	US EPA OPPTS 870.1200; OECD 402; Japanese MAFF 12-Nousan No. 8147; EC Directive 92/69/EEC, acute toxicity (dermal).
GLP:	Yes (Lab certified by National Authority)
Deviations:	None
Validity:	Yes

Material and methods:

<i>Test Material:</i>	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	Lot No.: ST-0811G (Batch specification 01)
Purity:	93.4%
Stability of test compound:	Expiry date: 21 November 2011 (after completion of treatment) (see certificate p179, ROT-0022)

<i>Test animals:</i>	
Species:	Rat
Strain:	Wistar (Slc:WistarHannover/Rcc)
Age:	8 weeks (at start of study)
Weight at dosing:	Males 259-268 g, females 170-183 g
Source:	██
Diet:	CRF-1 Pellet diet from Oriental Yeast Co, Ltd. Tokyo, Japan; <i>ad libitum</i>

Healthy, acclimated animals of appropriate age and body weight were assigned to the study. Five male and five female animals were each treated with 2000 mg/kg bw undiluted S-2200 TG moistened with water. On the day of dosing, an appropriate amount of test compound was measured and spread on a gauze pad wet with water. The gauze pad was applied to the shaved skin in an area of approximately 4 × 5 cm², covered with an impermeable sheet, and held in place with surgical tape. After 24 hours, the pad was removed and the treated area cleaned with water and an absorbent pad to remove the test compound. Observations for clinical signs and mortality were performed at 10 and 30 minutes, and 1, 2, 4 hours after initiation of treatment. Observations continued daily for an additional 14 days. Body weights were taken on the day of dosing, and again at 7 and 14 days post-dosing. On day 14, animals were euthanized and the organs/tissues examined macroscopically.

Findings:

No mortality was observed, and all animals appeared normal throughout the study. Body weight was not affected by administration of the test compound. There were no findings at the gross pathological examination. No dermal irritation was recorded in this study.

Conclusion:

No mortality occurred after dermal administration of 2000 mg/kg bw. According to Regulation (EC) No. 1272/2008, classification of mandestrobin for acute dermal toxicity is not required.

B.6.2.3 Acute inhalation toxicity

Reference:	Acute Inhalation Toxicity Study of S-2200 TG in Rats
Author(s), year:	██████████ 2010
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0020
Guideline(s):	US EPA OPPTS 870.1300; OECD 403; EC Guideline 93/21/EEC, Acute Toxicity (inhalation); Japanese MAFF 12-Nousan-No. 8147
GLP:	Yes (Lab certified by National Authority)
Deviations:	None
Validity:	Yes

Material and methods:

<i>Test Material:</i>	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G (Batch specification 01)
Purity:	93.4%
Stability of test compound:	21 November 2011 (after completion of treatment) (see certificate p179, ROT-0022)

Test animals:

Species:	Rat
Strain:	Wistar (██████████:WistarHannover/Rcc)
Age:	8 weeks at dosing
Weight at dosing:	Males 270-281 g; females 175-189 g
Source:	██
Diet:	CRF-1 pellet diet (Oriental Yeast company); <i>ad libitum</i>

Acute inhalation toxicity of S-2200 TG was evaluated with ██████████ WistarHannover/Rcc rats (five per sex) in nose only inhalation chambers. The animals were exposed for 4 hours to a mean actual aerial concentration of 4964 mg/m³ (determined gravimetrically). The Mass Median Aerodynamic Diameter (MMAD) of the dust aerosol was 3.80 µm and the Geometric Standard Deviation of the dust particles was 2.65. Following exposure, animals were maintained for a 14 day observation period. Mortality, clinical signs, body weights, and gross pathological findings were evaluated.

Findings:

No mortality was observed. No treatment related clinical signs were observed during treatment and observation period. Wet fur was observed in both sexes of air control and treatment groups immediately after the exposure period, but disappeared within two hours post-dosing. This finding is considered to be a result of the restraint procedure and not of toxicity related to treatment. Body weights in the treatment groups were comparable to controls. No lesions were detected at the gross pathological examination at the end of the study period.

Conclusion:

The 4-hour inhalation LC₅₀ was determined to be > 4.96 mg/L (maximum attainable concentration) for male and female rats. According to Regulation (EC) No. 1272/2008, classification of mandestrobin for acute inhalation toxicity is not required.

B.6.2.4 Skin irritation

Reference:	Primary Skin Irritation Test of S-2200 TG in Rabbits
Author(s), year:	██████ 2010a
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0015
Guideline(s):	OECD 404; EPA OPPTS 870.2500; Japanese MAFF Notification No. 8147, Skin irritation study (2-1-4); EU Council Regulation No.440/2008, Part B, Method B4.
GLP:	Yes (Lab certified by National Authority)
Deviations:	None
Validity:	Yes

Material and methods:

<i>Test Material:</i>	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G (Batch specification 01)
Purity:	93.4%
Stability of test compound:	Expiry date: 21 November 2011 (after completion of treatment) (see certificate p179, ROT-0022).

Test animals:

Species:	Rabbit (males only)
Strain:	New Zealand White
Age:	14 weeks at dosing
Weight at dosing:	2578.5 – 2765.6 g
Source:	████████████████████
Diet:	LRC4, Lot No. 090305, Oriental Yeast Co., Ltd, Japan

The primary skin irritation potential of S-2200 TG was evaluated in three male rabbits. A dose of 0.5 g of S-2200 TG (moistened with corn oil) on a gauze patch (2.5 × 2.5 cm) was applied for 4 hours under semi-occlusive bandage to clipped intact dorsal skin.

The animals were monitored for skin reactions (erythema and oedema) using the Draize method at 1, 24, 48, and 72 hours after patch removal. All animals were observed daily for clinical signs during the experimental period.

Findings:

No signs of ill health or toxicity were observed in any of the animals during the experimental period. No skin irritation reactions were observed in any animal at any timepoint during the observation period.

Conclusion:

The dermal irritation score was 0.0 (non-irritating). According to Regulation (EC) No. 1272/2008, classification of mandestrobin as skin irritant is not required.

B.6.2.5 Eye irritation

Reference:	Amended Final Report: Primary Eye Irritation Test of S-2200 TG in Rabbits
Author(s), year:	██████ 2010b
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0016
Guideline(s):	OECD 405; EPA OPPTS 870.2400; EU Council Regulation No.440/2008,

Material and methods:

Prior to treatment, on the day of application, both eyes of each rabbit were examined using a hand slit-lamp and fluorescein stain, and six rabbits with normal eyes were selected for the study.

A volume of 0.1 mL (0.062 g by weight) of test compound was placed into the conjunctival sac of one eye in each animal, and the upper and lower eyelids were held together for one second after administration. The other eye in each animal served as a control. After 30 seconds, the treated eye of three of the six animals was flushed with about 180 – 200 mL of sodium chloride solution isotonic for 30 seconds to wash out the test compound (these three animals are referred to as the washed group). Eyes of the remaining three animals were not flushed (these three animals were referred to as the unwashed group). Ocular lesions were recorded at 1, 24, 48, and 72 hours after application of test compound. The grading and scoring of irritation reactions were performed according to the Draize scale (1944). A hand slit-lamp was used to score the irritation, and fluorescein was used at 24, 48, and 72 hours to further examine the eyes.

Findings:

No signs of ill health or toxicity were observed in any of the animals during the experimental period. One hour after application, conjunctival redness (grade 1), chemosis (grade 1), and discharge (grade 1) was observed in all three rabbits, and congestion (grade 1) in the iris was observed in 2 out of 3 animals in the unwashed group. 24 hours after application, opacity (grade 1) of the cornea and conjunctival redness (grade 1) were observed in all 3 rabbits, and 2 out of 3 rabbits showed chemosis (grade 1) and discharge (grade 1-2) in the conjunctiva. 48 hours after application, corneal opacity (grade 1) was observed in one animal. All ocular findings were reversible within 72 hours after application.

In the washed group, the following ocular findings were present at the 24 hour timepoint: redness grade 1 in all three animals, chemosis grade 1 in one animal. There were no findings at later timepoints.

Table B.6.2.5-1: Eye irritation scores (unwashed group)

		Rabbit Nr.	1 hour	24 hours	48 hours	72 hours	Mean (24-72 h)
Conjunctiva	Chemosis	1	1	1	0	0	0.33
		2	1	1	0	0	0.33
		3	1	0	0	0	0.00
	Redness	1	1	1	0	0	0.33
		2	1	1	0	0	0.33
		3	1	1	0	0	0.33
Iris	Congestion	1	0	0	0	0	0.00
		2	1	0	0	0	0.00
		3	1	0	0	0	0.00
Cornea	Opacity	1	0	1	1	0	0.67
		2	0	1	0	0	0.33
		3	0	1	0	0	0.33

Table B.6.2.5-2: Eye irritation scores (washed group)

		Rabbit Nr.	1 hour	24 hours	48 hours	72 hours	Mean (24-72 h)
Conjunctiva	Chemosis	1	1	0	0	0	0.00
		2	1	0	0	0	0.00
		3	1	1	0	0	0.33
	Redness	1	1	1	0	0	0.33
		2	1	1	0	0	0.33
		3	1	1	0	0	0.33
Iris	Congestion	1	0	0	0	0	0.00
		2	0	0	0	0	0.00
		3	0	0	0	0	0.00
Cornea	Opacity	1	0	0	0	0	0.00
		2	0	0	0	0	0.00
		3	0	0	0	0	0.00

Conclusion:

Mandestrobin caused only mild transient ocular irritation and therefore does not require classification and labelling for eye irritation according to Regulation (EC) No. 1272/2008.

B.6.2.6 Skin sensitisation

Reference:	Skin Sensitization Test of S-2200 TG in Guinea Pigs
Author(s), year:	2010c
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0019
Guideline(s):	EPA OPPTS 870.2600; EU Council Regulation No.440/2008, Part B, Method B6; OECD 406; Japanese MAFF Notification No. 12 Nohsan 8147 Skin

	Sensitization Studies (2-1-6)
GLP:	Yes (Lab certified by National Authority)
Deviations:	None
Validity:	Yes

Material and methods:

<i>Test Material:</i>	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G (Batch specification 01)
Purity:	93.4%
Stability of test compound:	Analytically determined (Test compound considered stable for the duration of the study).
Positive control:	α -hexylcinnamaldehyde (HCA)

Test animals:

Species:	Guinea pig (females only)
Strain:	■:Hartley
Age:	6 weeks at main study
Weight at dosing:	313.4 – 390.5 g at start of main study
Source:	■
Diet:	Labo G Standard, Lot No. 20090577 and 20090676, Nosan Corp., Japan

A dose-finding study using six animals found slight erythema from use of 2% and 5% test compound for intradermal injection, so a 5% concentration in corn oil was selected for intradermal induction. No dermal reaction was observed at topical exposed sites with 25% and 10% test compound in acetone. The test compound at a 50% (w/v) concentration in acetone was not able to be uniformly prepared. Therefore, 25% concentration in acetone was considered to be the maximum practical concentration, used for 25% topical induction and as the maximum non-irritant concentration for the challenge. For the main study dorsal regions of 40 animals were clipped free of hair (approximately 4 cm × 6 cm); 20 animals were designated for treatment with the test compound, 10 animals were used as control group, 5 animals were designated for treatment with HCA, and 5 animals were designated for the HCA control group.

First (intradermal) induction: Three pairs of intradermal injections of 0.1 mL/site were given to the hairless region with one of each pair on either side of the midline. Top injection: 1:1 mixture (v/v) of Freud's complete adjuvant (FCA) and distilled water. Medial injection: corn oil (control groups); corn oil with 5% test compound or corn oil with 5% HCA. Caudal injection: 1:1 mixture (v/v) of FCA and distilled water (controls); 1:1 mixture (v/v) of FCA and water with 5% test compound or 1:1 mixture (v/v) of FCA and water with 5% HCA.

Six days after the first induction, 0.5 mL of 10% sodium dodecyl sulphate in petrolatum was applied to all animals so as to create skin irritation.

Second (topical) Induction - One week after the first induction, a lint patch (2 cm × 4 cm) was saturated with 0.4 mL of test compound at 25% concentration in acetone or 0.4 mL of a undiluted HCA, and placed against the skin of the scapular area of the sensitized animals for 48 hours. The patches were held in place and covered with surgical tape. Control animals were treated similarly using acetone alone.

Challenge: Two weeks after the second induction the right flank of each guinea pig was clipped free of hair. A lint patch (2 cm × 2 cm) was saturated with 0.2 mL of test compound at 25% concentration in acetone or 0.2 mL of HCA at 10% concentration in acetone. This was then taped onto the right flank for 24 hours. After the patch was removed, the application area was wiped with absorbent cotton dipped in acetone. The application site was assessed 24 and 48 hours after patch removal on a scale of 0-3 where 0 is no reaction and 3 is a severe reaction.

Findings:

No signs of ill health or toxicity were observed in any of the animals during the experimental period. The body weights of all animals increased normally during the experimental period.

After challenge, no dermal reaction was observed in any of the 20 animals of the test group. No skin reactions were observed in the control groups. Slight to moderate erythema and oedema were observed in all 5 animals treated with HCA. From these results the sensitisation rate for S-2200 was estimated to be 0%.

Conclusion:

S-2200 does not require classification and labelling for sensitization according to 1272/2008/EEC.

B.6.2.7 Potentiation/interactions of multiple active substances or products

Not an EU data requirement.

B.6.2.8 Summary of acute toxicity

All conducted studies are compliant with the EU and OECD testing guidelines and Good Laboratory Practice (GLP).

Mandestrobin is of very low acute toxicity in the rat by the oral and dermal route and by inhalation. It is not a skin irritant and induced only mild eye irritation in the rabbit. Mandestrobin did not cause sensitisation in test animals. Therefore, no classification and labelling is required for acute toxicity, skin and eye irritation, and sensitisation.

Table B.6.2.8-1: Summary of acute toxicity studies with Mandestrobin

Type of study (reference)	Species	Sex	Vehicle	Results
Oral route [REDACTED] 2010a	Rat	♀	Aqueous methylcellulose	LD ₅₀ > 2000 mg/kg bw
Dermal route [REDACTED] 2010b	Rat	♂/♀	-	LD ₅₀ > 2000 mg/kg bw
Inhalation route [REDACTED] 2010	Rat	♂/♀	-	LC ₅₀ at 4 hours > 4.964 mg/m ³ (nose only)
Primary skin irritation [REDACTED] 2010a	Rabbit	♂	Corn oil	Non irritating
Eye irritation [REDACTED] 2010b	Rabbit	♂	-	Non irritating
Skin sensitization (M&K-test) [REDACTED] 2010c	Guinea pig	♀	Corn oil/ acetone	Non sensitising

B.6.3 Short term toxicity (Annex IIA 5.3)

B.6.3.1 Oral 28-day toxicity (rodents)

No 28 days study was submitted, not mandatory.

B.6.3.2 Oral 90-day toxicity (rodents)

Reference:	S-2200 Technical Grade: 13 Week Oral (Dietary) Administration Toxicity Study in the Rat.
Author(s), year:	██████████ 2011a
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0025
Guideline(s):	OECD 408 (1998), Directive 96/54/EC B.26 with Additional Testing for Neurotoxicity, EPA OPPTS 870.3100, and Japanese MAFF 12, Mousan 8147 (2000)
GLP:	Yes (laboratory certified by National Authority)
Deviations:	Minor deviations not considered to affect the validity of the study.
Validity:	Yes

Material and methods:

<i>Test Material:</i>	S-2200 Technical Grade (S-2200-TG)
Lot/Batch:	ST-0811G
Purity:	93.4%
Stability of test compound:	Expiry date after completion of treatment; stability in diet checked in preliminary study (Covance study number 0333/289)
Vehicle:	None. Test material was mixed directly into diet.
<i>Test animals:</i>	
Species:	Rat
Strain:	Wistar, ████████:WI(Han)
Age:	7 weeks at start of treatment
Weight at dosing:	170.1-214.7 g for males and 127.5-179.2 g for females
Source:	████████████████████
Diet:	SQC Rat and Mouse Maintenance Diet No1, Ground Fine <i>ad libitum</i>

The purpose of this study was to assess cumulative toxicity of mandestrobin when administered to rats in their diets for a period of 13 weeks. The results of the study should indicate potential target organs and should identify chemicals with neurotoxic potential.

Animal assignment and treatment:

Twelve rats per sex were allocated to each of 5 groups, and given mandestrobin in the diet at dietary concentrations of 0 (control), 800, 4000, 10000 and 20000 ppm. Doses were selected based on a prior three month feeding study (Sumitomo Chemical Co., Ltd., Osaka, Japan, Study number S1370).

Diet preparation, analysis and administration:

Dietary formulations were prepared weekly and the stability of the test article in diet was determined at 800 and 20000 ppm from a trial preparation prepared before the start of this study in ██████████ Study Number ██████████. Achieved concentration and homogeneity analyses, for all concentrations of the dietary formulations, were performed in Weeks 1, 5 and 13 and were considered acceptable as the mean concentrations were between 90 and 110% of the target concentration.

Clinical observations:

All animals were observed at the beginning and the end of the working day to ensure they were in good health and displaying no signs of overt toxicity. Each animal was given a detailed physical examination at weekly intervals. An individual record was maintained of the clinical condition of each animal.

Food consumption and body weight:

Individual body weights were recorded on day -7, before treatment on the first day of dosing, at weekly intervals thereafter and before necropsy.

The amount of food consumed by each cage of animals was measured once weekly from Week -1. For each cage of animals, consumption was calculated as g/animal/week and the average daily consumption over the entire treatment period (g/animal/day) was calculated for each group.

Weekly compound consumption was calculated as mg/kg bw/day and also as a total consumption over the entire treatment period (mg/kg bw/day).

Ophthalmoscopic observation:

Investigations were performed on all animals pre-treatment and on control (Group 1), and high dose (Group 5) animals in Week 12. A mydriatic agent was instilled into the eyes before examinations.

Functional Observation Battery:

All animals were subjected to a battery of behavioural tests and observations before initiation of treatment and at weekly intervals thereafter.

In Week 13, an assessment was made of sensory reactivity to stimuli, grip strength and motor activity.

Observations (weekly):

Before removal from the home cage, each animal was observed and evaluated for the following: Posture, Activity, Gait, Arousal upon opening cage, Convulsion, Excessive vocalisation, Tremor.

After removal from the home cage, each animal was observed for the following: Ease of removal, Ease of handling, Excessive vocalisation, Tremor, Convulsion, Palpebral closure, Exophthalmus, Lacrimation, Lacrimation type, Salivation, Respiration, Piloerection, Appearance of fur.

Open field observations performed weekly: Each animal was placed into an open field arena for 2 minutes and the following observations were recorded: Latency to first step, Posture, Arousal, Circling, Gait type, Gait type severity, Stereotypy, Tremor, Convulsion.

The number of rears, faecal boli and urine pools, faecal consistency and the presence of polyuria during this 2-minute period were also recorded.

Open field (Week 13 only): Approach response, touch response, tail pinch, air righting ability, pupillary response, corneal tactile reflex test, auditory startle response, hindlimb foot splay, forelimb and hindlimb grip strength.

Locomotor activity (Week 13 only): Locomotor activity of each animal was assessed in an automated photocell activity recorder for 30 minutes. Activity counts were recorded at 2 minute intervals.

Haematology and clinical chemistry:

Blood samples (nominally 2 x 0.5 mL) were withdrawn from all animals in Week 13, two days after completion of the functional observational battery investigations. Samples were collected from the lateral caudal vein after an overnight period without food.

Due to a number of clotted samples or samples of insufficient volume at the scheduled bleed it was necessary to re-sample several animals at necropsy in order to measure all required parameters. These samples were obtained from the abdominal aorta. The re-sampled data were excluded from the calculation of group mean, standard deviation and statistical analyses.

The following parameters were determined for blood taken into EDTA anticoagulant: haemoglobin concentration (Hb), packed cell volume (PCV), mean cell volume (MCV), mean cell haemoglobin

concentration (MCHC), total and differential white blood cell count, red blood cell count, absolute reticulocytes, mean cell haemoglobin (MCH), and platelet count.

In addition, the following parameters were determined on plasma derived from whole blood taken into trisodium citrate anticoagulant: prothrombin time, activated partial thromboplastin time.

Blood smears were routinely prepared from all blood samples taken for haematology appraisal. Manual examination was not undertaken on these samples. Bone marrow smears were prepared at necropsy. They were fixed in methanol but not examined.

Clinical chemistry:

The following parameters were determined on plasma derived from whole blood collected into lithium heparin anticoagulant: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyltransferase (GGT), sodium (Na), potassium (K), calcium (Ca), inorganic phosphorus (P), chloride (Cl), total protein, albumin, globulin, albumin/globulin ratio total cholesterol, glucose, urea, total bilirubin, creatinine, triglycerides.

Urine analysis:

Urine samples were collected from the first six numbered males and females in each group in Week 12. Samples were collected over a six-hour daytime period. Food and water were removed during collection. The urine collected from animal 99F (Group 4) in Week 12 (day 83) was of insufficient volume to perform all required assays. Therefore, repeat sampling was performed during Week 13 (day 85).

The following parameters were determined:

Colour, turbidity, microscopy of sediment volume, specific gravity, pH, protein, glucose, ketones, bilirubin, blood, urobilinogen.

Sacrifice and pathology:

All animals, including the one decedent (animal number 92, found dead day 33), were subjected to necropsy. Males were killed on day 95 and females were killed on day 96.

The scheduled necropsies were performed after overnight fasting. Following anaesthesia by sodium pentobarbitone, the animals were exsanguinated by the severing of major blood vessels. A full macroscopic examination was performed under the general supervision of a pathologist and all lesions were recorded.

All tissues in the list below from all animals were preserved in the appropriate fixative.

adrenals (†) (š)	ovaries (†) (š)
aorta (š)	pancreas (š)
bone marrow smear (femur) (a)	Peyer's patches (š)
brain (e) (†) (š)	Pituitary (š)
caecum (š)	prostate (š)
colon (š)	rectum (š)
duodenum (š) (f)	salivary glands—mandibular-sublingual-parotid (š)
eyes (b) (š)	sciatic nerves (š)
femur with bone marrow and articular surface (š)	seminal vesicles (š)
gross lesions (š)	skin (š)
harderian glands (š)	spinal cord cervical (š)
heart (†) (š)	spinal cord lumbar (š)
ileum (š)	spinal cord thoracic (š)
jejunum (š)	spleen (†) (š)
kidney (†) (š)	sternum with bone marrow (š)
larynx (š)	stomach (š)

liver (†) (š)	testes + epididymides (c) (†) (š)
lungs with mainstem bronchi and bronchioles (š)	thymus (†) (š)
mammary (š)	thyroids + parathyroids (š)
mandibular lymph nodes (š)	tongue (š)
mesenteric lymph nodes (š)	trachea (š)
muscle (quadriceps) (š)	urinary bladder (š)
nasal turbinates (š)	uterus (†) (š)
nasopharynx / nares (š)	vagina (š)
oesophagus (š)	
optic nerves (š)	

(†) organs were dissected free from fat and other contiguous tissue and weighed before fixation. Left and right organs were weighed together

(š) tissues from the control and high dose and from the decedent animals were embedded, sectioned, H&E stained, and examined microscopically by the study pathologist.

Fixative = neutral-buffered 10% formalin except where indicated by:

- a – methanol
- b - Davidson's fluid
- c Bouin's fixative

e - cerebrum, mid-brain and cerebellum/medulla

f - proximal region to include pyloric-duodenal junction

In addition, liver, spleen, thyroid, kidney (males), and gross lesions in the intermediate dose groups were microscopically examined by the study pathologist.

In addition, immunohistochemistry slides for the kidney for $\alpha_2\mu$ globulin from two control (3M and 9M) and two high dose males (49M and 59M) were examined.

Statistical analysis:

ANOVA and Levene's tests were used to screen if data qualified (on the basis of heterogeneity) for parametric analysis using Dunnett's test. Statistical significance reported for Hb, MCH, MCHC, neutrophils (N), AST, ALT, Na, K, Ca, total protein, albumin, total cholesterol, creatinine are the results of pairwise comparison by Dunnett's test. Where data were too heterogeneous for parametric testing, or contained values of "0", non-parametric analysis was applied (Kruskal-Wallis, Terpstra-Jonckheere, and Wilcoxon). Statistical results for monocytes (M), bilirubin and gamma-glutamyltransferase are Wilcoxon pairwise comparisons.

Although a trend test was performed, no-statistically-significant difference in pairwise comparison was basically considered to negate a positive trend test.

Findings:

Clinical signs and mortality:

There were no clinical signs noted that were considered to be directly attributable to S-2200 TG. There were no post-dosing observations.

There was one female decedent from the 4000 ppm dose group (animal 92F). Cause of death was not established, but there were no microscopic findings suggesting death was due to effects of S-2200 TG.

Body weight, body weight gain, and food consumption:

Males given 20000 ppm gained notably less weight (approximately 15%) over the duration of the treatment period than control. Suppression of absolute body weight (approximately 9% less than control) was also observed at the end of treatment for males given 20000 ppm.

In females, statistical significances from control in absolute body weight were only observed at some of the collection points. At the end of treatment, the body weight difference for females given 20000 ppm to controls

was approximately 4%. Suppression of body weight gain, although not statistically significant, was also observed at 20000 ppm (approximately 11%).

There was inter-group variation in weekly food consumption, but there was no consistent pattern in the data to indicate an effect of treatment.

Table B.6.3.2-1: Mean compound intake, body weight, body weight gain, and food consumption

Diet concentration (ppm)	Males					Females				
	0	800	4000	10000	20000	0	800	4000	10000	20000
Mean substance intake (mg test material/kg bw/day, week 1-13)	-	54	282.6	742.7	1544.6	-	61.6	320.1	788.5	1886.5
Group mean body weights (in g, week 13)	383.4	397.4	381.2	373.8	349.9	233.7	225.0	225.6	231.6	223.5
Group mean body weight gains (in g, start to week 13)	188.1	204.1	193.0	183.9	160.2	83.7	79.3	78.6	85.5	74.2
Mean food consumption (g/animal/day, week 1-13)	22.3	22.7	22.9	23.2	23.0	17.4	16.5	17.3	17.0	19.5

Ophthalmoscopy:

There were no treatment-related pathological findings in the ophthalmoscopic examinations.

Functional Observation Battery data:

There were no overt effects of treatment or differences from control in the indices of:

Posture, activity, gait, tremors, convulsions, excessive vocalisations, arousal, ease of removal or handling, palpebral closure, exophthalmus, lacrimation, type of lacrimation, salivation, respiration, piloerection, appearance of fur, or other changes.

In the open field observations, there were no effects on latency to first step, posture, arousal, circling, gait type/severity, stereotypy, tremors, convulsions, number of rears, number of faecal boli, number of urine pools, faecal consistency, or polyuria.

There were no overt effects on the approach response, touch response, tail pinch, air righting, pupillary response, corneal tactile response, auditory response, hindlimb foot splay, or hindlimb grip strength, or locomotor activity.

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 gender was considered incidental.

Table B.6.3.2-2: Functional observation battery data: Forelimb and hindlimb grip strength

Diet concentration (ppm)	Males					Females				
	0	800	4000	10000	20000	0	800	4000	10000	20000
Mean forelimb grip strength (kg) (SD)	1.023 (0.270)	1.008 (0.253)	1.052 (0.292)	1.070 (0.149)	0.760* (0.235)	0.751 (0.207)	0.787 (0.178)	0.707 (0.205)	0.757 (0.264)	0.742 (0.233)

	Males					Females				
Mean hindlimb grip strength (kg) (SD)	0.613 (0.126)	0.642 (0.191)	0.625 (0.178)	0.732 (0.132)	0.665 (0.118)	0.620 (0.124)	0.612 (0.157)	0.607 (0.161)	0.620 (0.117)	0.633 (0.373)

*p < 0.05 (ANOVA)

Haematology and clinical chemistry:

Some changes in haematological parameters reached statistical significance in the highest dose group. The changes were within the ranges of historical control data.

Historical control data was available from studies performed between March 2005 and January 2008.

For haematology and clinical chemistry, 15 different studies were available (203 and 223 animals). For organ weights, 16 different studies with 163 analysed animals were available.

Table B.6.3.2-3: Haematological findings

	Males						Females					
Diet conc. (ppm)	0	800	4000	10000	20000	HCD 95% range	0	800	4000	10000	20000	HCD 95% range
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	
Mono cytes (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	
Eosinophils (10 ⁹ /L)	0.2	0.2	0.2	0.1	0.1		0.1	0.1	0.1	0.1	0.1	
PLT (10 ⁹ /L)	891	953	914	1000	1014	688–1211	932	865	903	974	1004	
PT (s)	20.9	21.6	20.7	20.2	21.2		20.5	21.2	20.6	19.6	19.5	17.6–21.8

APTT (s)	24.1	21.2	23.3	28.5	27.6	16.0-26.5	20.5	20.2	21.7	22.1	22.7	14.0-23.5
Hb	Haemoglobin concentration											
MCH	mean cell haemoglobin											
MCHC	mean cell haemoglobin concentration											
PLT	platelets											
PT	prothrombin time											
APTT	activated partial thromboplastin time											
HCD	historical control data (95% interval)											
*	p < 0.05											
**	p < 0.01											

The changes in clinical chemistry parameters were mild and within the range of historical control data (if available).

A dose dependent increase in cholesterol levels was observed in both genders that reached statistical significance at 10000 and 20000 ppm.

Table B.6.3.2-4: Clinical chemistry parameters

	Males						Females					
Diet conc. (ppm)	0	800	4000	10000	20000	HCD	0	800	4000	10000	20000	HCD
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	
ALP (IU/L)	66	66	66	66	66		34	35	30	33	30	
γGT (IU/L)	2	2	2	2	5**		2	2	2	3	4**	
Total Protein (g/L)	69	70	70	71	71		71	70	70	74	77**	63–78
Albumin (g/L)	45	45	46	47	47		48	49	47	50	51*	34–56
Globulin (g/L)	24	25	24	24	24		24	21	23	24	25	
Albumin/Globulin ratio	1.9	1.8	1.9	2.0	2.0		2.0	2.3	2.1	2.1	2.0	
Total Bili rubin (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
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
Cl (mmol/L)	103	103	102	102	101		103	104	103	102	103	
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
Triglycerides (mmol/L)	0.96	0.93	0.81	0.83	0.64		0.73	0.78	0.71	0.7	1.17	

* p < 0.05, ** p < 0.01, *** p < 0.001

Urine analysis:

There were no differences between control and any treated group/sex in urine volume and specific gravity.

Sacrifice and pathology:

Organ weight increases were noted in the liver of treated males and females. Mean unadjusted and relative liver weights were outside historical control ranges for animals of this strain and age housed at this laboratory in males given ≥ 4000 ppm and females given ≥ 10000 ppm.

Large liver was recorded among males given 4000, 10000 or 20000 ppm and females given 20000 ppm. Mottled appearance was also recorded for the liver of males given 4000, 10000 or 20000 ppm. These changes generally correlated with findings seen microscopically.

Historical control data was available from studies performed between March 2005 and January 2008. For organ weights, 16 different studies with 163 analysed animals were available.

A slight increase in relative kidney weight was observed in males at the highest dose group.

In the thyroid gland, increased follicular cell hypertrophy was observed from 4000 ppm onwards in both sexes.

No significant weight changes or other gross pathological lesions attributable to treatment were detected in other organs.

Microscopic findings in controls were generally infrequent, of a minor nature and consistent with the usual pattern of findings in animals of this strain and age.

In the liver, hepatocyte hypertrophy was recorded for males and females given 4000, 10000 or 20000 ppm, with incidence and severity increasing with dose, and correlated with large and/or mottled liver. Hepatocyte hypertrophy was characterised by hepatocytes with increased levels of pale, slightly granular cytoplasm. In the most severely affected livers, the hypertrophy involved hepatocytes in all zones of the liver. In less severely affected livers, there was no clear zonal distribution of the enlarged hepatocytes.

The agonal congestion/haemorrhage recorded for some treated males was considered to be a consequence of the liver hypertrophy by the study authors.

	Males					Females				
Diet concentration (ppm)	0	800	4000	10000	20000	0	800	4000	10000	20000
Liver										
Organ weight (absolute and relative)										
Absolute 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 weight (Ratio %)	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										
No. examined	12	12	12	12	12	12	12	11	12	12
Large	0	0	2	4	10	0	0	0	0	4
mottled	0	0	1	3	5	0	0	0	0	0
Histopathology: hepatocyte hypertrophy										
No examined	12	12	12	12	12	12	12	11	12	12
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
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	0	0	1	3	5	2	0	0	0	0
Thyroid										
Follicular cell hypertrophy										
No. examined	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
Kidney										

Organ weight (absolute and relative)										
weight (g)	1.934	1.948	1.988	1.923	1.897	1.363	1.252	1.275	1.277	1.288
Relative weight (Ratio %)	0.524	0.512	0.546	0.545	0.560	0.599	0.569	0.578	0.575	0.589
Hyaline droplets										
No. examined	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
α 2u globulin positive reaction by immunohistochemistry										
No. examined	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

* p < 0.05, ** p < 0.01, *** p < 0.001

Grade 1 = minimal, Grade 2 = slight, Grade 3 = moderate, Grade 4 = moderately severe, Grade 5 = severe

Conclusion:

In the original study report, the study authors set the NOAEL at 10000 ppm. Increases in liver weight, hepatocellular hypertrophy, and follicular cell hypertrophy in the thyroid gland observed from 4000 ppm were considered to be secondary to liver enzyme induction and increased catabolism of thyroid hormones in a manner similar to the observed effects induced by phenobarbital (position paper ██████████ 2012a and study A ██████████ 2012e).

The hyaline droplets (increased incidence from 4000 ppm onwards) in the kidneys of male rats contained α 2u-globulin and were thus considered to be not relevant for human risk assessment.

The RMS shares the opinion of the study authors on the mechanistic basis of the observed effects. There is clear evidence (summarized in the position paper 'The toxicological relevance of the liver and thyroid alterations observed in rats treated with S-2200 TG based on mode of action' by ██████████ 2012a) that the liver and thyroid alterations are caused by a phenobarbital like mode of action and are not of relevance for human risk assessment.

However, the RMS proposes to set the study NOAEL for rats at 4000 ppm (282.6 ♂ and 320.1 ♀ mg/kg bw/day) based on liver weight increase (21.8% absolute and 27.9% relative increase in males, and 17% absolute and 19.6% relative increase in females), hepatocellular hypertrophy, and thyroid follicular cell hypertrophy, increased cholesterol levels in both genders at 10000 ppm.

Reference:	Amended Final Report 1: S-2200 Technical Grade: 13 Week Oral (Dietary) Administration Toxicity Study in the Mouse
Author(s), year:	██████████ 2011b
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0023
Guideline(s):	OECD 408, Directive 2001/59/EC (Annex 5D) B.26, EPA OPPTS 870.3100, and Japanese MAFF 12 Nousan 8147 (2000), 2-1-9, 90 day repeated oral toxicity studies
GLP:	Yes (laboratory certified by National Authority)

Deviations:	No
Validity:	Yes

Material and methods:

Test Material: S-2200 Technical Grade (S-2200 TG)

Lot/Batch: ST-0811G

Purity: 93.4%

Stability of test compound: The stability of the test article in diet was confirmed before the start of this study under Covance Study Number 0333/289

Vehicle: None. Test material was mixed directly into diet.

Test animals:

Species: Mouse

Strain: CD-1 (ICR)

Age: approximately 7 weeks at start of treatment

Weight at dosing: 30.6 – 38.1 g (males); 22.2–28.9 g (females)

Source: [REDACTED]

Diet: Finely ground SQC Rat and Mouse Maintenance Diet No 1 (Special Diets Services Ltd, Witham, UK) *ad libitum*

Animal assignment and treatment:

Twelve mice per sex were allocated to each of four dose groups, and offered mandestrobin in the diet at fixed concentrations of 0 (control), 1750 ppm, 3500 ppm, and 7000 ppm for 13 weeks. Animals were housed in groups of three and were provided with environmental enrichment.

Table B.6.3.2-7: Diet concentration and daily dose levels

	Males				Females			
Diet concentration (ppm)	0	1750	3500	7000	0	1750	3500	7000
mg test material/kg bw/day	-	204.1	404.9	807.3	-	251.8	529.1	1111.2

Diet preparation, analysis and administration:

The test article was formulated weekly as a diet mix in SQC Rat and Mouse Maintenance Diet No 1 (Ground Fine). Due to unused diet being discarded in error during Week 1, a new batch of each formulation (all groups) was prepared on day 4, in order to provide sufficient diet to complete Week 1. Duplicate samples were taken from the top middle and bottom of the mixer after diet preparation on week 1, and week 13 of the dosing period, and achieved concentrations were between 90 and 110% of the target concentration.

Clinical observations:

All animals were observed at the beginning and the end of the working day to ensure they were in good health.

All animals were observed daily for signs of ill health or overt toxicity. In addition, each animal was given a detailed physical examination at weekly intervals. An individual record was maintained of the clinical condition of each animal.

Food consumption and body weight:

Individual body weights were recorded on day -7, before treatment on the first day of dosing, at weekly intervals thereafter, and before necropsy. In addition, body weights were inadvertently recorded for all animals on day 4. These data are maintained within the raw data but not reported.

The amount of food consumed by each cage of animals was measured once weekly from Week -1.

Consumption was calculated as g/animal/week and as average daily consumption over the entire treatment period (g/animal/day).

Weekly compound consumption was calculated as mg/kg bw/day and also as a total consumption over the entire treatment period (mg/kg bw/day).

Haematology and clinical chemistry:

Blood samples were withdrawn in Week 13 from non-fasted, anaesthetised animals. Samples from the first six numbered males and females in each group were analysed for haematology parameters and blood taken from the last six numbered animals were analysed for blood biochemistry parameters. Due to samples being clotted or of insufficient volume, it was necessary to re-sample animals 14 (Group 2M), 30 (Group 4M) and 65 (Group 2F) at necropsy.

The following parameters were determined on blood taken into EDTA anticoagulant: red blood cell count, absolute reticulocytes, packed cell volume (PCV), mean cell volume (MCV), haemoglobin concentration (Hb), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), platelet count (PLT), and total and differential white blood cell count.

Blood smears were routinely prepared from all haematology samples. Manual examination was not undertaken on these samples.

The following parameters were determined on plasma derived from whole blood collected into heparin anticoagulant: aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma glutamyltransferase, sodium, potassium, calcium, inorganic phosphorus, chloride, total protein, albumin, globulin, albumin/globulin ratio, total cholesterol, glucose, urea, total bilirubin, creatinine, triglycerides.

Urine analysis:

Not performed.

Sacrifice and pathology:

All animals were subjected to necropsy.

A full macroscopic examination was performed under the general supervision of a pathologist and all lesions were recorded.

All tissues in the tissue list below from all animals were preserved in the appropriate fixative.

adrenals (†) (š)	ovaries (†) (š)
aorta (š)	pancreas (š)
brain (†) (š)	Peyer's patch (š)
caecum (š)	pituitary (š)
colon (š)	prostate (š)
duodenum (š)	rectum (š)
eyes (š)	salivary glands – mandibular, sublingual,
femur with bone marrow and stifle joint (š)	parotid (š)
gall bladder (š)	sciatic nerves (š)
gross lesions (š)	seminal vesicles (š)
harderian glands (š)	skin and subcutaneous tissue (š)
heart (†) (š)	spinal cord cervical (š)

ileum (š)	spinal cord lumbar (š)
jejunum (š)	spinal cord thoracic (š)
kidney (†) (š)	spleen (†) (š)
larynx (š)	sternum with bone marrow (š)
liver (†) (š)	stomach (š)
lungs (š)	testes + epididymides (e) (†) (š)
mammary (š)	thymus (†) (š)
mandibular lymph nodes (š)	thyroids + parathyroids (š)
mesenteric lymph nodes (š)	tongue (š)
muscle (quadriceps) (š)	trachea (š)
nasal cavity (š)	urinary bladder (š)
nasopharynx / nares (š)	uterus including cervix (†) (š)
oesophagus (š)	vagina (š)
optic nerves (š)	zymbal glands (g)

Organ weights

Animals were weighed before necropsy. The organs denoted by (†) from all animals in the tissue list were dissected free from fat and other contiguous tissue and weighed before fixation. Left and right organs were weighed together.

Histology

Gross lesions from all animals and tissues denoted by (š) in the tissue list from control (Group 1) and high dose (Group 4) animals were embedded in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin. Liver, spleen, kidney, thyroid and caecum from all animals in Groups 2 and 3 were processed to slide stage.

Additional tissues were also included to complete standard tissue blocks.

Pathology

Gross lesions from all animals and tissues denoted by (š) in the tissue list from control and high dose animals were examined microscopically by the Study Pathologist. In addition, the liver and thyroid from all low and intermediate dose (Groups 2 and 3, respectively) animals were examined.

Statistics:

Absolute body weights, body weight gains, necropsy body weights, organ weights and organ to necropsy body weight ratios were analysed using one-way analysis of variance (ANOVA), separately for each sex. Levene's test for equality of variances among the groups was performed. Where this showed no evidence of heterogeneity ($p \geq 0.01$), pairwise comparisons with control were made using Dunnett's test. A linear contrast was used to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was reported only where none of the pairwise comparisons was significant. Food consumption, haematology and blood biochemistry variables were analysed using two-way analysis of variance (ANOVA). Levene's test for equality of variances across groups, between sexes and for any interaction was performed and where these tests showed no evidence of heterogeneity ($p \geq 0.01$ for all 3 tests), pairwise comparisons with control were made, for each sex separately, using Dunnett's test. For each sex separately, a linear contrast was used to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was only reported where none of the pairwise comparisons was significant.

Where Levene's test showed evidence of a sex effect only ($p < 0.01$), the data were analysed using one-way ANOVA for each sex separately.

Non-parametric analyses were performed for clinical pathology parameters with values above or below the limit of the assay and for Monocytes, Eosinophils, Basophils and Large Unstained Cells. The non-parametric

methods employed were the Kruskal-Wallis ANOVA, the Terpstra-Jonckheere test for a dose related trend and the Wilcoxon rank sum test for pairwise comparisons. Where the Kruskal-Wallis ANOVA was not significant, the pairwise comparisons were not reported in order to protect the Type I error. Macroscopic and microscopic findings data were supplied for analysis. Male and female data were analysed separately. All tests were performed with a two-sided risk. For each macroscopic finding, comparisons were made between the incidence in each of the treated groups and that in the control group, using Fisher's Exact test. For microscopic findings, comparisons were made between the incidence in each of the treated groups and that in the control group. Where a given finding had more than two categories, the Wilcoxon-Mann-Whitney rank sum test was performed; where only two categories were observed, Fisher's Exact test was used.

Findings:

Clinical signs and mortality:

All animals survived to the scheduled sacrifice. There were no clinical signs noted that were considered to be directly attributable to S-2200 TG. There were no post-dosing observations.

Food consumption:

Over the entire treatment period, there were no notable effects on food consumption (see Table B.6.3.2-8).

Body weight and body weight gain:

There were no statistically significant effects on body weight or body weight gain for males.

In females, when compared to controls, mean weight gain was lower in the treated groups (90%, 89% and 73% of controls for animals offered 1750, 3500 and 7000 ppm, respectively) with differences for animals offered 7000 ppm being statistically significant at the $p < 0.05$ level.

The animals did not show signs of systemic toxicity.

Table B.6.3.2-8: Food consumption, body weight and body weight gain

	Males				Females			
Diet concentration (ppm)	0	1750	3500	7000	0	1750	3500	7000
Food consumption, mean weeks 0-13 (g/mouse/day)	5.2	5.0	4.9	5.0	4.6	4.5	4.8	4.9
Terminal bodyweight (g)	43.6	43.7	42.7	44.4	33.5	32.3	32.1	30.7
Bodyweight gain, weeks 0-13 (g)	10.5	9.6	8.9	9.7	8.3	7.5	7.4	6.1*

Level of statistical significance: * $p < 0.05$

Haematology and clinical chemistry:

S-2200 TG had no obvious or adverse effects on haematology parameters.

Statistically significant decreases in haemoglobin concentration (Hb) and packed cell volume (PCV) were seen in males offered 3500 ppm. In the absence of a dose response and mean values being within historical control ranges, these changes are considered to be incidental.

Statistically significant increased platelet counts (PLT) in males offered 1750 or 7000 ppm were considered not to be treatment related, since the increased mean platelet values were not dose-responsive and the value at 7000 ppm was within the historical control range.

The significant dose-responsive changes seen for mean cell haemoglobin concentration (MCHC) were considered not to be treatment related as the differences observed were small, and none of the pairwise comparisons were significant.

S-2200 TG had no obvious or adverse effects on blood biochemistry test results. The following statistically significant difference between control and treated female animals was observed:

The apparent statistically significant decrease (15.7%) in mean glucose for females given 7000 ppm was considered not to be toxicologically significant, since the value at 7000 ppm was within the historical control range.

The significant dose-responsive changes in chloride (Cl), urea (UREA) and total bilirubin (T Bili) were considered not to be treatment related, since none of the pairwise comparisons were significant.

Table B.6.3.2-9: Haematology and clinical chemistry parameters

Diet concentration (ppm)	Males					Females				
	0	1750	3500	7000	HCR	0	1750	3500	7000	HCR
Haematology										
Hb (g/dL)	14.6	14.3	13.2 **	14.1	12.0-15.1	14.6	14.9	14.8	14.1	-
PCV (%)	44.8	44.6	41.6 *	44.9	38.0-50.4	45.1	45.8	45.8	44.3	-
PLT (10 ⁹ /L)	1292	1756 **	1450	1666 *	941-1721	1555	1351	1390	1526	-
MCHV (g/dL)	32.3	32.1	31.7	31.5	-	32.4	32.5	32.3	31.9	-
Clinical pathology										
GLUC (mmol/L)	8.9	9.7	9.3	9.1	7.9-12.0	10.8	10.3	9.3	9.1*	-
Cl (mmol/L)	109	109	109	107	-	112	112	112	112	-
Urea (mmol/L)	6.4	6.3	6.7	7.3	-	7.1	6.8	6.6	6.6	-
T Bili (μmol/L)	2.5	1.8	2.1	1.8	-	1.7	1.7	1.7	1.9	-

* p < 0.05, ** p < 0.01, ***p < 0.001. HCR : Historical Control Range

Sacrifice and pathology:

Organ weight increases were noted in the liver of treated males and females.

Mean unadjusted and relative liver weights were increased in all treated groups (both sexes) and, in the majority of cases, were outside historical control ranges for animals of this strain and age housed at this laboratory.

Although there were no histopathological findings in the liver, this finding is considered to be an adaptive change related to drug metabolism and not an adverse toxic effect. The mode of action for the liver weight increase is considered to be due to liver enzyme induction (CYP2B), via activation of the constitutive androstane receptor by S-2200 TG, as evidenced by mode of action work. Because an adaptive mechanism is ascribable for the increase in liver weights rather than a pathological effect, the increases may be considered non-adverse, particularly in the absence of any biochemical or histological markers of liver pathology. A statistically significant increase in relative to body (24%) spleen weight was seen in males offered 3500 ppm, when compared to control. However, in the absence of a dose response, these changes are considered not to be treatment related.

Statistically significant differences between the control and treated animals for all other organs were considered incidental as they reflect normal individual variation.

Weights of all other organs were not affected by treatment.

Most tissues were macroscopically and microscopically unremarkable and the findings seen were generally consistent with the usual pattern of findings in mice of this strain and age.

There were no macroscopic and microscopic findings due to effects of mandestrobin.

Table B.6.3.2-10: Organ weights and histopathology of the liver

Diet concentration (ppm)	Males				Females			
	0	1750	3500	7000	0	1750	3500	7000
Organ weights								
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
Ratio (%) to body 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								
Focal necrosis	2	1	4	5	3	2	6	3
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
Haemopoiesis	0	1	0	0	0	0	0	0

* p < 0.05, ** p < 0.01, ***p < 0.001

Conclusion:

In a 13 week dietary toxicity study in the mouse (0, 1750, 3500 and 7000 ppm S-2200 TG), liver weight increases were noted in all treated groups in both sexes. Because an adaptive mechanism is ascribable for the increase in liver weights rather than a pathological effect, the increases may be considered non-adverse, particularly in the absence of any biochemical or histological markers of liver pathology.

In the absence of other treatment-related adverse findings, the No-Observed-Adverse-Effect-Level (NOAEL) is considered to be 7000 ppm (807.3 mg/kg bw/day for males and 1111.2 mg/kg bw/day for females, respectively).

B.6.3.3 Oral 90-day toxicity (dog)

Reference:	Amended Final Report 2: S-2200 Technical Grade: 13 Week Oral (Dietary) Administration Toxicity Study in the Dog
Author(s), year:	█ 2012d
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0024
Guideline(s):	OECD 409, US EPA OPPTS 870.3150, JMAFF 2-1-9, 90-day repeated oral toxicity studies
GLP:	Yes (Lab certified by National Authority)
Deviations:	Minor deviations not affecting the validity of the study
Validity:	Yes

Material and methods:

Test Material:	S-2200 Technical Grade (S-2200 TG)
Lot/Batch No.:	ST-0811G
Purity:	93.4%
Stability of test compound:	Expiry date stated as 21 November 2011 (after end of treatment)
Vehicle:	None
Test animals:	
Species:	Dog
Strain:	Beagle
Age:	20 – 24 weeks at initiation of dosing (males and females)
Weight at dosing:	Males: 10.21 – 12.01 kg; Females: 8.38 – 10.74 kg
Source:	[REDACTED]
Diet:	5L66 Certified High Density Canine Diet (IPS Product Supplies Ltd, London, UK); 300 g/day

Animal assignment and treatment:

Sixteen healthy animals per sex were selected based on the results of pre-test examinations. Animals were randomly distributed into 4 animals/sex/group based on the most recent body weight data, except for separation of familial animals.

Diet preparation, analysis and administration:

The test article was administered orally by incorporation into the diet. Each animal had *ad libitum* access to the dietary formulations for approximately 8 hours/day for at least 13 weeks. Control animals received untreated diet. The high dose was selected as a limit dose of approximately 1000 mg/kg bw/day.

Fresh batches of dietary formulation were prepared weekly. All diets were hand mixed with mains water into clumps of irregular size and shape. The formulations were stored at room temperature in the dark before administration.

Accuracy and homogeneity of preparation was confirmed by analysis. Homogeneity of mixing was determined at the top, middle, and bottom of each mixture. Samples were taken before treatment, and on weeks 1 and 13. Stability in diet for at least 9 days at ambient temperature was established before the study started.

Table B.6.3.3-1: Diet concentration and daily dose levels

	Males				Females			
Diet concentration (ppm)	0	4000	12000	40000	0	4000	12000	40000
mg test material/kg bw/day	0	90.9	267.8	933.1	0	102.7	304.4	820.4

Clinical observations:

All animals were observed daily for signs of ill health or overt toxicity. In addition, each animal was given a daily detailed physical examination. Animals were observed approximately 1 and 4 hours after presentation of the diet. One control male (animal number 3) received topical and oral antibiotics for presumed bites.

All animals were subjected to a battery of behavioural tests and observations before initiation of treatment and at once weekly intervals thereafter. The unrestrained behaviour of each animal was observed in an examination room for approximately 1 to 2 minutes.

Observations included the following:

Level of consciousness	General level of activity
Head posture	Head co-ordination
Neck posture	Circling
Limb posture (standing)	Gait
Tremors	Unusual behaviour

Visual fixating response test and auditory startle tests were assessed at the end of the observation period.

Following the unrestrained observations all animals were subjected to neurological examinations each week.

Examinations included the following:

Proprioceptive paw positioning	Righting postural reaction
Hemihopping postural reactions	Wheel barrowing postural reactions
Muscle tone	Palpebral closure
Nictitating membrane	Palpebral reflex
Eye position	Eye movement
Pupil size	Pupillary light reflex
Lacrimation	Salivation
General reactions	General clinical observations

Body weight, general clinical signs, heart rate and rectal temperatures were taken as part of the data set for assessment of neurobehavioral effects of test articles.

Food consumption and body weight:

Food consumption for all animals was measured daily from the time of allocation until termination of the dosing period.

Weekly compound consumption was calculated as mg/kg bw/day and also as a total consumption over the entire treatment period (mg/kg bw/day).

Body weight was measured in all animals once weekly throughout the dosing period, from Week -3 until termination.

Ophthalmoscopic observation:

A mydriatic agent was instilled into the eyes before examinations. On day -2, a mydriatic agent was administered to all animals but no ophthalmic examination took place. Eyes of all animals were observed once pre-test (day -1) and at Week 12.

Haematology and Clinical Chemistry:

Blood was sampled from all animals before the start of treatment (Week -1) and at weeks 4, 8, and 13 of the administration period (sampling was performed after animals were fasted overnight). Samples were taken from jugular vein and collected into EDTA (for cell parameters), trisodium citrate (for clotting function), and lithium heparin (blood chemistry).

The blood samples collected into trisodium citrate anticoagulant from one male and three female in Week 8 (day 52) were unsuitable for analysis, so blood samples were recollected and re-examined on day 56. Additionally, the prothrombin time results from two females were estimated due to the analysis software having difficulties interpreting the data. The blood sample collected into lithium heparin anticoagulant in Week 13 (day 87) for one male was unsuitable for analysis; so sampling was repeated on day 92.

The following parameters were examined:

Haematology: Haemoglobin concentration (HGB), Red blood cell count (RBC), Mean cell haemoglobin (MCH), Mean cell haemoglobin concentration (MCHC), Mean cell volume (MCV), Packed cell volume (PCV),

Platelet count (PLT), Total and differential white blood cell count, Reticulocytes, Prothrombin time (PT), and Activated partial thromboplastin time (APTT)

Blood Chemistry: Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), γ -Glutamyl transferase (GGT), Sodium (Na), Potassium (K), Calcium (Ca), Inorganic phosphorus (IP), Chloride (Cl), Triglycerides (TG), Total protein (TP), Albumin (Alb), Globulin (G), Albumin-globulin ratio (A/G), Total cholesterol (Cho), Glucose (Glu), Urea (U), Total bilirubin (Bil), and Creatinine (Cre).

Urine analysis:

Urine samples were collected overnight from all animals before the start of treatment (Week -1) and in Weeks 3, 7, and 12 of the administration period; food and water were removed during collection. Due to spillage, urine volume was not measured for one male in Week 3.

The following parameters were examined: Urinary volume (Vol), Colour (Col), Turbidity, Specific Gravity (S.G.), pH, Protein (Pro), Glucose (Glu), Ketones (Ket), Urobilinogen (Uro), Bilirubin (Bil), Blood, and microscopic sediment.

Sacrifice and pathology:

All animals survived until scheduled sacrifice, and were sacrificed with sodium thiopentone, and exsanguinated from the major blood vessels.

The following organs were weighed: adrenals, brain, heart, kidneys, liver, ovaries, pituitary, prostate, spleen, testes, epididymides, thymus, thyroids (with parathyroids), and uterus (including cervix). Bilateral organs were weighed together.

Histopathological examination: After necropsy, the following organs and tissues collected from all animals were fixed in 10% neutral buffered formalin, except bone marrow smears which were fixed in methanol, testes and epididymides which were fixed in Bouin's solution and eyes/optic nerves fixed in Davidson's fluid.

Adrenals	Oesophagus
Aorta	Ovaries
Bone and marrow (sternum)	Oviducts
Brain	Pancreas
Caecum	Pituitary
Colon	Prostate
Duodenum	Rectum
Eyes and Optic nerves	Salivary gland – mandibular
Bone with marrow (femur) and articular surface	Salivary gland – Sublingual, parotid
Gallbladder	Sciatic nerves
Gross lesions	Skin and subcutaneous tissue
Heart	Spinal cord (cervical, thoracic, lumbar)
Ileum	Spleen
Jejunum	Sternum with bone marrow
Kidneys	Stomach
Lacrimal glands	Testes and epididymides
Larynx	Thymus
Liver	Thyroids and parathyroids
Lungs with bronchi and bronchioles	Tongue
Mammary glands	Trachea
Mandibular lymph nodes	Ureters
Mesenteric lymph nodes	Urinary bladder
Muscle	Uterus (including cervix)
Nasal cavity	Vagina
Nasopharynx	

Statistical analysis:

Absolute body weights, body weight gains, necropsy body weights, food consumption, heart rates, body temperatures, haematology, blood biochemistry and urine analysis variables were analysed using two-way analysis of variance (ANOVA). Levene's test for equality of variances across groups, between sexes and for any interaction was performed. Where these tests showed no evidence of heterogeneity ($p \geq 0.01$ for all 3 tests), pairwise comparisons with control were made, for each sex separately, using Dunnett's test. For each sex separately, a linear contrast was used to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was reported only where none of the pairwise comparisons was significant.

Where Levene's test showed evidence of group effects, sex effects or a sex by group interaction ($p < 0.01$), the data were analysed either using the same methods after applying a log-transformation or using non-parametric methods. The non-parametric methods employed were the Kruskal-Wallis ANOVA, the Terpstra-Jonckheere test for a dose related trend and the Wilcoxon rank sum test for pairwise comparisons. Where the Kruskal-Wallis ANOVA was not significant, the pairwise comparisons were not reported in order to protect the Type I error.

The non-parametric analyses described above were also performed for clinical pathology parameters with values above or below the limit of the assay and for Monocytes, Eosinophils, Basophils and Large unstained cells.

Organ weights and organ to necropsy body weight ratios were analysed using oneway analysis of variance (ANOVA), separately for each sex. Levene's test for equality of variances among the groups was performed. Where this showed no evidence of heterogeneity ($p \geq 0.01$), pairwise comparisons with control were made using Dunnett's test. A linear contrast was used to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was reported only where none of the pairwise comparisons was significant.

Where Levene's test showed evidence of heterogeneity ($p < 0.01$), the data were analysed either using the same methods after applying a log-transformation or using non-parametric methods, as previously described.

Organ weights were also analysed using Analysis of Covariance (ANCOVA) and Dunnett's test, for each sex separately, using the necropsy body weight as covariate. This analysis depends on the assumption that the relationship between the organ weights and the covariate is the same for all groups and the validity of this assumption was tested. Where the test for equality of slopes failed (male thyroid/parathyroid weights; $p < 0.01$), no analysis was performed. Levene's test for equality of variances across the groups was also performed. Where this showed evidence of heterogeneity (male prostate weights; $p < 0.01$), no further analysis was performed.

Macroscopic and microscopic findings data were supplied for analysis. Male and female data were analysed separately. All tests were performed with a two-sided risk. For each macroscopic finding, comparisons were made between the incidence in each of the treated groups and that in the control group, using Fisher's Exact test.

For microscopic findings, comparisons were made between the incidence in each of the treated groups and that in the control group. Where a given finding had more than two categories, the Wilcoxon-Mann-Whitney rank sum test was performed; where only two categories were observed, Fisher's Exact test was used.

Findings:

Clinical signs and mortality:

All animals survived the duration of the study.

Thin appearance was observed in three males and one female offered 40000 ppm.

Signs noted at the daily clinical examinations, included red eyes/ears/mouth, sores/lesions and earwax.

These were considered incidental as they were either infrequent or were 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 offered ≥ 4000 ppm, up to eight hours following presentation of the diets. Emesis was seen in two males offered 40000 ppm in Week 1, up to eight hours following presentation of the diets.

Body weight, body weight gain, and food consumption:

There were statistically significant decreases in mean body weight or reductions in mean body weight gain for males and females at doses of 40000 ppm.

Body weight suppression from concurrent control values became statistically significant from Week 6 for males at doses offered 40000 ppm and from Week 5 for females offered 40000 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% to a gain of 11.3%. Following 13 weeks of treatment, most females at doses of 40000 ppm had similar or slightly lower body weights than initial values. However, there was an overall decrease in mean body weight principally as a result of one female exhibiting a loss of 21%.

No statistically significant alterations in mean body weight were noted for males or females offered 4000 or 12000 ppm, when compared with concurrent controls.

Food consumption in the control group, and for both sexes at doses of 4000 or 12000 ppm, was comparable and relatively stable over the treatment period (Week 1 to Week 13). When compared to concurrent controls, there was an overall reduction in mean food consumption for males and females at doses of 40000 ppm, despite a high degree of inter-animal variability, this correlated with reductions in body weight or body weight gain.

In males offered 40000 ppm, mean consumption was similar to concurrent controls over Week 1 to Week 4. Reduced food consumption was apparent in subsequent weeks and this was statistically significant ($p < 0.001$) when compared to concurrent controls.

Consumption was consistently lower than with concurrent controls at all weekly measurement intervals for females offered 40000 ppm. Over the duration of the treatment period, the difference in consumption was statistically significant ($p < 0.001$) and marked.

Table B.6.3.3-2: Mean body weight, body weight gain, and food consumption

	Males				Females			
	Dose (ppm)				Dose (ppm)			
	0	4000	12000	40000	0	4000	12000	40000
Terminal body weight (kg)	14.07	13.57	13.61	11.16**	12.36	11.94	11.93	9.03***
Bodyweight gain (kg)	2.84	2.86	2.55	0.14***	3.01	2.38	2.47	-0.57***
Food consumption (g/animal/week)	2110	2111	2111	1947***	2100	2109	2058	1430***
Thin appearance	0/4	0/4	0/4	3/4	0/4	0/4	0/4	1/4

*** $p < 0.001$ different from control

** $p < 0.01$ different from control

Ophthalmoscopy:

There were no ophthalmic abnormalities noted pre-treatment or in Week 12.

Functional Observation Battery data:

Occasional statistically significant differences in mean body temperature were recorded for both sexes at doses of 12000 ppm and in males at doses of 40000 ppm, when compared to concurrent controls. In addition, in Week 3, there was a statistically significant relationship between increasing dose and response for females. These changes were transient, inconsistent and considered not to be related to treatment by the study authors.

Statistically significant differences from concurrent controls in mean heart rate were recorded sporadically for males at doses of 4000 ppm, females at doses of 12000 ppm, and there was a statistically significant relationship between increasing dose and response for females in Week 13. These changes were transient, inconsistent and considered not to be related to treatment.

There were no overt differences between control and treated animals in the incidences of observations recorded during unrestrained behaviour.

Weekly neurological examinations revealed incidences of abnormal proprioceptive paw positioning, abnormal righting postural reactions, abnormal hemihopping postural reactions, abnormal wheel barrowing postural reactions and decreased muscle tone in a few males and/or females at doses of 40000 ppm, and protrusion of the nictitating membrane in males and females at doses \geq 4000 ppm. In addition, there were single occurrences of abnormal hemihopping/wheel barrowing postural reactions in one female at doses of 4000 ppm in Week 9, and abnormal proprioceptive paw positioning or abnormal hemihopping postural reactions in animals offered 12000 ppm in Week 4. These recorded observations were generally infrequent, occasionally observed in control animals and are considered in some cases likely to be a result of reduced body condition.

Overall, there were no findings from any assessment indicative of any neurotoxicological effect of the test article.

Haematology and clinical chemistry:

Haematology:

Mean platelet count displayed a statistically significant increase in males at doses of 40000 ppm in Week 4 (35%) and in both sexes at doses of 40000 ppm in Weeks 8 and 13 (45 – 69%), when compared to concurrent controls.

Sporadic differences reached statistical significance in other parameters, however, these changes were not considered treatment-related since they were slight or comparable to pre-test values.

Blood clinical chemistry:

At the mid dose and above, in males and/or females, statistically significant increases in alanine aminotransferase (178-435%), alkaline phosphatase (74-249%), and γ -glutamyl transferase (67-167%) activities were observed during weeks 4, 8 and 13. Statistically significant increases in aspartate aminotransferase activity (ca. 56%) and triglyceride concentration (76-82%) were observed at 40000 ppm only, during weeks 4, 8 and/or 13. Statistically significant decreases were seen mean albumin (ca. 25% males and females), A/G ratio (ca. 44% males and females), total cholesterol (52% males, 27% females) and glucose (21% males, 12% females) at 40000 ppm during weeks 4, 8 and/or 13.

There were no other blood chemistry parameters which showed a significant association with treatment.

Table B.6.3.3-3: Haematology and clinical chemistry parameters

	Males				Females			
	Dose (ppm)				Dose (ppm)			
	0	4000	12000	40000	0	4000	12000	40000
Haematology (week 13)								
Mean Cell Volume (fL)	64.5	66.1	67.4	68.2	67.4	66.9	67.1	69.2
White blood cell count (10 ⁹ /L)	9.4	9.6	9.9	12.6*	9.1	9.6	10.2	10.6
Neutrophils (10 ⁹ /L)	4.7	5.1	4.9	7.3**	4.9	5.2	5.2	5.4
Monocytes (10 ⁹ /L)	0.6	0.6	0.6	1.1*	0.6	0.5	0.5	0.6
Platelets (10 ³ /μL)	314	375	366	454	285	364	348	481*
Blood clinical 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***
γGT (IU/L)	4	3	3	10*	3	4	5	8*
TG (mmol/L)	0.34	0.43	0.42	0.62**	0.38	0.39	0.42	0.67**
ALB (g/L)	36	32	34	27***	37	37	35	29***
A/G	1.8	1.6	1.7	1.0***	2.4	2.0	2.0	1.4***
CHO (mmol/L)	6.7	5.5	5.6	3.2***	6.7	6.1	5.2	4.9*
G (g/L)	20	22	20	29**	16	18	18	22
BIL (μmol/L)	2.2	2.2	1.8	2.9	2.0	2.0	1.4	2.9
Cre (μmol/L)	62	66	64	54	57	63	68*	61
GLU (mmol/L)	5.8	5.8	5.9	4.6***	5.7	5.9	5.9	5.0*
K (mmol/L)	4.3	4.4	4.5	4.8	4.2	4.1	4.5	4.7
Ca (mmol/L)	2.80	2.72	2.75	2.64**	2.76	2.78	2.75	2.72

* p < 0.05 different from control,

** p < 0.01 different from control,

*** p < 0.001 different from control

Urine analysis:

In the highest dose group in week 12, urine volume was decreased in male animals, and specific gravity was slightly increased in both sexes (statistically significant in a dose response test).

Table B.6.3.3-4: Urine analysis (week 12)

	Males				Females			
	Dose (ppm)				Dose (ppm)			
	0	4000	12000	40000	0	4000	12000	40000
Volume (ml)	339	356	255	179	175	169	136	108
Specific gravity	1.024	1.024	1.026	1.059	1.037	1.035	1.039	1.053

Sacrifice and pathology:

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 40000 ppm generally correlated with minimal gain or body weight loss, reduced food consumption, and decreased muscle tone recorded during the functional observational battery.

Pathological findings in the liver were consistent with increases in the weight of this organ in S-2200 TG-treated males and females, and with increased aspartate aminotransferase activities, triglyceride levels seen in both sexes offered 40000 ppm, increased gamma glutamyl transferase activities in females offered 12000 ppm and both sexes offered 40000 ppm, increased alanine aminotransferase and alkaline phosphatase levels seen in both sexes offered 12000 or 40000 ppm and the decreases in total cholesterol seen in females offered 12000 ppm and in both sexes offered 40000 ppm. Therefore, the hepatic changes observed at 12000 and 40000 ppm were considered toxicologically significant. The increased globulin and total bilirubin levels seen in one male (14M) offered 40000 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 for animals offered 4000 ppm were considered not to be toxicologically significant.

Organ weight changes in the spleen were considered not to be toxicologically significant by the notifier as there were no macroscopic or microscopic correlates, and mean values were generally within the 95% historical control reference ranges (absolute: 21.440 g to 81.380 g, relative to body weight: 0.1883% to 0.6848%; n = 103). Changes in relative kidney weight, relative brain weight and relative pituitary weight were considered not to be toxicologically significant and are probably related to the body weight loss in these animals, since the absolute weights were not statistically different, no changes were observed for related clinical chemistry parameters and there were no macro or microscopic correlates.

Decreased heart weights were considered not to be toxicologically significant, and were probably related to body weight loss in these animals, since the relative weights were not statistically significant and there were no macroscopic or microscopic correlates.

Thymus involution/atrophy was characterised by decreased cortex:medulla ratio, and in some cases an increase in tingible body macrophages. As no microscopic findings were seen at 4000 ppm and no macroscopic or microscopic findings were seen at 12000 ppm, the macroscopic change in the thymus at 4000 ppm is considered not to be related to treatment.

The testes were immature in two males at 40000 ppm. Testes were pubescent in one or two males from each group, including controls. Severe oligospermia was present in the epididymides of three males at 40000 ppm and minimal oligospermia, due to pubescence, was present in one male at doses of 12000 ppm. Immaturity was characterised by the absence of spermatids within seminiferous tubules and oligospermia in the epididymus. Pubescence was characterised 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 40000 ppm were immature. The ovaries of all other animals in the study were pubescent. Immaturity was characterised 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 (29F and 32F) offered 40000 ppm were considered not to be toxicologically significant by the notifier and likely to be related to the body weight losses in these animals, since the absolute and relative weights were within background ranges (absolute: 1.622 g to 51.683 g, relative to body weight: 0.0185% to 0.4667%; n = 96) and there were no gross and/or histopathological findings observed.

Dogs used in 13 week toxicity studies are generally not fully sexually mature at sacrifice, which makes interpretation of prostate, testis and ovary findings in these studies difficult (Ronald D Hood, Developmental and Reproductive Toxicology, A practical approach, Second Edition, Taylor and Francis).

The study was started with dogs that were 20-24 weeks of age (5-6 months). At study termination, the dogs were 33-37 weeks old (about 8-9 months). Dogs achieve sexual maturity by about 7-10 months (9-10 months: Rehm 2000; 7 – 8 months: Goedkin et al 2008). Terminal sacrifice in this study therefore occurred approximately at the time dogs would be undergoing puberty, with delayed puberty at the top dose level being secondary to retarded growth and detected as immaturity.

Immaturity or delayed development of prostate, testes and ovaries can be associated with stress, weight loss and non-specific toxicity (Greaves P. Histopathology of Preclinical Toxicity Studies 2nd Edition Elsevier Science 2000, Harleman et al 1997).

'Pubescent' in prostate, testis and ovary and 'immature' in ovary were spontaneously observed in this laboratory. Organ weights of testes/epididymides, ovaries and uterus were within historical control ranges in control and treated groups; only absolute and relative prostate weights at 40000 ppm were slightly below historical control ranges (see historical control data submitted by notifier after request for clarification). Accordingly, the immaturity of reproductive organs that was seen at 40000 ppm (a dose level which caused marked reductions in body weight, body weight gain, and food consumption) can be considered to be an indirect effect of the test article.

	Males				Females			
Diet concentration (ppm)	0	4000	12000	40000	0	4000	12000	40000
Liver and gall bladder								
Liver weight (g)	496.0	504.6	570.1	565.1	364.3	451.5 ^{***}	453.2 ^{***}	387.5 ^{**}
Ratio (%) to body weight	3.5	3.7	4.2	5.1 ^{***}	2.9	3.8 ^{***}	3.8 ^{***}	4.3 ^{***}
Macroscopic findings and histopathology liver								

	Males				Females			
Diet concentration (ppm)	0	4000	12000	40000	0	4000	12000	40000
Liver and gall bladder								
Liver weight (g)	496.0	504.6	570.1	565.1	364.3	451.5 ^{***}	453.2 ^{***}	387.5 ^{**}
Ratio (%) to body weight	3.5	3.7	4.2	5.1 ^{***}	2.9	3.8 ^{***}	3.8 ^{***}	4.3 ^{***}
Macroscopic findings and histopathology liver								

Dark	0/4	0/4	2/4	4/4	0/4	0/4	2/4	4/4
Large	0/4	0/4	3/4	3/4	0/4	0/4	1/4	0/4
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
Macroscopic and histopathology gall bladder								
Cholelithis (macrosc)	0/4	0/4	0/4	4/4	0/4	0/4	0/4	3/4
Distention	0/4	0/4	0/4	2/4	0/4	1/4	0/4	1/4
Cholelithis (histopath)	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
Kidney								
Kidney weight (g)	65.689	60.980	62.900	67.725	51.663	52.828	51.586	49.328
Ratio (%) to body weight	0.467	0.449	0.465	0.608	0.416	0.447	0.432	0.552*
Brain								
Brain weight (g)	82.8	84.3	81.8	85.2	78.9	75.8	75.8	71.8
Ratio (%) to body weight	0.6	0.6	0.6	0.8**	0.6	0.6	0.6	0.8*
Thymus								
Thymus weight (g)	15.820	13.172	12.840	6.914**	15.375	15.532	15.237	6.602
Ratio (%) to body weight	0.112	0.097	0.094	0.060*	0.122	0.132	0.128	0.071
Macroscopic findings and histopathology								
Small	0/4	1/4	0/4	2/4	0/4	0/4	0/4	1/4
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
Spleen								
Spleen weight (g)	47.152	44.380	46.152	69.905	46.109	44.659	53.655	40.606
Ratio (%) to body weight	0.334	0.327	0.336	0.640*	0.367	0.381	0.456	0.463
Heart								
Heart weight (g)	108.0	100.2	107.7	79.6**	90.2	96.3	93.9	71.4
Ratio (%) to body weight	0.8	0.7	0.8	0.7	0.7	0.8	0.8	0.8
Pituitary								
Pituitary weight (g)	0.094	0.097	0.084	0.077	0.070	0.077	0.076	0.065
Ratio (%) to body weight	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001**
Prostate								
Prostate weight (g)	4.471	3.884	2.962	1.168*	-	-	-	-

Ratio (%) to body weight	0.032	0.029	0.022	0.011*	-	-	-	-
Macroscopic findings and histopathology								
Small	0/4	2/4	2/4	4/4	-	-	-	-
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 and epididymides								
Test./epid. weight (g)	28.861	21.880	24.518	20.531	-	-	-	-
Ratio (%) to body weight	0.205	0.161	0.181	0.183	-	-	-	-
Ratio (%) to brain weight	34.9	26.0	30.0	24.1	-	-	-	-
Histopathology 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	-	-	-	-
Histopathology epididymides								
Oligospermia/spermatocytes	0/4	0/4	1/4	3/4	-	-	-	-
Ovaries								
Ovaries weight (g)	-	-	-	-	0.758	0.999	0.833	0.744
Ratio (%) to body weight	-	-	-	-	0.006	0.008	0.007	0.008
Histopathology								
Immature	-	-	-	-	0/4	0/4	0/4	2/4
Pubescent	-	-	-	-	4/4	4/4	4/4	2/4
Uterus								
Uterus weight (g)	-	-	-	-	4.198	5.623	5.631	2.702
Ratio (%) to body weight	-	-	-	-	0.034	0.047	0.048	0.030

* p < 0.05 different from control; ** p < 0.01 different from control; *** p < 0.001 different from control.

Table B.6.3.3-6: Historical control data submitted: Jan 1998-Mar 2007, 27-52 weeks of age

	Males				Females			
	Mean	n	95% ref range low	95% ref range high	Mean	n	95% ref range low	95% ref range high
Terminal Body Weight (g)	11543	148	7013	15603	10172	147	6865	13719
Testes/Epididymides (g)	21.279	140	5.313	35.359	-	-	-	-
Testes/Epididymides ratio (%) to bw	0.1807	140	0.0731	0.2643	-	-	-	-

Testes/Epididymides ratio (%) to brain weight	25.97	140	7.20	43.51	-	-	-	-
Ovaries (g)	-	-	-	-	1.171	146	0.525	3.630
Ovaries ratio (%) to bw	-	-	-	-	0.0114	146	0.0058	0.0345
Thymus (g)	14.908	72	5.331	26.766	13.850	72	6.270	28.299
Thymus ratio (%) to bw	0.1320	72	0.0576	0.2458	0.1398	72	0.0560	0.2692
Prostate (g)	5.589	144	1.289	13.730	-	-	-	-
Prostate ratio (%) to bw	0.0472	144	0.0113	0.1093	-	-	-	-
Uterus (g)	-	-	-	-	n.a.	96	1.622	51.683

n.a. not available

Conclusion:

In the 90 days study in dog the NOAEL for males and females is proposed at 4000 ppm (90.9 mg/kg bw/day for male and 102.7 mg/kg bw/day for female animals, based on increased liver weight, histopathological changes in the liver, and increased alkaline phosphatase levels. Liver weight increases in females dosed at 4000 ppm (> 20%) were not observed in the 52 week toxicity study in dogs and were therefore not considered for the derivation of the NOAEL of the 90 day study.

B.6.3.4 Oral 1-year toxicity (dog)

Reference:	S-2200 Technical Grade: 52 Week Oral (Dietary) Administration Toxicity Study in the Dog
Author(s), year:	██████████ 2012a
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0071
Guideline(s):	OECD 452, US EPA OPPTS 870.4100, JMAFF 2-1-14, EC No 440/2008 B30
GLP:	Yes (Laboratory certified by national authority)
Deviations:	Minor deviations not affecting the validity of the study
Validity:	Yes

Material and methods:

Test Material:	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G
Purity:	93.4%
Stability of test compound:	Expiry date stated as 21 November 2011 (after end of treatment)
Vehicle:	none
Test animals:	
Species:	Dog
Strain:	Beagle
Age:	27 – 34 weeks at beginning of dosing
Weight at dosing:	Males: 10.02 – 13.42 kg; Females: 8.86 – 12.20 kg
Source:	████████████████████
Diet:	5L66 Certified High Density Canine Diet (IPS Product Supplies Ltd,

London, UK); 300 g per day

Animal assignment and treatment:

Twenty healthy animals per sex were selected based on the results of pre-test examinations. Animals were randomly distributed into groups of 4 animals/sex/group based on the most recent body weight data, except for separation of familial animals.

The test article was administered orally by incorporation into the diet. Each animal had ad libitum access to the dietary formulations for approximately 8 hours/day for at least 52 weeks. Control animals received untreated diet.

Diet preparation, analysis and administration:

Fresh batches of dietary formulation were prepared weekly. All diets were hand mixed with mains water into clumps of irregular size and shape. The formulations were stored at room temperature in the dark before administration.

Accuracy and homogeneity of preparation was confirmed by analysis. Stability in diet for at least 9 days at ambient temperature was established before the study started.

Although there were some deviations to the protocol with regards to the analysis of the test formulations, the data was sufficient to confirm homogeneity and stability, and there is considered to be no impact to the study integrity.

Table B.6.3.4-1: Diet concentration and daily dose levels

	Males					Females				
Diet concentration (ppm)	0	200	800	4000	8000	0	200	800	4000	8000
mg test material/kg bw/day	0	4.3	19.2	92.0	180.7	0	4.5	20.4	92.0	225.7

Clinical observations:

All animals were observed daily for signs of ill health or overt toxicity. In addition, each animal was given a daily detailed physical examination. Animals were observed approximately 1 and 4 hours after presentation of the diet. A number of animals from both the control and treatment groups received veterinary treatments throughout the study period. The need for veterinary treatment was not dose related.

All animals were observed daily for signs of ill health or overt toxicity. In addition, each animal was given a daily detailed physical examination. Animals were observed approximately 1 and 4 hours after presentation of the diet. One control male (animal number 3) received topical and oral antibiotics for presumed bites.

All animals were subjected to a battery of behavioural tests and observations before initiation of treatment and at once weekly intervals thereafter. The unrestrained behaviour of each animal was observed in an examination room for approximately 1 to 2 minutes.

Observations included the following:

Level of consciousness

Head posture

Neck posture

Limb posture (standing)

Tremors

General level of activity

Head co-ordination

Circling

Gait

Unusual behaviour

Visual fixating response test and auditory startle tests were assessed at the end of the observation period.

Following the unrestrained observations all animals were subjected to neurological examinations each week.

Examinations included the following:

Proprioceptive paw positioning	Righting postural reaction
Hemihopping postural reactions	Wheel barrowing postural reactions
Muscle tone	Palpebral closure
Nictitating membrane	Palpebral reflex
Eye position	Eye movement
Pupil size	Pupillary light reflex
Lacrimation	Salivation
General reactions	General clinical observations

Body weight, general clinical signs, heart rate and rectal temperatures were taken as part of the data set for assessment of neurobehavioral effects of test articles.

Food consumption and body weight:

Food consumption for all animals was measured daily from the time of allocation until termination of the dosing period.

Weekly compound consumption was calculated as mg/kg bw/day and also as a total consumption over the entire treatment period (mg/kg bw/day).

Body weight was measured in all animals once weekly throughout the dosing period, from Week -3 until termination.

Ophthalmoscopic observation:

A mydriatic agent was instilled into the eyes before examinations. Eyes of all animals were observed once pre-test and at Week 51.

Haematology and clinical chemistry:

Blood was sampled from all animals before the start of treatment (Week -1) and at weeks 13, 26, and 52 of the administration period (sampling was performed after animals were fasted overnight). Samples were taken from jugular vein and collected into EDTA (for cell parameters), trisodium citrate (for clotting function), and lithium heparin (blood chemistry).

1.0 mL (nominal) of blood was collected from each animal for haematology (0.5 mL for coagulation tests and 0.5 mL for other tests). The blood sample collected into trisodium citrate anticoagulant from one female in Week 52 was unsuitable for analysis, so blood samples were recollected and re-examined on week 53.

The following parameters were examined:

Haematology: Haemoglobin concentration (HGB), Red blood cell count (RBC), Mean cell haemoglobin (MCH), Mean cell haemoglobin concentration (MCHC), Mean cell volume (MCV), Packed cell volume (PCV), Platelet count (PLT), Total and differential white blood cell count, Reticulocytes, Prothrombin time (PT), and Activated partial thromboplastin time (APTT)

Blood chemistry: Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), γ -Glutamyl transferase (GGT), Sodium (Na), Potassium (K), Calcium (Ca), Inorganic phosphorus (IP), Chloride (Cl), Triglycerides (TG), Total protein (TP), Albumin (Alb), Globulin (G), Albumin-globulin ratio (A/G), Total cholesterol (Cho), Glucose (Glu), Urea (U), Total bilirubin (Bil), and Creatinine (Cre).

Urine analysis:

Urine samples were collected overnight from all animals before the start of treatment (Week -1) and in Weeks 12, 25, and 51 of the administration period; food and water were removed during collection. In Week

25, two of the male urine samples showed a blue discolouration. Following a thorough investigation, this was considered due to trace contamination of the apparatus with a dyestuff and not considered to impact the study integrity. Fresh samples were obtained overnight from all males in Week 25. Additionally, in Week 51, two further male urine samples showed a blue discolouration. The cages were visually inspected prior to collection and considered to be clean. Prior to the female collection, all cages were thoroughly power washed after being left to soak in detergent. Subsequently, the discoloured male samples were repeated in Week 52.

Parameters examined: Urinary volume (Vol), Colour (Col), Turbidity, Specific Gravity (S.G), pH, Protein (Pro), Glucose (Glu), Ketones (Ket), Urobilinogen (Uro), Bilirubin (Bil), Blood, and Microscopic sediment.

Sacrifice and pathology:

All animals survived until scheduled sacrifice, and were sacrificed with sodium thiopentone, and exsanguinated from the major blood vessels.

The following organs were weighed: adrenals, brain, heart, kidneys, liver, ovaries, pituitary, prostate, spleen, testes (with epididymides), thymus, thyroids (with parathyroids), and uterus (including cervix). Bilateral organs were weighed together.

Histopathological examination: After necropsy, the following organs and tissues collected from all animals were fixed in 10% neutral buffered formalin, except bone marrow smears which were fixed in methanol, testes and epididymides which were fixed in Bouin's solution and eyes/optic nerves fixed in Davidson's fluid.

Adrenals	Ovaries
Aorta	Oviducts
Bone and marrow (sternum)	Pancreas
Brain	Pituitary
Caecum	Prostate
Colon	Rectum
Duodenum	Salivary gland – mandibular
Eyes and Optic nerves	Salivary gland – Sublingual, parotid
Bone with marrow (femur) & articular surface	Sciatic nerves
Gallbladder	Skin and subcutaneous tissue
Gross lesions	Spinal cord (cervical, thoracic, lumbar)
Heart	Spleen
Ileum	Sternum with bone marrow
Jejunum	Stifle joint
Kidneys	Stomach
Larynx	Testes and epididymides
Liver	Thymus
Lungs with bronchi and bronchioles	Thyroids and parathyroids
Mammary glands	Tongue
Mandibular lymph nodes	Trachea
Mesenteric lymph nodes	Ureters
Muscle	Urinary bladder
Nasal cavity	Uterus (including cervix)
Nasopharynx	Vagina
Oesophagus	

Statistical analysis:

Necropsy body weights, organ weights and organ weight to body weight ratios were analysed using one-way analysis of variance, separately for each sex. Levene's test for equality of variances among the groups was performed. Where this showed no evidence of heterogeneity ($p \geq 0.01$), pairwise comparisons with control were made using Dunnett's test. A linear contrast was used to determine whether there was a relationship

between increasing dose and response. A significant trend ($p < 0.05$) was reported only where none of the pairwise comparisons was significant.

Absolute body weights, body weight gains, food consumption, body temperatures, heart rates, haematology, blood biochemistry and urine analysis variables were analysed using two-way ANOVA. Levene's test for equality of variances across groups, between sexes and for any interaction was performed. Where these tests showed no evidence of heterogeneity ($p \geq 0.01$ for all 3 tests), pairwise comparisons with control were made, for each sex separately, using Dunnett's test. For each sex separately, a linear contrast was used to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was reported only where none of the pairwise comparisons was significant.

Where Levene's test showed evidence of a sex effect only ($p < 0.01$ for both untransformed and log-transformed data), the data were analysed using one-way ANOVA for each sex separately, as previously described.

Where Levene's test showed evidence of group effects, sex effects or a sex by group interaction ($p < 0.01$), the data were analysed either using the same methods after applying a log-transformation or using non-parametric methods. The non-parametric methods employed were the Kruskal-Wallis ANOVA, the Terpstra-Jonckheere test for a dose related trend and the Wilcoxon rank sum test for pairwise comparisons. Where the Kruskal-Wallis ANOVA was not significant, the pairwise comparisons were not reported in order to protect the Type I error.

Clinical pathology parameters with values above or below the limit of the assay were analysed using non-parametric methods, as described above. Where such values occurred in one sex only, data for the other sex were analysed using one-way ANOVA.

All macroscopic and microscopic findings data, for all tissues examined were supplied for analysis. Male and female data were analysed separately. All tests were performed with a two-sided risk.

For each macroscopic finding, comparisons were made between the incidence in each of the treated groups and that in the control group, using Fisher's Exact test.

For microscopic findings, comparisons were made between the incidence in each of the treated groups and that in the control group. Where a given finding had more than two categories, the Wilcoxon-Mann-Whitney rank sum test was performed; where only two categories were observed, Fisher's Exact test was used.

Findings:

Clinical signs and mortality:

All animals survived the duration of the study.

Thin appearance was observed in one female offered 8000 ppm, and this was considered to be correlated with body weight loss and occasional incidences of decreased muscle tone recorded during the functional observation battery. Other clinical observations noted at the daily examinations in treated animals were considered incidental as they were infrequent, associated with the female sexual cycle or typical findings of dogs held under laboratory conditions.

Body weight, body weight gain, and food consumption:

No statistically significant alterations in mean body weights or mean body weight gains were noted in treated animals, when compared to concurrent controls.

Following 52 weeks of treatment, females offered 8000 ppm displayed a body weight gain lower than concurrent controls. This reduced growth rate was due to one female animal exhibiting an overall bodyweight loss of 6.0%.

Table B.6.3.4-2: Mean body weight, body weight gain, and food consumption

	Males					Females				
	Dose (ppm)					Dose (ppm)				
	0	200	800	4000	8000	0	200	800	4000	8000
Terminal body weight (kg)	14,64	15,22	13,29	14,10	13,70	12,92	12,71	12,63	13,36	11,05
Bodyweight gain (kg)	2,97	3,88	1,82	2,58	2,66	2,68	2,36	2,57	3,19	0,81
Food consumption (g/animal/week)	2107	2110	2112	2110	2111	2067	1920	2089	1993	2094

Ophthalmoscopy:

There were no ophthalmic abnormalities noted pre-treatment or in Week 52.

Functional observation battery data:

Overall, there were no findings from any assessment indicative of any neurotoxicological effect of the test article.

Occasional statistically significant differences in mean body temperature were recorded for males offered ≥ 800 ppm and females offered ≥ 200 ppm, when compared to concurrent controls. In addition, there was a statistically significant relationship between increasing dose and response for males in Weeks 15, 18, 28, 34 and 43 and for females in Weeks 7, 8, 9, 11 and 15. These changes were transient, inconsistent and considered not to be related to treatment.

Statistically significant differences from concurrent controls in mean heart rate were recorded sporadically for males offered ≥ 200 ppm and females offered 800 or 4000 ppm. These changes were transient, inconsistent and considered not to be related to treatment.

There were no overt differences between concurrent control and treated animals in the incidences of observations recorded for unrestrained behaviour.

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/dilation of pupil size, abnormal pupillary light reflex, increased lacrimation, increased salivation and untypical general reactions. These observations were infrequent and occasionally observed in concurrent control animals or before treatment was initiated.

Haematology and clinical chemistry:

For clinical biochemistry, several statistically significant differences between controls and treated groups were observed.

Statistically increased aspartate aminotransferase activity in Week 26 for males offered 8000 ppm is considered not to be treatment-related as this finding is largely reflective of an increase for one male with values for the other animals in this group being within historical control range. Furthermore, the plasma activity for the same male in Week 52 was similar to that determined before treatment. The changes at Week 52 for another male offered 8000 ppm are considered to be treatment related.

Disturbances to alanine aminotransferase, alkaline phosphatase (800 ppm only), gamma glutamyltransferase and/or total cholesterol for animals offered 200 or 800 ppm were considered not to be toxicologically significant as they were small in magnitude, inconsistent over time and between sexes and/or

lacked a histopathological correlate. However, the increased alkaline phosphatase activities for males offered 4000 ppm at Week 52, and males and females offered 8000 ppm at Weeks 13, 26 and 52, are considered to be treatment-related.

The statistically higher triglyceride level observed at Week 26 in males offered 4000 ppm was considered not to be toxicologically significant since the mean value is within the historical control range. The increased triglyceride level for one male offered 4000 ppm and two males offered 8000 ppm at Week 13 are considered not to be toxicologically significant since the values for these animals at Week 52 were similar to that determined before treatment was initiated. The changes for one male offered 4000 ppm at Week 13 and Week 52, and one male offered 8000 ppm at Week 52, are considered to be treatment related.

Albumin levels were statistically significantly decreased in females offered 8000 ppm at Weeks 26 and 52, when compared to concurrent controls.

Statistically decreased total protein and calcium levels in females offered 8000 ppm at Weeks 13 and 26 and at Weeks 13, 26 and 52, respectively, are considered not to be treatment-related as it may be in part reflective of decreased albumin concentrations (Meuten DJ, Relationship of serum total calcium to albumin and total protein in dogs. J Am Vet Med Assoc. 1982;180(1):63-7). Additionally, all individual values were within the historical control ranges and/or differences were similar to those recorded pre-treatment.

Total cholesterol was statistically decreased at Week 26 in females offered 200, 800 or 8000 ppm and at Week 52 in females offered 800 or 8000 ppm, when compared to 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.

Occasional differences reached statistical significance in other parameters, however, these changes were not considered treatment-related since they were slight, within the historical control range, comparable to pre-test values or there were no significant differences between sexes and collection points.

Table B.6.3.4-3: Haematology and clinical chemistry parameters (weeks 13, 26, and 51)

	Males					Females				
	Dose (ppm)					Dose (ppm)				
	0	200	800	4000	8000	0	200	800	4000	8000
AST (IU/L)	32	38	29	34	42	28	28	26	32	36
	30	30	29	29	61*	26	30	28	23	28
	29	30	29	27	37	26	24	29	28	30
ALT (IU/L)	38	42	44	46	136	29	36	38	39	79
	42	48	48	43	113	31	36	46	31	57
	40	53	51	47	128	29	35	50	33	59
ALP (IU/L)	63	68	103	120	178*	91	76	70	91	207*
	53	52	100	120	140*	75	79	66	78	198**
	41	43	73	112**	147**	55	64	53	69	185**
γGT (IU/L)	2	2	3	3	4	2	3	3	2	3
	3	5	3	5	3	2	2	4	3	4
	3	3	4	4	4	3	3	3	4	5
TP (g/L)	55	55	56	57	57	60	58	57	56	55**
	58	58	59	59	58	62	62	60	61	57*
	58	59	56	59	59	61	61	59	59	57
Alb (g/L)	35	36	36	37	36	39	37	38	37	35
	36	35	34	35	33	39	38	36	37	34*

	35	35	33	37	33	39	37	37	36	34**
A/G ratio	1.8	1.9	1.8	1.9	1.6	1.9	1.9	1.9	2.1	1.8
	1.6	1.6	1.4	1.4	1.4	1.6	1.6	1.5	1.6	1.5
	1.5	1.5	1.5	1.7	1.3	1.8	1.6	1.6	1.6	1.5
Cho (mmol/L)	7.1	5.9	6.6	6.9	6.1	8.3	7.0	6.1	5.9	6.4
	6.7	5.7	6.7	6.7	5.8	8.2	6.3*	5.9**	6.6	6.0*
	6.0	5.1	5.7	6.3	5.5	7.5	6.1	5.5*	5.9	5.4*
TG (mmol/L)	0.37	0.28	0.45	0.53	0.48	0.59	0.63	0.60	0.49	0.51
	0.33	0.30	0.44	0.49*	0.45	0.51	0.48	0.48	0.52	0.50
	0.40	0.36	0.42	0.53	0.52	0.55	0.60	0.60	0.58	0.52
Ca (mmol/L)	2.83	2.79	2.76	2.81	2.80	2.89	2.88	2.86	2.84	2.70*
	2.66	2.67	2.60	2.66	2.61	2.74	2.76	2.72	2.74	2.59*
	2.63	2.63	2.57	2.69	2.57	2.71	2.70	2.66	2.66	2.53*

* p < 0.05 different from control, ** p < 0.01 different from control

Urine analysis:

There were no treatment-related effects on urinary parameters measured at the Week 12, 25 or 51.

The mean volume of urine voided by females offered 200 or 800 ppm at Week 12 was statistically decreased when compared to concurrent controls. However, as the volumes were similar to those measured before treatment, no dose response was observed and no statistical differences from concurrent controls were observed in males at Week 12 or in both sexes at Weeks 25 or 51, changes are considered to be incidental and not related to treatment.

Sacrifice and pathology:

Organ Weights:

Increased mean liver weights were found in males offered ≥ 4000 ppm and females offered 8000 ppm, with a statistically significant increase in the relative liver-to-bodyweight for females offered 8000 ppm compared to controls.

Decreases in absolute kidney weight were considered not to be toxicologically significant since the relative weights were not statistically different, all individual values were within historical control limits, no changes were observed for related clinical chemistry parameters and there were no macroscopic or microscopic correlates.

Organ weight changes in the adrenals and testes/epididymides were considered not to be toxicologically significant as they were either inconsistent between sexes, there were no macroscopic or microscopic correlates, values were within historical control limits or there was a lack of a clear dose-response relationship.

Gross and Histopathology:

Most tissues were macroscopically unremarkable and the findings seen were generally consistent with the usual pattern of findings in animals of this strain and age.

Dark liver was observed for one male and three females offered 8000 ppm, and correlated with findings recorded microscopically.

There were no other macroscopic findings suggestive of treatment-related effects.

Microscopic findings were generally infrequent, of a minor nature and consistent with the usual pattern of findings in animals of this strain and age.

In the liver, hepatocyte hypertrophy was recorded for males and females offered 4000 or 8000 ppm.

Increased levels of hepatocyte pigment were recorded for males offered 4000 or 8000 ppm and females

offered 8000 ppm, when compared with controls. The incidence of hepatocyte hypertrophy and hepatocyte pigment achieved statistical significance ($p < 0.05$) in males and females offered 8000 ppm, when compared with the controls. There was also a marginal increase in pigmented macrophages in males and females offered 8000 ppm, when compared with the controls. Additionally, centrilobular degeneration was recorded for one male and one female offered 8000 ppm, and portal fibrosis/bile duct proliferation was recorded for one male offered 8000 ppm.

Agonal congestion/haemorrhage was recorded for one male and three females offered 8000 ppm, this finding correlated with the observed dark liver that was recorded during the macroscopic examination. Hepatocyte hypertrophy was characterised by hepatocytes in the centrilobular/mid-zonal area with increased pale eosinophilic staining cytoplasm. Hepatocyte pigment was characterised by the presence of small golden brown cytoplasmic granules, primarily in hepatocytes in the centrilobular and periportal zones. Pigmented macrophages were characterised by macrophages in the portal area and in the hepatic sinusoids with dark brown cytoplasmic pigment. The hepatocyte pigment and macrophage pigment was identified as lipofuscin using Schmorl's and Long Ziehl Neelsen stains. Centrilobular degeneration was characterised by the presence of degenerating hepatocytes in the centrilobular zone, with occasional single cell necrosis. Portal fibrosis/bile duct proliferation was characterised by the presence of proliferating bile duct cells and fibroblasts, with an overall increase in fibrous tissue in the portal tracts. The increase in lipofuscin on this study was considered to be related to hepatocyte hypertrophy and/or cellular degeneration. The liver of animals offered 200 or 800 ppm was comparable to the controls. There were no other microscopic findings suggestive of treatment-related effects.

Table B.6.3.4-4: Organ weights and histopathology

	Males					Females				
Diet concentration (ppm)	0	200	800	4000	8000	0	200	800	4000	8000
Liver										
Liver weight (g)	437	425	409	484	488	429	437	437	448	455
Ratio (%) to body weight	3.0	2.8	3.1	3.4	3.6	3.3	3.4	3.5	3.4	4.2*
Macroscopic and histopathology liver										
Dark	0/4	0/4	0/4	0/4	1/4	0/4	0/4	0/4	0/4	3/4
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

Kidney										
Kidney (g)	73.9	65.0	61.7	60.6*	60.0*	57.3	58.5	58.4	53.1	54.9
Ratio (%) to body weight	0.508	0.428	0.469	0.431	0.437	0.442	0.460	0.467	0.400	0.509
Adrenals										
Ratio (%) to body weight	0.010	0.010	0.010	0.010	0.011	0.013	0.015	0.017	0.009*	0.017*
Testes/ epididymides										
Testes/epididymides (g)	32.3	25.2	24.3*	27.5	25.3	-	-	-	-	-
Ratio (%) to body weight	0.220	0.166*	0.186	0.196	0.184	-	-	-	-	-

* p < 0.05 different from control; ** p < 0.01 different from control; *** p < 0.001 different from control.

Conclusion:

Mandestrobin administration of 8000 ppm was associated with increased absolute and relative liver weights, hepatocyte hypertrophy and hepatocyte pigment and disturbances to clinical biochemistry parameters (increased alkaline phosphatase, γ -glutamyltransferase and triglycerides) in both genders. At 4000 ppm, male animals showed increased ALP-activity and hepatocyte hypertrophy and pigmentation. The livers of female animals at this dose level were comparable to controls.

Based on these indicators of changes in the liver, the No Observable Adverse Effect Level (NOAEL) for males was considered to be 800 ppm (19.2 mg/kg bw/day), and the NOAEL for females was concluded to be 4000 ppm (92.0 mg/kg bw/day).

B.6.3.5 Repeated dose (28-day) inhalation toxicity (rodents)

No repeated dose inhalation study (28-day) with mandestrobin has been submitted, not mandatory.

B.6.3.6 Repeated dose (90-day) inhalation toxicity (rodents)

No repeated dose inhalation study (90-day) with mandestrobin has been submitted, not mandatory.

B.6.3.7 Repeated dose (28-day) percutaneous toxicity (rodents)

Reference:	A 28-Day Repeated Dose Dermal Toxicity Study of S-2200 Technical Grade in Rats
Author(s), year:	██████████ 2011
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0022
Guideline(s):	OECD 410, EC Guideline B.9 OJEC No L 383 A/144, EPA OPPTS 870.3200, and Japanese MAFF 12 Nousan 8147 (2000)
GLP:	Yes (lab certified by National Authority)
Deviations:	No
Validity:	Yes

Material and methods:

<i>Test Material:</i>	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G
Purity:	93.4%
Stability of test compound:	The stability of the test compound during the study period was confirmed
Vehicle:	Water for injection

Test animals:

Species:	Rat
Strain:	Wistar
Age:	8 weeks at start of treatment
Weight at dosing:	Males: 123.2 – 146.8 g; Females: 97.5 – 125.6 g (upon receipt)
Source:	
Diet:	CRF-1 pellet diet (Oriental Yeast Co., Ltd) <i>ad libitum</i>

Animal assignment and treatment:

10 animals per sex were assigned to each group by the stratified sequenced randomization method on the basis of body weight of the day, and it was confirmed that the weight variation of animals used was within $\pm 20\%$ of the mean weight for each sex.

About 24 hours before the first administration, hair on the administration sites (dorsal region, approximately 5 cm \times 6 cm) of animals was clipped off with an electric clipper (Speedik DC-6, Blade: 0.1 mm, Shimizu E.C.) while taking care not to damage the skin. After that, hair clipping was performed for all animals on days 7, 14, 21, and 28.

Dose levels were 100, 300 and 1000 mg/kg bw/day, applied to the skin for 6 hours/day.

The required amount of test compound was weighed on a piece of medical paper based on the latest body weights.

The administration area was equivalent to 10% of the total body surface (approximately 4 cm \times 5 cm). The required amount of dosing formulation (test compound) was uniformly transferred on a gauze sheet (approximately 4 cm \times 5 cm, 1 piece, Yamato Kojo Co., Ltd.) lined with an impermeable sheet (BlendermTM, 3M Health Care), and the gauze sheet was moistened sufficiently with about 0.3 mL vehicle (water for injection). The administration site was covered with the gauze sheet and occluded with an elastic bandage (SILKYTEX, ALCARE Co., Ltd.) for at least 6 hours. The skin from the axillary to the chest region was covered with a gauze sheet to protect it from the bandage.

After about 6-hour occlusive application, the test compound, gauze sheet, and elastic bandage were removed.

The administration area was wiped once with a sheet of gauze (approximately 4 cm \times 5 cm, 1 piece) immersed in lukewarm water (this operation was repeated 3 times), and then wiped with dry tissue paper to remove fluid on the skin. The animals were restrained with neck collars during the administration period, except during the body weight measurement, ophthalmology, and pooled urine sampling. The animals in the control group were treated with vehicle only, and were handled in the identical manner to the test group subjects.

Clinical observations:

Clinical signs and mortality were observed twice daily, before administration and after the end of administration (after removal of dosing formulation).

Detailed clinical observation was conducted before the start of administration and in weeks 1, 2, 3, and 4 of administration (after removal of dosing formulation). Animals were observed in an open field for 2 minutes. The observation results were evaluated according to scoring criteria.

Cageside observations included posture, convulsions, stereotypies and bizarre behaviour, and tremors. Hand held observations included reactivity to handling, vocalizations, tremors, twitches, convulsions, respiration, salivation, lacrimation, pupil size, exophthalmos, ocular or nasal secretions, skin, piloerection, soiled fur, skin colour, incontinence of urine, muscle tone, and body temperature. Open field observations included arousal, gait, stereotypies and bizarre behaviour, ptosis, diarrhoea, defecation, and urination.

Food consumption and body weight:

Body weights were measured for all animals on days 1, 8, 15, 22, and 28, and at necropsy. Food consumption was measured for all animals on days 1, 8, 15, 22, and 28.

Ophthalmoscopic observation:

Ophthalmoscopy was conducted before the start of administration and in week 4 before dosing. Light reflexes were confirmed using a direct ophthalmoscope (HEINE alpha Ophthalmoscope). A slit lamp (SL-15, Kowa) was used with mydriatic solution to inspect the cornea, iris, lens and vitreous body, and a binocular indirect ophthalmoscope (OMEGA2000, Heine Optotechnik GmbH & Co. KG) was used to inspect the fundus oculi.

Haematology and clinical chemistry:

The animals were examined at necropsy after the end of the administration period. All animals were anesthetized with intraperitoneal injection of sodium pentobarbital and blood was collected from posterior vena cava.

For examination of the coagulation system, 0.9 mL of blood was collected into a glass tube containing trisodium citrate. For the examination of other items, remaining blood was collected into a container containing an anticoagulant (EDTA-2K). The animals were fasted for 16 to 22 hours before blood sampling. Parameters measured were:

Haematology:

Leukocytes, Differential leukocyte count: (Neutrophils, Lymphocytes, Monocytes, Eosinophils, Basophils) Erythrocytes (RBC), Haemoglobin concentration (Hgb), Haematocrit, Mean corpuscular volume (MCV), Mean corpuscular haemoglobin (MCH), Mean corpuscular haemoglobin concentration (MCHC), Reticulocytes, Platelets, Prothrombin time (PT), Activated partial thromboplastin time (APTT)

Blood biochemistry:

Total protein, Albumin, Albumin/globulin ratio (A/G ratio), Total bilirubin (T. bilirubin), Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), γ -Glutamyl transpeptidase (γ -GTP), Alkaline phosphatase (ALP), Total cholesterol (T. Cholesterol), Triglycerides, Phospholipids, Glucose, Blood urea nitrogen (BUN), Creatinine, Inorganic phosphorus (IP), Calcium (Ca), Sodium (Na), Potassium (K), Chloride (Cl).

Urinalysis:

Urinalysis was conducted in week 4.

Fresh urine samples were collected in the morning before dosing, using metabolic cages, and urine samples were collected successively for about 24 hours (pooled urine). The following parameters were measured: Volume, Gravity, and Colour were all examined using 24 hour pooled samples. pH, protein, glucose, ketone body, occult blood, and urobilinogen were examined using fresh urine samples with Pretest 8all (Wako Pure Chemical Industries Ltd.). Urine sediments were obtained by centrifugation and examined for epithelial cells, erythrocytes, leukocytes, casts, and crystals (phosphates and oxalates).

Sacrifice and pathology:

At the end of the administration period, animals were euthanized by exsanguination after blood sampling. All organs and tissues were immediately examined macroscopically.

After necropsy, organs were weighed (absolute weight) and the ratio of organ weight to body weight (relative weight) was calculated on the basis of body weight measured on the day of necropsy. Paired organs were measured separately and the total weight was also calculated.

Organs and tissues were fixed in 10% neutral buffered formalin (the eyes, optic nerves and Harderian gland were pre-fixed in Davidson's solution, and the testes and epididymides were pre-fixed in Bouin's solution). In all animals in control and high dose groups, embedded block specimens (submaxillary lymph node: left side, parathyroid gland: left side (Nos. 376 and 377: right side), other bilateral organs/tissues: left side) were prepared, sectioned with paraffin and stained with haematoxylin and eosin (HE) for microscopic examination. In addition, all gross lesions were examined microscopically in the same manner.

Since examination of the high dose group showed no changes related to the test compound administration, the histopathological examination of the middle and low dose groups, except for the examination of gross lesions, was not conducted.

Sampling and examination of organs were performed as follows:

Tongue, Larynx/Pharynx, Oesophagus, Stomach, Duodenum, Jejunum, Ileum (with Peyer's patch), Caecum, Colon, Rectum, Submaxillary gland (with sublingual gland), Parotid gland, Liver, Pancreas, Trachea, Lung (with bronchi), Thymus, Submaxillary lymph node, Mesenteric lymph node, Spleen, Heart, Aorta, Kidney, Urinary bladder, Prostate, Seminal vesicle, Testis, Epididymis, Ovary, Uterus, Vagina, Mammary gland, Pituitary, Thyroid (with parathyroid) *¹, Adrenal, Brain *², Spinal cord (cervical to lumbar) *³, Optic nerve, Sciatic nerve, Eye, Harderian gland, M. biceps femoris, Sternum (with bone marrow), Femur (with bone marrow), Integument (lower abdominal), Nasal cavity, Treated site, Other gross lesions.

*¹: The parathyroid was examined on either side

*²: Cerebrum, cerebellum and medulla/pons

*³: Cervical, thoracic and lumbar region

Findings:

Clinical signs and mortality:

All animals survived until the scheduled sacrifice. No treatment-related clinical signs were detected.

Body weight and body weight gain:

There were no differences in bodyweight and bodyweight gain between treated groups and control animals during and at the end of the study.

Food consumption:

Food consumption was unaffected by treatment.

Ophthalmoscopy:

No abnormalities were seen in any treated group.

Haematology and clinical chemistry:

No changes related to the administration of the test compound were seen in any group.

Sacrifice and pathology:

No changes related to the administration of the test compound were seen in any group.

Conclusion:

There was no toxicological change related to administration of the test compound in any parameter. Therefore, the NOAEL (no-observed-adverse-effect levels) of S-2200 TG under the current study conditions is concluded to be 1000 mg/kg bw/day (the highest dose tested) for both males and females.

B.6.3.8 Repeated dose (90-day) percutaneous toxicity (rodents)

No repeated dose (90-day) percutaneous toxicity study with mandestrobin has been submitted.

B.6.3.9 Immunotoxicity

Reference:	S-2200 TG – A 28-Day Dietary Dose Range-Finding Study in Wistar Han Rats
Author(s), year:	██████████ 2011a
Report/Doc. number:	Sumitomo Chemical Co. Ltd. Report No. ROT-0035
Guideline(s):	In general accordance with US EPA OPPTS 870.7800
GLP:	No
Deviations:	-
Validity:	Yes

Material and methods:

Test Material:	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G
Purity:	93.4%
Stability of test compound:	Expiry date after completion of treatment
Vehicle:	None; test material was mixed directly into the diet
Test animals:	
Species:	Rat
Strain:	Wistar; ████████:WI(HAN)
Age:	Approximately 7 weeks at start of treatment
Weight at dosing:	188-216 g for males and 141-172 g for females
Source:	██
Diet:	Certified Rodent LabDiet® 5002 (meal) (PMI Nutrition International, LLC) <i>ad libitum</i>

Animal assignment and treatment:

Groups of randomly assigned female and/or male rats were fed diets containing S-2200 for 28 days. For the T-cell dependent antibody response (TDAR) groups (1-5), each group consisted of 8 females. Each neurotoxicity group (Groups 1A-4A) consisted of 5 males and 5 females.

On study day 24, all animals in the TDAR groups were immunized via an intravenous lateral tail vein injection with 0.5 mL of 2×10^8 sRBC in EBSS with HEPES. In addition, all positive control group animals (Group 5) were administered CPS (cyclophosphamide monohydrate) via intraperitoneal injection once daily on study days 24 through 27 at a dose level of 50 mg/kg/day and dose volume of 10 mL/kg/day. Individual animal positive control doses were based on the most recently recorded body weights to provide the correct mg/kg/day dose. The sRBC immunizations were administered prior to administration of CPS (cyclophosphamide monohydrate) on study day 24.

Rats in Group 5 were administered CPS, a known immune suppressant, at 50 mg/kg/day on study days 24-27 by intraperitoneal injection. The dose volume was 10 mL/kg/day; therefore the concentration of the dosing formulation was 5 mg/mL. Group 5 served as a positive control group and was included to aid in the interpretation of the results and verification of the assay sensitivity.

Table B.6.3.9-1: Animal assignment and treatment

Group number	Group ^a	Dietary concentration (ppm)	CPS Daily dose on days 24-27 (mg/kg/day i.p.)	sRBC on day 24 (mL/rat i.v.)	Males	Females
T-cell dependent antibody response (TDAR)						
1	Diet only	0	0	0.5	0	8
2	S-2200 TG Low	1500	0	0.5	0	8
3	S-2200 TG Medium	5000	0	0.5	0	8
4	S-2200 TG High	15000	0	0.5	0	8
5	Positive control: CPS	0	50	0.5	0	8
Neurotoxicity Groups						
1A	Vehicle	0			5	5
2A	S-2200 TG Low	1500			5	5
3A	S-2200 TG Medium	5000			5	5
4A	S-2200 TG High	15000			5	5

Clinical observations:

All animals were observed twice daily, once in the morning and once in the afternoon, for mortality and moribundity.

Clinical examinations were performed once daily. The absence or presence of findings was recorded for individual animals at the scheduled intervals. Observations included but were not limited to: changes in the skin, fur, eyes, and mucous membranes; respiratory circulatory, and autonomic and central nervous systems function; somatomotor activity and behaviour patterns. Detailed physical examinations were conducted on all animals approximately weekly, beginning approximately 1 week prior to randomization, at the time of randomization, once weekly during the study, and on the day of the scheduled necropsy. The animals were removed from their home cages and placed in a standard arena for observation of changes in gait, posture or clonic or tonic movements. Stereotypies (e.g. excessive grooming, repetitive circling), bizarre behaviour (e.g. self-mutilation, walking backwards) and permanent or semi-permanent signs, such as skin lesions and hair loss, were also recorded. Daily observations were not performed on days when detailed physical examinations were conducted.

All animals were checked for overt signs of toxicity by daily general cageside observations. Detailed clinical observations were performed during pre-test, and once weekly during the study.

Food consumption and body weight:

Individual body weights were recorded for all animals twice weekly beginning approximately 1 week prior to randomization, at the time of animal selection for randomization, on study day 0 and ending just prior to the scheduled necropsy.

Individual food consumption was recorded approximately weekly for all animals, beginning approximately 1 week prior to randomization, and ending just prior to the scheduled necropsy. Food consumption was calculated as g/animal/day for the corresponding body weight intervals. The mean amounts of S-2200 TG consumed (mg/kg bw/day) per dose group was calculated from the mean food consumed (g/kg bw/day) and the appropriate target concentration of test compound in the food (mg/kg).

Statistical analysis:

All statistical tests were performed using [REDACTED] Data Management System™ unless otherwise noted. Analyses were conducted using two-tailed tests (except as noted otherwise) for minimum significance levels of 1% and 5%, comparing each test compound-treated group to the control group by sex. Body weight, body weight change, food consumption, and organ weight (adrenal glands and brain) data were subjected to a parametric one-way ANOVA to determine intergroup differences. If the ANOVA revealed statistically significant ($p < 0.05$) intergroup variance, Dunnett's test was used to compare the test compound-treated groups to the control group.

The AFC data was expressed as both Specific Activity, IgM antibody forming cells per million spleen cells (AFC/ 10^6 spleen cells), and as IgM Total Spleen Activity (AFC/spleen). Data was first tested for homogeneity of variances using Bartlett's Chi Square Test. Homogenous data were evaluated using a parametric one-way analysis of variance (ANOVA). When the ANOVA indicated a significant difference, the treatment groups were compared to the vehicle control group using Dunnett's test. Non-homogenous data were evaluated using non-parametric analysis of variance. When significant differences occurred, the treatment groups were compared to the vehicle control group using the Gehan-Wilcoxon Test when appropriate. The Jonckheere's Test was used to test for dose-related trends across the control and treated groups. The positive control group was compared to the vehicle control group using a Student's t-Test. The criterion for accepting the positive control in the AFC assay was a statistically significant ($p \leq 0.05$) decrease in the response as compared to the vehicle control group.

Ophthalmoscopic observation:

Not performed

Haematology and clinical chemistry:

Not performed

Urinalysis:

Not performed

Sacrifice and pathology:

A complete necropsy was conducted on all animals. Animals were not fasted overnight prior to the necropsy and were euthanized by carbon dioxide inhalation followed by blood sample collection for possible future IgM antibody analysis. The necropsies included, but were not limited to, examination of the external surface, all orifices, and the cranial, thoracic, abdominal, and pelvic cavities, including viscera.

The following organs were weighed from all animals in the TDAR groups (except as noted) at the scheduled necropsy: Adrenals, brain (weights recorded from all animals in the neurotoxicity groups), spleen, and thymus. The liver and kidneys were not weighed.

Organ (spleen and thymus) weights and final body weights were provided to ImmunoTox®, Inc. following the scheduled necropsy. Statistical analyses of spleen and thymus (absolute and relative) and final body weights were conducted by ImmunoTox®, Inc.

Serum Collection (TDAR groups)

Blood samples for possible IgM antibody analysis were collected from all animals at the scheduled necropsy (study day 28) and processed to serum. Serum samples were not analysed and were discarded following the completion of the AFC assay.

Splenic Antibody-Forming Cell (AFC) Assay (TDAR groups)

At the scheduled necropsy, the spleens from all animals were harvested immediately following blood collection for the IgM AFC assay, weighed and placed into individual tubes containing Earle's Balanced Salt Solution (EBSS) with 15 mM HEPES supplemented with gentamycin as a bacteriostat. Tubes containing the spleens were placed on crushed ice and shipped to ImmunoTox®, Inc., Richmond, VA. The AFC assay served to determine the number of specific IgM antibody-forming cells directed towards sRBC.

Findings:

Clinical signs and mortality:

All animals survived until the scheduled sacrifice. No treatment-related clinical signs were detected.

Body weight and body weight gain:

Reduced body weights gains (3 – 7%) in the 15000 ppm TDAR and neurotoxicity groups during the first week of administration correlated with lower food consumption. However, body weights and food consumption were similar to the vehicle control group throughout the remainder of the study, suggesting low palatability of the 15000 ppm test diet at the beginning of the study. Therefore, lower body weights and food consumption noted in the 15000 ppm groups for study day 0 to 7 were not considered to be adverse.

Otherwise there were no test compound-related body weight changes noted in the 1500 and 5000 ppm TDAR and neurotoxicity groups. Mean body weight gains were statistically significantly higher in the 5000 ppm TDAR group females from study day 21 to 24 when compared to the vehicle control group; however, this was considered transient, sporadic, and unrelated to test diet administration.

Statistically significant lower mean body weight gains than the vehicle control group were noted in the positive control group from study day 24 to 28. As a result, lower mean body weight (5.3%) than the vehicle control group was noted and lower mean cumulative body weight gain was noted from study day 0 to 28. Lower body weights are consistent with the known effects of CPS.

Food consumption:

Test compound-related lower mean food consumption was noted for the 15000 ppm TDAR and neurotoxicity groups from study day 0 to 7 when compared to the vehicle control group. However, no effect on food consumption was observed through the end of dosing, as food consumption for the 15000 ppm groups were similar to the vehicle control group from study day 7 to 28. There were no test compound-related effects on food consumption in the 1500 and 5000 ppm TDAR and neurotoxicity groups.

Table B.6.3.9-2: Average test substance consumption (mg/kg/day)

	Males	Females
ppm	TDAR Groups	
0		0
1500		135
5000		436
15000		1340
	Neurotoxicity Groups	
0	0	0
1500	132	135
5000	430	430
15000	1200	1305

Neurological findings:

Clinical findings in the test compound-treated groups were noted with similar incidence in the vehicle control group, were limited to single animals, were not noted in a dose-related manner, and/or were common findings for laboratory rats of this age and strain.

Sacrifice and pathology:

Organ weights:

There were no test-compound-related effects on absolute and relative (to body weight) brain, adrenal gland, spleen, and thymus weights. As anticipated, the positive control group produced statistically significant decreases in both absolute and relative spleen and thymus weights when compared to the vehicle control group.

Gross pathology:

There were no test compound-related macroscopic findings at the scheduled necropsy. All macroscopic findings noted were considered to be spontaneous and/or incidental in nature and unrelated to test diet administration.

Small thymus was noted for 4 of 8 animals in the positive control group. This finding was consistent with known immunosuppressive effects of CPS.

Splenic Antibody-Forming Cell Assay (TDAR groups):

There were no test compound-related effects in the splenic IgM specific activity (AFC/10⁶ spleen cells) or total spleen activity (AFC/spleen). Additionally, there were no test compound-related effects on spleen cell number.

As anticipated, the positive control group produced statistically significant decreases in specific activity, total spleen activity, and spleen cell number, as compared to the vehicle control group.

Conclusion:

In the absence of S-2200 TG-related effects on the AFC response, the no-observed-effect-level (NOEL) for the humoral immune response in the TDAR groups was considered to be 15000 ppm, equivalent to 1340 mg/kg of body weight/day, which is excess of the limit dose for such studies. No adverse effect was observed for the general toxicity parameters evaluated. A dose level of 15000 ppm was concluded to be appropriate for subsequent definitive immunotoxicity and neurotoxicity testing.

Reference:	S-2200 TG – A 28-Day Oral (Dietary) Immunotoxicity Study in Female Wistar Han Rats
Author(s), year:	██████████ 2011b
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0039
Guideline(s):	US EPA OPPTS 870.7800
GLP:	Partially: Yes ██████████ portion)/ Quality Assured (ImmunoTox data)
Deviations:	-
Validity:	Yes

Material and methods:

Test Material:	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G
Purity:	93.4%
Stability of test compound:	Expiry date after completion of treatment; homogeneity and 10-day stability of S-2200 TG in rodent diet was

also established in the dietary analysis in a previous study (Bodle, 2011, [REDACTED], ROA-0020).

Vehicle: None, test material was mixed directly into the diet

Test animals:

Species: Rat (females only)

Strain: Wistar [REDACTED] WI(HAN)

Age: Approximately 7 weeks at start of treatment

Weight at dosing: 137-175 g

Source: [REDACTED]

Diet: Certified Rodent LabDiet® 5002 (meal) (PMI Nutrition International, LLC) *ad libitum*

Animal assignment and treatment:

10 randomly assigned female rats/dose group were fed diets containing S-2200 for 28 days. On study day 24, all animals were immunized via an intravenous lateral tail vein injection with 0.5 mL of 2×10^8 sRBC in EBSS with HEPES. In addition, all positive control group animals (Group 5) were administered cyclophosphamide monohydrate (CPS) via intraperitoneal injection once daily on study days 24 through 27 at a dose level of 50 mg/kg/day and dose volume of 10 mL/kg/day. Individual animal positive control doses were based on the most recently recorded body weights to provide the correct mg/kg/day dose. The sRBC immunizations were administered prior to administration of CPS on study day 24.

Table B.6.3.9-3: Animal assignment and treatment

Group number	Group	Dietary concentration (ppm)	CPS Daily dose on days 24-27 (mg/kg/day i.p.)	sRBC on day 24 c (mL/Rat i.v.)	Females
T-cell dependent antibody response (TDAR)					
1	Diet only	0	0	0.5	10
2	S-2200 TG Low	1500	0	0.5	10
3	S-2200 TG Medium	5000	0	0.5	10
4	S-2200 TG High	15000	0	0.5	10
5	Positive control: CPS	0	50	0.5	10

Clinical observations:

All animals were observed twice daily, once in the morning and once in the afternoon, for mortality and moribundity.

Clinical examinations were performed once daily. The absence or presence of findings was recorded for individual animals at the scheduled intervals. Observations included but were not limited to; changes in the skin, fur, eyes, and mucous membranes; respiratory circulatory, and autonomic and central nervous systems function; somatomotor activity and behaviour patterns. Detailed physical examinations were conducted on all animals approximately weekly, beginning approximately 1 week prior to randomization, at the time of randomization, once weekly during the study, and on the day of the scheduled necropsy. The animals were removed from their home cages and placed in a standard arena for observation of changes in gait, posture or clonic or tonic movements Stereotypies (e.g. excessive grooming, repetitive circling), bizarre behaviour (e.g. self-mutilation, walking backwards) and permanent or semi-permanent signs, such as skin lesions and hair loss, were also recorded. Daily observations were not performed on days when detailed physical examinations were conducted.

Food consumption and body weight:

Individual body weights were recorded for all animals twice weekly beginning approximately 1 week prior to randomization, at the time of animal selection for randomization, on study day 0, and ending just prior to the scheduled necropsy. Final body weights were recorded on the day of the scheduled necropsy. Individual food consumption was recorded approximately weekly for all animals, beginning approximately 1 week prior to randomization, and ending just prior to the scheduled necropsy. Food consumption was calculated as g/animal/day for the corresponding body weight intervals. The mean amounts of S-2200 TG consumed (mg/kg bw/day) per dose group was calculated from the mean food consumed (g/kg bw/day) and the appropriate target concentration of test compound in the food (mg/kg).

Statistical analysis:

All statistical tests were performed using [REDACTED] Data Management System™ unless otherwise noted. Analyses were conducted using two-tailed tests (except as noted otherwise) for minimum significance levels of 1% and 5%, comparing each test compound-treated group to the control group.

Body weight, body weight change, food consumption and organ weight data (adrenal glands; absolute and relative to final body weight ratio) were subjected to a parametric one-way ANOVA to determine intergroup differences. If the ANOVA revealed statistically significant ($p < 0.05$) intergroup variance, Dunnett's test was used to compare the test compound-treated groups to the control group.

The positive control data were evaluated using the Student's t-Test and compared to the vehicle control group.

The AFC assay, terminal body weight, spleen and thymus organ weight (absolute and relative to terminal body weight), and spleen cellularity data obtained in this study were analysed by ImmunoTox®, Inc. All means were presented with standard errors. The AFC data was expressed as both specific activity (IgM antibody forming cells per million spleen cells [AFC/10⁶ spleen cells]) and as IgM total spleen activity (AFC/spleen). Data was first tested for homogeneity of variances using the Bartlett's Chi Square test. Homogeneous data was evaluated using a parametric one-way ANOVA. When significant differences occurred, the treatment groups were compared to the vehicle control group using Dunnett's test. Non-homogenous data was evaluated using a non-parametric ANOVA. When significant differences occurred, the treatment groups were compared to the vehicle control group using the Gehan-Wilcoxon test, when appropriate. The Jonckheere's test was used to test for dose-related trends across the vehicle control and treatment groups. The positive control data was evaluated using the Student's t-Test and compared to the vehicle control group. The criteria for accepting the results of the positive control group included a statistically significant ($p \leq 0.05$) decrease in the response compared to the of the vehicle control group. For the purpose of data interpretation, statistical significance was not considered automatically to imply immunotoxicological significance. Conversely, the absence of a statistically significant comparison was not considered solely to imply the lack of a biologically relevant effect.

Ophthalmoscopic observation:

Not performed

Haematology and clinical chemistry:

Not performed

Urinalysis:

Not performed

Sacrifice and pathology:

Animals were euthanized by carbon dioxide inhalation followed by exsanguination. A complete necropsy was conducted on all animals. The necropsies included, but were not limited to, examination of the external surface, all orifices, and the cranial, thoracic, abdominal, and pelvic cavities, including viscera. The following tissues and organs were collected and placed in 10% neutral-buffered formalin (except as noted) for possible future microscopic evaluation: both adrenals, lymph nodes (axillary (left), mandibular, mesenteric), Peyer's patches, spleen, and thymus.

The following organs were weighed from all animals at the scheduled necropsy: adrenals, spleen, and thymus. Liver and kidneys were not weighed.

IgM Antibody analysis:

Blood samples for possible IgM antibody analysis were collected from all animals at the scheduled necropsy (study day 28) and processed to serum. Serum samples were not analysed and were discarded on issuance of the final report.

Splenic Antibody-Forming Cell (AFC) Assay:

Spleens were collected from all animals at the scheduled necropsy immediately following blood collection. Individual spleens were placed into individual tared tubes, containing EBSS with 15 mM HEPES and supplemented with gentamycin as a bacteriostat. Each tube was then weighed to provide a "wet" weight for each spleen, maintained on ice, and shipped to ImmunoTox®, Inc., for AFC analysis. Spleen, thymus, and final body weight data previously recorded by [REDACTED] was provided to ImmunoTox®, Inc., for subsequent organ weight analysis. The spleen samples were processed into single-cell suspensions. The cell suspensions were centrifuged and resuspended. Spleen cell counts were performed using a Model Z1 Coulter Counter®. Viability of splenocytes was determined using propidium iodide and flow cytometry (Coulter® EPICS® XL-MCL). The AFC assay served to determine the number of specific IgM antibody-forming cells directed towards sRBC.

Findings:

Clinical signs and mortality:

All animals survived until the scheduled sacrifice. No treatment-related clinical signs were detected.

Body weight and body weight gain:

Body weights were unaffected by test compound administration. However, a statistically significantly higher mean body weight gain was noted at 15000 ppm (study day 3 to 7) and higher mean cumulative body weight gains were noted at 5000 (study days 0 to 7, 0 to 10, and 0 to 14) and 15000 ppm (study days 0 to 10 and 0 to 14) compared to the vehicle control group. Differences in body weight gain were probably due to biological variability and were not considered related to test compound administration due to 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 significant lower mean body weight gains and cumulative body weight gains were noted in the positive control group from study day 24 to 28 and 0 to 28, respectively, compared to the vehicle control group. These lower body weight gains were consistent with the known effects of CPS.

Food consumption:

Food consumption was unaffected by test compound administration. There were no statistically significant differences when the vehicle control and test compound-treated groups were compared.

Group mean compound consumptions (mg/kg bw/day), were based on theoretical dietary concentrations of

the test compound.

Table B.6.3.9-4: Average test substance consumption (mg/kg bw/day)

ppm	mg/kg bw/day
0	0
1500	147
5000	471
15000	1419

Neurological findings:

There were no observations of neurological findings in the test compound-treated groups.

Sacrifice and pathology:

There was statistically significant higher spleen weight relative to final body weight at 15000 ppm compared to the vehicle control group; however, absolute spleen weights were not statistically significantly different. There were no test compound-related effects on adrenal gland and thymus weights; values were not statistically significant when compared to the vehicle control group. As anticipated, statistically significant lower spleen and thymus weights (absolute and relative to body weight) were noted in the positive control group, when compared to the vehicle control group. These effects were consistent with the known immunosuppressive effects of CPS and validated the appropriateness of the assay.

Gross pathology:

There were no test compound-related macroscopic findings at the scheduled necropsy. All macroscopic findings noted were considered to be spontaneous and/or incidental in nature in the absence of a dose-response and unrelated to test compound administration.

A small thymus was noted in 9 of the 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.

Splenic Antibody-Forming Cell (AFC) Assay:

There were no statistically significant effects on spleen cell numbers in the S-2200 TG-treated groups when compared to the vehicle control group. In the functional evaluation of the IgM AFC response to the T-dependent antigen sheep erythrocytes, treatment with S-2200 TG did not significantly suppress the humoral immune response when evaluated as either specific activity (AFC/10⁶ spleen cells) or as total spleen activity (AFC/spleen).

As anticipated, statistically significant lower spleen cell numbers (87%), specific activity (100%), and total spleen activity (100%) were noted in the positive control group, CPS, when compared to the vehicle control group.

Conclusion:

S-2200 TG administered *ad libitum* in the diet for 28 consecutive days to 3 groups of female Crl:WI(HAN) rats at dosage levels of 1500, 5000, and 15000 ppm did not suppress the AFC response to the T cell-dependent antigen sheep red blood cells. Therefore, the no-observed-adverse-effect level (NOAEL) for the functional humoral immune response was considered to be 15000 ppm (equivalent to 1419 mg/kg/day), the highest dose level tested.

B.6.3.10 Summary of short-term toxicity studies

A series of studies was carried out to investigate the effects of orally administered mandestrobin in rats (one 90-day study), mice (one 90-day study) and dogs (one 90-day and one 1-year study) following repeated exposure via the oral route over subchronic periods. In addition, effects after repeated exposure via the dermal route were also investigated in the rat (one 28-day study). Furthermore, a 28 day immunotoxicity study in rats was performed.

In the 90-days rat study, the NOAEL was set at 4000 ppm (282.6♂ and 320.1♀ mg/kg bw/day) based on liver weight increase of more than or close to 20%, hepatocellular hypertrophy, increased cholesterol, and thyroid follicular cell hypertrophy at 10000 ppm.

The mechanistic basis for increases in liver weight, hepatocellular hypertrophy, and follicular cell hypertrophy in the thyroid gland observed from 4000 ppm is considered to be liver enzyme induction and increased catabolism of thyroid hormones in a manner similar to the observed effects induced by phenobarbital (see also position paper Yamada 2012a and study Asano 2012e). These effects are not considered relevant for human risk assessment.

In a 90-days toxicity study in the mouse (0, 1750, 3500 and 7000 ppm S-2200 TG), liver weight increases were noted in all treated groups in both sexes. Because an adaptive mechanism is ascribable for the increase in liver weights rather than a pathological effect, the increases were considered non-adverse, particularly in the absence of any biochemical or histological markers of liver pathology.

In the absence of other treatment-related adverse findings, the NOAEL is considered to be 7000 ppm (807.3 mg/kg bw/day for males and 1111.2 mg/kg bw/day for females, respectively).

In the 90-days study in dog the NOAEL is proposed at 4000 ppm (90.9 mg/kg bw/day for male and 102.7 mg/kg bw/day for female animals), based on increased liver weight, histopathological changes in the liver (pigmentation and centrilobular degeneration), and increased alkaline phosphatase levels.

In the 1-year dog study the NOAEL for males is proposed at 800 ppm (19.2 mg/kg bw/day), and the NOAEL for females at 4000 ppm (92.0 mg/kg bw/day). Mandestrobin administration of 8000 ppm was associated with increased absolute and relative liver weights, hepatocyte hypertrophy, hepatocyte pigment and disturbances to clinical biochemistry parameters (increased alkaline phosphatase, γ-glutamyltransferase and triglycerides) in both genders. At 4000 ppm, male animals showed increased ALP-activity, hepatocyte hypertrophy, and pigmentation. The livers of female animals showed similar changes at the next higher dose level (8000 ppm).

In the dermal 28-days study in rats, no toxicological change related to administration of the test compound was observed in any tested parameter. Therefore, the NOAEL is concluded to be 1000 mg/kg bw/day (the highest dose tested) for both males and females.

In an oral range finding study and in an immunotoxicity study, no treatment related effects were observed up to the highest dose tested. The NOAEL for immunotoxicity was therefore set at 15000 ppm (equivalent to 1419 mg/kg bw/day).

Table B.6.3.10-1: Summary of subchronic toxicity studies

Study	Dose Levels	NOAEL	Effects at the LOAEL
Rat, 90-days (oral) [REDACTED] 2011a	0, 800, 4000, 10000, 20000 ppm ♂: 0, 54, 282.6, 742.7, 1544.6 mg/kg bw/day ♀: 0, 61.6, 320.1, 788.5, 1886.5 mg/kg bw/day	4000 ppm ♂ 282.6 mg/kg bw/day ♀ 320.1 mg/kg bw/day	↑ absolute and relative liver weight Hepatocellular hypertrophy Follicular cell hypertrophy in the thyroid ↑ Cholesterol levels
Mouse, 90-days (oral) [REDACTED] 2011b	0, 1750, 3500, 7000 ppm ♂: 0, 204.1, 404.9, 807.3 mg/kg bw/day ♀: 0, 251.8, 529.1, 1111.2 mg/kg bw/day	7000 ppm ♂ 807.3 mg/kg bw/day ♀ 1111.2 mg/kg bw/day	No treatment related adverse effects observed at the highest tested dose level
Dog, 90-days (oral) [REDACTED] 2012d	0, 4000, 12000, 40000 ppm ♂: 0, 90.9, 267.8, 933.1 mg/kg bw/day ♀: 0, 102.7, 304.4, 820.4 mg/kg bw/day	4000 ppm ♂ 90.9 mg/kg bw/day ♀ 102.7 mg/kg bw/day	↑ liver weight Pigmentation of the liver Centrilobular degeneration ↑ alkaline phosphatase levels
Dog, 1-year (oral) [REDACTED] 2012a	0, 200, 800, 4000, 8000 ppm ♂: 0, 4.3, 19.2, 92.0, 180.7 mg/kg bw/day ♀: 0, 4.5, 20.4, 92.0, 225.7 mg/kg bw/day	♂ 800 ppm ♀ 4000 ppm ♂ 19.2 mg/kg bw/day ♀ 92.0 mg/kg bw/day	♂ hepatocyte hypertrophy, pigmentation ↑ alkaline phosphatase levels ♀ ↑ rel liver weight hepatocyte hypertrophy, pigmentation ↑ alkaline phosphatase levels
Rat, 28-days (dermal) [REDACTED] 2011	0, 100, 300, 1000 mg /kg bw/day	1000 mg/kg bw/day	No treatment related effects observed at the highest tested dose level
Rat, 28-days (oral), range finding study for immunotox and neurotox studies [REDACTED] 2011a	0, 1500, 5000, 15000 ppm ♂: 0, 132, 430, 1200 mg/kg bw/day ♀: 0, 135, 436, 1340 mg/kg bw/day	15000 ppm ♂ 1200 mg/kg bw/day ♀ 1340 mg/kg bw/day	No treatment related effects observed at the highest tested dose level
Rat, 28-days (oral), immunotox study [REDACTED] 2011b	0, 1500, 5000, 15000 ppm ♀: 0, 147, 471, 1419 mg/kg bw/day	15000 ppm ♀ 1419 mg/kg bw/day	No treatment related effects observed at the highest tested dose level

B.6.4 Genotoxicity (Annex IIA 5.4)

B.6.4.1 *In vitro* assays

B.6.4.1.1 Bacterial assay for gene mutation

Reference:	Reverse Mutation Test of S-2200 TG in Bacterial Systems
Author(s), year:	Kitamoto, S.; 2010a
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No.ROT-0012
Guideline(s):	US EPA OPPTS 870.5100; OECD 471; EU Council Regulation No.440/2008, Part B, Method B13/14; Japanese MAFF Notification No. 12 Notsan 8147.
GLP:	Yes (Lab certified by National Authority)
Deviations:	None
Validity:	Yes

Material and methods:

<i>Test Material:</i>	S-2200 Technical Grade
Lot/Batch:	ST-0811G
Purity:	93.4%
Stability of test compound:	Confirmed for the duration of the study by Certificates of Analysis spanning the study period. Stability was confirmed analytically in the solvent DMSO.
Solvent	DMSO

Test strains and chemicals used as positive controls

Without metabolic activation:

<i>S. typhimurium</i> TA100	0.01 µg/plate	2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
<i>S. typhimurium</i> TA1535	0.5 µg/plate	sodium azide
<i>E. coli</i> WP2uvrA	0.01 µg/plate	2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
<i>S. typhimurium</i> TA98	0.1 µg/plate	2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
<i>S. typhimurium</i> TA1537	80 µg/plate	9-aminoacridine

With metabolic activation:

<i>S. typhimurium</i> TA100	1 µg/plate	2-aminoanthracene
<i>S. typhimurium</i> TA1535	2 µg/plate	2-aminoanthracene
<i>E. coli</i> WP2uvrA	10 µg/plate	2-aminoanthracene
<i>S. typhimurium</i> TA98	0.5 µg/plate	2-aminoanthracene
<i>S. typhimurium</i> TA1537	2 µg/plate	2-aminoanthracene

Drug metabolizing enzyme system (S9 mix):

<i>S9-fraction:</i>	Oriental Yeast Co., Ltd. (Tokyo, Japan); Lot No. 09051502 Liver fraction prepared from male Sprague Dawley rats induced by phenobarbital and 5,6-benzoflavone
<i>S9-mix:</i>	10% (v/v) S9-fraction 8 µmol MgCl ₂ 33 µmol KCl 5 µmol glucose-6-phosphate 4 µmol NADPH 4 µmol NADH 100 µmol Na-phosphate buffer (pH 7.4)

S-2200 TG was evaluated for its mutagenic potential by a reverse mutation test with four strains of *Salmonella typhimurium* (TA100, TA98, TA1535, and TA1537) and one strain of *Escherichia coli* (WP2uvrA). The test was conducted by the preincubation method in the presence and absence of metabolic activation (rat liver S9 mix).

In a dose-finding assay the doses ranged from 4.88-5000 µg/plate. Cytotoxicity in strains TA100, TA1535 and TA1537 was observed at, and above, 313 µg/plate without S9 mix and at, and above, 1250 µg/plate with S9 mix. No cytotoxicity was seen at any dose for the other strains with or without S9 mix. Precipitation of the test compound was observed at and above 1250 µg/plate with and without S9 mix.

In the main assays (I and II), S-2200 TG was tested in triplicate at doses ranging from 9.77-313 µg/plate for TA100, TA1535 and TA1537 without S9 mix, from 39.1-1250 µg/plate for TA100, TA1535, TA1537 with S9 mix, and from 156-5000 µg/plate for WP2uvrA and TA98 with and without S9 mix.

Findings:

There was no statistically significant dose-related increase in the number of revertant colonies to at least twice as many as that of the solvent control with or without S9 mix. The negative result was replicated (Assay II). Positive controls showed the appropriate increase in the number of revertant colonies.

Table B.6.4.1-1: Results of reverse mutation test in bacterial systems

Substance (µg/plate)	S9- mix	Revertant colonies/plate (mean)									
		TA100		TA1535		WP2uvrA		TA98		TA1537	
		Assay I	Assay II	Assay I	Assay II	Assay I	Assay II	Assay I	Assay II	Assay I	Assay II
0	-	85	82	8	8	23	27	21	24	10	14
9.77	-	92	81	8	10	NT	NT	NT	NT	7	9
19.5	-	86	86	9	8	NT	NT	NT	NT	10	12
39.1	-	85	84	8	12	NT	NT	NT	NT	11	10
78.1	-	91	86	8	9	NT	NT	NT	NT	11	10
156	-	87	93	9	9	25	17	25	23	11	11
313	-	65*	66*	5	5*	27	25	27	21	6*	7*
625	-	NT	NT	NT	NT	28	23	23	22	NT	NT
1250 [†]	-	NT	NT	NT	NT	23	26	23	24	NT	NT
2500 [†]	-	NT	NT	NT	NT	22	25	19	22	NT	NT
5000 [†]	-	NT	NT	NT	NT	19	22	16	20	NT	NT
PC	-	519	566	250	263	102	101	303	330	366	326
0	+	81	78	8	8	29	28	24	28	15	15
39.1	+	96	81	5	8	NT	NT	NT	NT	16	11
78.1	+	88	94	9	9	NT	NT	NT	NT	7	11
156	+	93	96	6	10	29	33	34	25	11	13
313	+	79	85	7	6	33	30	24	27	11	12
625	+	82*	72*	6*	8*	25	28	25	26	12*	13*
1250 [†]	+	78*	74*	5*	9*	27	27	25	20	7*	11*
2500 [†]	+	NT	NT	NT	NT	19	26	29	23	NT	NT
5000 [†]	+	NT	NT	NT	NT	20	22	24	20	NT	NT
PC	+	701	611	208	184	516	503	207	197	103	110

PC positive control

NT not tested

* toxic effects observed

† precipitation

Conclusion:

S-2200 TG is not mutagenic under the test conditions.

B.6.4.1.2 Test for clastogenicity in mammalian cells

Reference:	<i>In vitro</i> Chromosomal Aberration Test on S-2200 TG in Chinese Hamster Lung Cells (CHL/IU)
Author(s), year:	Kitamoto, S; 2010b
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No.: ROT-0013
Guideline(s):	US EPA OPPTS 870.5375; OECD 473; EU Council Regulation No.440/2008, Part B, Method B10; Japanese MAFF Notification No. 12 Nousan 8147
GLP:	Yes (Lab certified by national authority)
Deviations:	No
Validity:	Yes

Material and methods:

Test Material:	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G
Purity:	93.4%
Stability of test compound:	Confirmed for the duration of the study by Certificates of Analysis spanning the study period. Stability was confirmed analytically in the solvent DMSO.
Solvent:	DMSO

Control Materials:

Negative:	Solvent (DMSO)
Positive:	
- without activation (-S9):	Mitomycin C (MMC)
- with activation (+S9):	Cyclophosphamide (CP)

Activation: S9 was derived from liver homogenates of male SD rats induced with phenobarbital and 5,6-benzoflavone, and obtained commercially (Oriental Yeast Co, Tokyo)

- S9 Mix composition:	S9 fraction	30%
	MgCl ₂	5 mM
	Glucose-6-phosphate	5 mM
	NADPH	4 mM
	KCl	33 mM
	HEPES buffer (pH 7.2)	4 mM

Test cells: CHL/IU cells from Dainippon Pharmaceutical Co, Ltd (Osaka, Japan) were obtained on 29 June 1987, and stored frozen in liquid nitrogen. Cells were confirmed not to have mycoplasma contamination. One ampoule of cell stock was cultured for this experiment; the number of cell passage was from 9 to 16 in this study. Doubling time for these cells is approximately 15 hours, a check of stock cells within two months of this study showed a doubling time of 13 hours.

Culture medium: Eagle's minimum essential medium (MEM; Nissui Pharmaceutical Co, Ltd, Tokyo, Japan) supplemented with 10% bovine serum (Lot

No.: 731681 Life Technologies, Inc., USA) in plastic dishes under a humidified atmosphere of 5% CO₂ at 37°C

Test concentrations:

Preliminary cytotoxicity assays:

With and without S9 mix: 0, 3.91, 7.81, 15.6, 31.3, 62.5, 125, 250, 500*, 1000* µg/mL

(* - Precipitate seen at these concentrations)

Chromosomal aberration assay, (6 hours):

Without S9 mix: 0, 20, 35, 50, 65, 80 µg/mL (first trial)

Without S9 mix: 0, 10, 20, 40, 60, 80, 100, 120 µg/mL (second trial)

With S9 mix: 0, 25, 50, 75, 100, 125, 150 µg/mL

Chromosomal aberration assay, (24 hours, repeat 6 hours):

Without S9 mix: 0, 1.95, 3.91, 7.81, 15.6, 31.3 µg/mL

With S9 mix: 0, 25, 50, 75, 100, 125, 150 µg/mL

The test material was dissolved in DMSO. CHL/IU cells from frozen stock were seeded at approximately 3 x 10³ cells/mL, 5 mL per 60 mm-diameter culture dish, 3 days prior to each experiment. Duplicate monolayer plates were exposed to a range of concentrations of test compound with or without concurrent S9 mix for either 6 hours (then washed and held in test compound-free culture for 18 hours), or exposed to the test compound for 24 hours.

Cytotoxicity was tested with and without S9 fraction, using test compound at nine concentrations starting at 1000 µg/mL and decreasing sequentially by a factor of 2. Cells were incubated in test compound for either 6 hours (then washed and held in fresh medium for 18 hours) or for 24 hours. The plates were then trypsinized and the cell suspension counted on a Coulter Counter. Growth rate (% cells in treated group compared to corresponding control) and precipitation of test compound were recorded.

In the assays for chromosomal aberration, cell plates were treated as before but colcemid was added to the cultures to a final concentration of 0.1 µg/mL 1.5 hours prior to harvest. Cells were trypsinized then examined for cell growth and preparation of metaphase spreads. To examine metaphase spreads, cells from the lowest dose at which the growth rate was 50% or lower were fixed and spread on clean glass slides.

After aging for at least one day, the slides were stained with Giemsa. The highest three doses that yielded 200 cells for analysis were used. All slides were read blind with positive and negative controls included. To examine structural aberrations, one hundred metaphases with standard karyotype were evaluated from each duplicate culture. Types of chromosome aberrations were recorded as chromatid gaps, chromosome gaps, chromatid breaks, chromatid exchanges, chromosome breaks, chromosome exchanges (including chromosome rings and dicentric chromosomes) and fragmentation. A gap was defined as an achromatic lesion smaller than the width of one chromatid, and with minimum misalignment of the chromatids. When a cell contained more than 9 chromosomal aberrations, the individual aberrations were not enumerated and the cell was scored as bearing 10 aberrations. To examine numerical aberrations, one hundred metaphases were evaluated from each duplicate culture. Polyploid cells and endoreduplicated cells were recorded.

Incidence of structurally aberrant cells (excluding gaps) and that of numerically aberrant cells were classified according to the criteria of Ishidate: Negative (-) < 5%, Marginal (±) ≥ 5 – < 10%, and Positive (+) ≥ 10%. The test compound was concluded to induce chromosomal aberrations when both of the following criteria were fulfilled: a) incidence of cells with structural aberrations (excluding gaps) and/or with numerical aberrations are Marginal or Positive, and b) a dose/response relationship or reproducibility is observed.

Findings:

Based on the solubility limit in 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 concentrations of 125 µg/mL and at the end of treatment at and above concentrations of 500 µg/mL.

Marked growth inhibition was seen after exposure of CHL cells (+/- S9) to S-2200 TG. The dose-response was steep: (i) without S9, from 48.2% growth at 62.5 µg/mL to 15.1% growth at 125 µg/mL for 6 hours treatment, and from 25.9% growth at 62.5 µg/mL to 3.3% growth at 125 µg/mL, for 24 hours treatment, and (ii) with S9, from 77.0% growth at 62.5 µg/mL to 42.0% at 125 µg/mL, to 1.6% at 250 µg/mL.

In the initial 6 hour exposure assay, in the absence of S9 no precipitate was seen in the medium at any concentrations and because the growth rate at 80.0 µg/mL was not less than 50% a metaphase spread was not prepared in this test and the second trial was performed. In the second trial precipitates were seen in the medium at the beginning of treatment at concentrations of 120 µg/mL. Slides from 40, 60 and 80 µg/mL were analysed. A concentration of 80 µg/mL caused slightly greater than 50% cell growth inhibition, so this concentration and two lower concentrations were analysed. In the presence of S9, precipitates were seen in the medium at the beginning of treatment at and above concentrations of 125 µg/mL. Slides from 100, 125 and 150 µg/mL were analysed. A concentration of 150 µg/mL caused more than 50% cell growth inhibition so this concentration and two lower concentrations were analysed. The test compound induced no increase in the incidence of chromosomally aberrant cells (structural, numerical or polyploid) in any treatment groups with or without S9.

In a 24 hour exposure assay, CHL cells without S9 were treated with 5 doses, starting with 31.3 µg/mL and decreasing sequentially by a factor of 2. A concentration of 15.6 µg/mL caused more than 50% cell growth inhibition, so slides from 3.91, 7.81 and 15.6 µg/mL were analysed, and showed no increases in the incidence of structurally aberrant cells or polyploid cells. This study was considered confirmatory of the negative response seen in the absence of S9 after the 6-hour exposure.

In a repeat 6 hour exposure assay with S9 mix using exposures from 25 – 150 µg/mL, no increases in the incidence of structurally aberrant cells or polyploid cells was observed.

Table B.6.4.1: Results of the *in vitro* Chromosome aberration test

Group	Dose	Rel growth	N	Structural Aberrations										Ishdate Judgement	Polyploid
				No. of Aberrations								Cells with abs			
				gap	ctb	cte	csb	cse	Mul	Tot		(%)			
	(µg/mL)	(%)								+G	-G	+G	-G		(%)
6-Hour Exposure, without S9															
Control	0	100	200	0	1	0	0	0	0	1	1	0.5	0.5	-	0.0
S-2200 TG	40	56.6	200	2	1	0	0	0	0	3	1	1.5	0.5	-	2.5
	60	52.9	200	0	0	0	0	0	0	0	0	0.0	0.0	-	2.0
	80	43.7	200	0	3	0	0	0	0	3	3	1.5	1.5	-	1.5
MMC	0.06	75.0	200	6	23	35	0	1	1	75	69	26.0	24.0	+	0.0
6-Hour Exposure, with S9															
Control	0	100	200	0	0	0	0	0	0	0	0	0.0	0.0	-	0.5
S-2200 TG	100	62.9	200	1	2	6	0	0	0	9	8	2.0	1.5	-	2.0
	125 ^p	55.6	200	1	2	0	0	0	0	3	2	1.5	1.0	-	2.0
	150 ^p	31.8	200	3	4	5	0	0	0	12	9	4.5	3.5	-	3.0
CP	10	53.3	200	13	64	55	0	0	0	132	119	41.5	38.5	+	0.0
24-Hour Exposure, without S9															
Control	0	100	200	1	2	0	0	0	0	3	2	1.5	1.0	-	0.5
S-2200	3.91	70.7	200	2	1	0	0	1	0	4	2	2.0	1.0	-	0.0

TG	7.81	65.1	200	3	4	1	0	0	0	8	5	4.0	2.5	-	0.0
	15.6	48.3	200	2	1	0	0	0	0	3	1	1.0	0.5	-	0.0
MMC	0.02	85.8	200	4	29	10	0	1	0	44	40	19.5	18.0	+	0.0
6-Hour Exposure, with S9, first repeat															
Control	0	100	200	2	0	0	0	0	0	2	0	1.0	0.0		0.0
S-2200 TG	100	77.1	200	1	0	1	0	0	0	2	1	1.0	0.5	-	0.5
	125 ^p	61.6	200	1	4	3	0	1	0	9	8	3.0	2.5	-	2.5
	150 ^p	46.2	200	1	4	0	0	0	0	5	4	2.5	2.0	-	1.5
CP	10	56.3	200	6	58	61	0	0	0	125	119	46.0	45.5	+	0.0

N – number of metaphases analysed
 - Negative, + Positive, ± Marginal as determined by criteria of Ishidate
^p – Precipitation observed at the beginning of the experiment
 Rel growth – % of controls, cytotoxicity
 ctb – chromatid break
 cte – chromatid exchange
 csb – chromosome break
 cse – chromosome exchange
 Mul – multiple aberrations (cells with more than 9 aberrations)
 Tot – total aberrations
 +G – aberrations including gaps; -G – aberrations excluding gaps
 PolypI – polyploid cells and cells with endoreduplication

All the negative control cultures gave values of chromosome aberrations (structural aberration and polyploidy) within the expected range. Positive control chemicals MMC and CP displayed clear and expected increases in the incidence of cells with structural aberrations.

Conclusion:

Mandestrobin has no potential to induce chromosomal aberrations in Chinese hamster lung cells in culture under the conditions tested.

B.6.4.1.3 Test for gene mutation in mammalian cells

Reference:	Gene Mutation Assay in Chinese Hamster V79 Cells <i>in vitro</i> (V79/HPRT) with S-2200 TG
Author(s), year:	Wolny, H.E.; 2010
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0021
Guideline(s):	US EPA OPPTS 870.5300; OECD 476; EU Council Regulation No.440/2008, Part B, Method B17
GLP:	Yes (laboratory certified by national authority)
Deviations:	No
Validity:	Yes

Material and methods:

Test Material:	S-2200 Technical Grade (S-2200TG)
Lot/Batch:	ST-0811G
Purity:	93.4%
Stability of test compound:	Confirmed for the duration of the study by Certificates of Analysis spanning the study period. Stability was confirmed analytically in the solvent DMSO.
Solvent	DMSO

Control Materials:

Negative:	Culture medium and solvent (DMSO)
Positive:	
- Without activation (-S9):	Ethylmethane sulfonate (EMS)
- With activation (+S9):	7,12-dimethylbenz(a)anthracene (DMBA)

Activation:

S9 mix was prepared from phenobarbital/ β -naphthoflavone-induced male rat liver (8-12 weeks old) according to standard protocols. Aliquots were frozen and stored at -80°C, then thawed and mixed with standard S9 co-factor solution prior to use (8 mM MgCl₂, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP in 100 mM sodium-phosphate buffer, pH 7.4).

Test cells:

V79 cell stocks are supplied by the Laboratory for Mutagenicity Testing, Technical University, 64287 Darmstadt, Germany. Each batch is screened for mycoplasma, and checked for karyotype stability and spontaneous mutant frequency. Thawed stock cultures are propagated at 37°C in 80 cm² plastic flasks seeded with 5×10^5 cells.

Culture medium:

Minimal essential medium (MEM)-Hanks' supplemented with 10% fetal calf serum and 1% neomycin. For selection of mutant cells, 11 µg/mL 6-thioguanine (6-TG) was added to the medium.

Locus examined:

Hypoxanthine-guanine phosphoribosyl transferase (HPRT)

Test concentrations:

Preliminary cytotoxicity (range finding) assay:

Without S9 mix, 4 hours treatment: 0, 7.8, 15.6, 31.3, 62.5, 125.0, 250.0[†], 500.0[†], 1000.0[†] µg/mL
 Without S9 mix, 24 hours treatment: 0, 7.8, 15.6, 31.3, 62.5, 125.0, 250.0, 500.0[†], 1000.0[†] µg/mL
 With S9 mix, 4 hours treatment: 0, 7.8, 15.6, 31.3, 62.5, 125.0, 250.0, 500.0[†], 1000.0[†] µg/mL
 († precipitate formation)

Mutation assay I:

Without S9 mix, 4 hours treatment: 0, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0 µg/mL
 With S9 mix, 4 hours treatment: 0, 8.0, 16.0, 32.0, 64.0, 128.0, 172.0 µg/mL
 With S9 mix, 4 hours treatment: 0, 16.0, 32.0, 64.0, 128.0, 144.0, 160.0, 172.0 µg/mL

Mutation assay II:

Without S9 mix, 24 hours treatment: 0, 1.88, 3.75, 7.5, 15.0, 30.0, 40.0, 50.0, 60.0, 70.0 µg/mL
 With S9 mix, 4 hours treatment: 0, 16.0, 32.0, 64.0, 128.0, 144.0, 160.0, 172.0 µg/mL

Doses were selected on the basis of a cytotoxicity pre-test. The highest dose for mutagenicity testing was set based on toxicity of the test material (reducing survival to 10-20%) after 4 hour and 24 hour treatments.

Three day old sub-cultivation stock cultures were trypsinized, rinsed, then used to plate at 1.5×10^6 cells (single culture) and 5×10^2 cells (duplicate cultures) in MEM-Hanks' with 10% FCS (complete medium). After 24 hours the medium was replaced with serum-free medium (or, for 24 hour treatment, with complete medium) containing S-2200 TG, either without S9 mix or with S9 mix (50 µL/mL). Negative and positive controls were treated in parallel. After the designated treatment period, the cultures were rinsed and complete medium returned to the cell flasks. Three days after treatment 1.5×10^6 cells per experimental point were subcultured, and allowed to grow another 7 days. Cells were again recultured and for each duplicate culture, 10 flasks seeded with about $3 - 5 \times 10^5$ cells in medium containing 6-TG. Two additional flasks were seeded with approximately 500 cells each in non-selective medium to determine cell viability. Cultures were incubated for about 8 days, and then stained with methylene blue. Stained colonies with more than 50 cells were counted. Evaluation was performed on at least four concentrations per assay, of which the highest concentration was one producing approximately 10-20% cell survival.

Findings:

Preliminary cytotoxicity assay:

After 4 hour treatment cell survival 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.

Mutation assays:

Experiment I (4 hour treatment): Nine doses from 0.25 – 12.0 µg/mL were chosen for treatment without S9 mix, and the six treatments from 1.0 – 10.0 µg/mL were selected for evaluation. Six doses from 8.0 – 172.0 µg/mL were chosen for treatment with S9 mix, and the five treatments from 8.0 – 128.0 µg/mL were selected for evaluation. The maximum dose was selected based on cytotoxicity.

Experiment IA (4 hour treatment): Seven doses from 16.0 – 172.0 µg/mL were chosen for treatment with S9 mix, and the five treatments from 16.0 – 144.0 µg/mL were selected for evaluation. The maximum dose was selected based on cytotoxicity.

Experiment II (24 hour treatment without S9 mix and 4 hour treatment with S9 mix): Nine doses from 1.88 – 70.0 µg/mL were chosen for treatment without S9 mix, and five doses from 7.5 – 50.0 µg/mL were selected for evaluation. Seven doses from 16.0 – 172.0 µg/mL were chosen for treatment with S9 mix, and five doses from 16.0 – 144.0 µg/mL were selected for evaluation. The maximum dose was again selected based on cytotoxicity.

Negative and positive controls were within the expected historical range. No relevant and reproducible increase in mutant colony number/ 10^6 cells were noted in the main experiments up to the maximum concentration. Isolated increases of the mutation frequency, exceeding the threshold of three 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 to the corresponding negative controls, the 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 of the mutation frequency indicated by a p value <0.05 in all of the experimental groups.

Table B.6.4.1: Results of *In Vitro* Mammalian Gene Mutation (V79-HPRT)

Substance	Dose	Relative cloning efficiency I - survival	Relative cloning efficiency II - viability	Mutant colonies per 10^6 cells
-----------	------	--	--	----------------------------------

	(µg/mL)	Culture 1	Culture 2	Culture 1	Culture 2	Culture 1	Culture 2
Experiment I (without S9, 4 hours treatment)							
Negative control	-	100.0	100.0	100.0	100.0	20.1	12.9
Solvent control (DMSO)	0	100.0	100.0	100.0	100.0	22.7	9.3
Positive control (EMS)	150.0	90.8	89.2	82.5	92.8	168.2	84.2
S-2200 TG	0.25	93.4	96.4	#	#	#	#
	0.5	91.7	93.2	#	#	#	#
	1.0	90.7	87.6	109.0	91.6	15.0	7.7
	2.0	87.3	86.3	107.4	81.2	19.8	11.9
	4.0	65.6	83.2	100.2	103.8	24.8	6.0
	6.0	25.8	58.6	96.9	90.4	11.5	9.7
	8.0	5.9	42.4	99.3	92.5	13.3	7.8
	10.0	4.3	11.8	101.9	79.5	4.4	20.9
	12.0	##	##	##	##	##	##
Experiment I (with S9, 4 hours treatment)							
Negative control	-	100.0	100.0	100.0	100.0	7.8	23.2
Solvent control (DMSO)	0	100.0	100.0	100.0	100.0	11.4	7.9
Positive control (DMBA)	1.1	63.0	70.8	83.4	83.5	528.9	849.8
S-2200 TG	8.0	97.6	100.5	89.1	94.0	12.9	25.0
	16.0	94.7	97.8	82.2	90.9	9.0	13.6
	32.0	98.7	96.8	93.5	100.5	8.2	11.7
	64.0	96.3	96.0	111.0	98.4	21.0	13.3
	128.0	67.4	81.5	86.6	105.1	15.5	22.3
	172.0	0.1	0.2	##	##	##	##
Experiment IA (with S9, 4 hours treatment)							
Negative control	-	100.0	100.0	100.0	100.0	17.6	16.2
Solvent control (DMSO)	0	100.0	100.0	100.0	100.0	6.5	12.6
Positive control (DMBA)	1.1	75.0	80.0	69.6	80.7	761.8	827.8
S-2200 TG	16.0	91.8	99.7	97.7	92.2	9.7	9.6
	32.0	85.7	100.4	81.9	99.9	20.2	17.3
	64.0	99.9	101.7	85.8	88.3	8.7	29.4
	128.0	11.0	64.5	97.8	87.9	9.4	15.8
	144.0	2.8	7.2	82.7	85.0	2.7	5.1
	160.0	0.0	0.0	#	#	#	#
	172.0	0.0	0.0	#	#	#	#
Experiment II (without S9, 24 hours treatment)							
Negative control	-	100.0	100.0	100.0	100.0	12.4	18.4
Solvent control (DMSO)	0	100.0	100.0	100.0	100.0	15.5	11.6
Positive control (EMS)	150.0	166.2	97.8	97.1	98.2	352.2	396.4
S-2200 TG	1.88	185.8	101.2	#	#	#	#

	3.75	104.3	97.3	#	#	#	#
	7.5	120.9	97.1	97.9	96.3	10.3	8.7
	15.0	176.4	94.3	101.6	98.1	12.7	6.4
	30.0	76.0	56.0	99.1	99.2	8.2	10.5
	40.0	46.0	35.3	98.2	95.5	11.7	11.4
	50.0	20.0	8.9	96.5	93.0	15.8	15.2
	60.0	0.0	0.0	##	##	##	##
	70.0	0.0	0.0	##	##	##	##
Experiment II (with S9, 4 hours treatment)							
Negative control	-	100.0	100.0	100.0	100.0	10.8	18.2
Solvent control (DMSO)	0	100.0	100.0	100.0	100.0	9.3	7.8
Positive control (DMBA)	1.1	38.3	41.4	97.8	76.6	675.2	816.8
S-2200 TG	16.0	98.8	105.2	103.1	98.8	10.0	18.8
	32.0	99.2	107.5	98.7	96.1	9.1	11.6
	64.0	98.3	104.3	100.3	93.9	13.6	8.8
	128.0	60.1	79.3	98.2	100.8	10.9	22.5
	144.0	10.9	27.5	102.7	94.4	6.3	38.0
	160.0	6.2	13.6	##	##	##	##
	172.0	0.0	0.0	##	##	##	##

Relative: as % of controls

^p – Precipitate

- Culture was not continued, five higher concentrations were selected to be evaluated at the end of the experiment

- Culture was not continued due to strong toxic effects

Conclusion:

Mandestrobin did not induce gene mutations at the HPRT locus in V79 cells.

B.6.4.2 In vivo assays

B.6.4.2.1 In vivo studies in somatic cells

Reference:	Micronucleus Test on S-2200 TG in CD-1 Mice
Author(s), year:	██████████ 2010c
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0014
Guideline(s):	US EPA OPPTS 870.5395; OECD 474; EU Council Regulation No.440/2008, Part B, Method B12; Japanese MAFF Notification No. 12 Nousan 8147.
GLP:	Yes (laboratory certified by national authority)
Deviations:	No
Validity:	Yes

Material and methods:

Test Material:	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G
Purity:	93.4%
Stability of test compound:	Confirmed for the duration of the study by Certificates of Analysis spanning the study period. Stability and homogeneity were confirmed analytically in the 0.5%

Solvent:	aqueous methylcellulose solvent. 0.5% w/v aqueous methylcellulose
<i>Control Materials:</i>	
Negative control:	0.5% aqueous methylcellulose
Positive control:	Cyclophosphamide (CP)
<i>Test animals:</i>	
Species:	Mouse
Strain:	CD-1 (ICR)
Age:	8 weeks at the time of dosing
Weight:	Range-finding: 35.2 – 14.7 g (males) and 24.8 – 30.9 g (females) Micronucleus test: 32.4 – 39.2 g (males)
Source:	
Number of animals per dose:	Range finding: 5/sex/group, 4 groups Micronucleus test: 5 males/group, 4 groups
<i>Dose levels:</i>	Range finding test: 0, 500, 1000, 2000 mg/kg bw administered by single gavage doses (10 mL/kg) based on weights taken on the day of administration Micronucleus assay: 0, 500, 1000, 2000 mg/kg bw administered by single gavage doses (10 mL/kg) based on weights taken on the day of administration

A range-finding toxicity assay was conducted in which groups of 5 mice per gender received doses of 0 (vehicle control), 500, 1000, or 2000 mg/kg bw S-2200 TG. Mice were observed for clinical signs and mortality immediately after dosing, again at 2.5 hours post-dosing, and daily for 2 days. Body weights were recorded once daily for 2 days. No abnormal sign was observed in any animals, but male mice administered the highest dose showed a slight decrease in body weight gain. This difference in the genders was considered slight and to cause no toxicological concern, so the micronucleus test was conducted in males only. For the micronucleus assay, groups of 5 male mice received a single gavage dose of 0, 500, 1000, or 2000 mg/kg and bone marrow smears prepared 24 hours later. Another group received 2000 mg/kg and smears were prepared at 48 hours. There was an additional cyclophosphamide (CP) positive control group (smears prepared at 24 hours) and two solvent negative control groups (smears prepared at 24 and 48 hours). Animals were sacrificed by cervical dislocation. Bone marrow was extracted from femurs, and centrifuged to obtain cell pellets. The pellets were resuspended and cells were smeared on clean glass slides, fixed with methanol, and Giemsa stained. Incidence of micronuclei in 2000 polychromatic erythrocytes (PCEs), and incidence of PCEs in 1000 erythrocytes (including normochromatic erythrocytes (NCEs), and PCEs) was recorded for each animal. Comparison of the incidence of micronucleated PCE cells was performed using the Kastenbaum-Bowman tables based on the binomial distribution. Levels of significance were tested at p 0.05 and p 0.01. The t-test was used for analysis of the ratio of PCEs to total erythrocytes. The Student's t-test was used if $p \geq 0.05$ in the F-test, and Welch's t-test was used if $p < 0.05$ in the F-test. Levels of significance were tested at p 0.05 and p 0.01 (two tailed).

Findings:

In confirmation of the initial toxicity test, no mice died as a result of administration of S-2200 TG and no abnormal signs were observed in the S-2200 TG treated animals. There was no dose-related increase in micronuclei as a result of S-2200 TG administration. The positive control showed appropriate increase in micronuclei formation to validate the sensitivity of the assay. There was no decrease in the PCE/(PCE+NCE) ratio after exposure to S-2200 TG. Although no change in the PCE/NCE ratio was found, a slight decrease of

body weight gain at 2000 mg/kg bw in males in the range finding test indicates systemic absorption. In addition, sufficient exposure of bone marrow was confirmed in the single dose ADME studies in rats (Sumitomo Chemical Co. Ltd. Report No. ROM-0033). In the 5 mg/kg dose group, S-2200 derived radioactivities in bone marrow were 234.1 (male) and 216.9 (female) ng equivalents/g at 0.5 hours post-dose. In 1000 mg/kg dose group, S-2200 derived radioactivities in bone marrow were 7455 (male) and 3828 (female) ng equivalents/g at 8 hours post-dose. Both S-2200 and its metabolites were detected in plasma at the timepoints stated above.

Table B.6.4.2: Results of the *in vivo* Micronucleus Test

Treatment	Dose (mg/kg)	Sampling time (hr)	Micronucleated PCE (% , mean \pm SD)	PCE ratio (% , mean \pm SD)
Control	0	24	0.35 \pm 0.158	52.0 \pm 4.66
S-2200 TG	500		0.28 \pm 0.057	54.7 \pm 5.10
	1000		0.42 \pm 0.211	55.9 \pm 2.08
	2000		0.42 \pm 0.160	57.1 \pm 2.41
Cyclophosphamide	60	48	4.09 \pm 0.783	44.5 \pm 5.18*
Control	0		0.29 \pm 0.185	50.3 \pm 3.91
S-2200 TG	2000		0.33 \pm 0.120	51.4 \pm 6.67

PCE - polychromatic erythrocytes
NCE – normochromatic erythrocytes
*p < 0.05; **p \leq 0.01
Micronuclei: 2000 PCE were examined from each animal
PCE ratio: PCE/(PCE+NCE), 1000 erythrocytes examined from each animal

Conclusion:

Mandestrobin did not cause an increase in number of micronucleated PCE in male mice.

B.6.4.2.2 *In vivo* studies in germ cells

Based on the results of the *in vitro* and *in vivo* studies as reported above, no further mutagenicity studies in germ cells are necessary.

B.6.4.3 Summary and overall conclusions of genotoxicity studies

Mandestrobin was tested in a standard battery of genotoxicity and mutagenicity tests *in vitro* and *in vivo*. There was no indication of induction of gene mutation either in the presence or absence of metabolic activation in both the bacterial reverse mutation and the mammalian gene mutation tests (HPRT). A negative response for chromosomal aberrations was observed *in vitro* in the Chinese hamster lung cells (CHL/IU). A mouse micronucleus test *in vivo* gave clear negative results. It can be concluded that mandestrobin has no genotoxic potential.

Table B.6.4.3-1: Summary of genotoxicity tests

Test	Test Object	Concentration	Result	Report Ref.
Bacterial reverse mutation	<i>Salmonella typhimurium</i> (TA100, TA98, TA1535, and TA1537) and <i>Escherichia coli</i> (WP2uvrA)	9.77-5000 µg/plate (-S9) 39.1-5000 µg/plate (+S9)	negative	Kitamoto, S. (2010a)
Chromosomal aberration	Chinese Hamster Lung cells (CHL/IU)	1.95-120 µg/mL (-S9) 25-150 µg/mL (+S9)	negative	Kitamoto, S. (2010b)
HPRT forward mutation	Chinese Hamster V79 Cells	0.25-70.0 µg/mL (-S9) 8.0-172.0 µg/mL (+S9)	negative	Wollny, H.E. (2010)
Micronucleus	Mouse (CD-1)	500-2000 mg/kg	negative	██████████ (2010c)

B.6.5 Long term toxicity and carcinogenicity (Annex IIA 5.5)

B.6.5.1 Long term oral toxicity and carcinogenicity study in the rat

Reference:	S-2200 Technical Grade: 104 Week Oral (Dietary) Administration Combined Toxicity/Carcinogenicity Study in the Rat
Author(s), year:	██████████ 2012b
Report/Doc. number:	Sumitomo Chemical Co. Ltd. Report No. ROT-0072
Guideline(s):	OECD 453 (1981), EPA OPPTS 870.4300 (1998), Japanese MAFF 12 Nohsan 8147 (2-1-16) (2001), Reg. (EC) No. 440/2008, B33 (2008)
GLP:	Yes (laboratory certified by National Authority)
Deviations:	Thyroid was not weighed although it is one of the organs which is mandatory to investigate according to OECD 453
Validity:	Yes

Material and methods:

Test Material:	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G
Purity:	93.4%
Stability of test compound:	Expiry date: 21 November 2011 (after completion of treatment)
Vehicle:	None. Test material was mixed directly into diet.
Test animals:	
Species:	Rat
Strain:	██████████:Wl(Han)
Age:	approximately 6 weeks at the start of treatment
Weight at dosing:	140.6 to 225.0 g for males and 113.0 to 178.6 g for females
Source:	██
Diet:	Finely ground SQC Rat and Mouse Maintenance Diet No 1 (Special Diets Services Ltd, Witham, UK) <i>ad libitum</i>

The purpose of this study was to assess the chronic toxicity as well as carcinogenic potential of mandestrobin after dietary administration to rats for 52 weeks (chronic toxicity cohort; 20 animals per sex and dose group) and 104 weeks (carcinogenicity cohort; 50 animals per sex and dose group).

Animal assignment and treatment:

70 animals per sex were assigned to each treatment group using a total randomisation procedure. 20 animals per sex (i.e. chronic toxicity cohort) in each treatment group were used for interim sacrifice and examination at 52 weeks. Following the first full weighing (day -7), group mean body weights and standard deviations were calculated and inspected to ensure there were no unacceptable differences between the groups. The rats received S-2200 TG in the diet at concentrations of 0 (control), 400, 2000, 7000 and 15000 ppm.

Diet preparation and analysis:

Formulations were prepared weekly. The test article was formulated as a diet mix in SQC Rat and Mouse Maintenance Diet No 1 (ground fine).

Formulations were analysed for their test article content and homogeneity using High Performance Liquid Chromatography with Ultra Violet detection (HPLC-UV), based on a method supplied by the Sponsor, and was validated in [REDACTED] Study Number [REDACTED]

The test article was demonstrated to be stable in diet at 100 and 20000 ppm when stored for 15 days at ambient temperature [REDACTED] Study Number [REDACTED].

Table B.6.5.1-1: Group mean compound intakes (mg/kg bw/d) following 52 (chronic toxicity cohort) and 104 (carcinogenicity cohort) weeks of treatment

Group	Dose level (ppm)	Mean compound consumption (mg/kg bw/d)			
		Chronic toxicity cohort		Carcinogenicity cohort	
		Male	Female	Male	Female
1 (control)	0	-	-	-	-
2 (low)	400	25.5	31.3	21.0	26.7
3 (intermediate I)	2000	130.3	151.4	105.1	135.2
4 (intermediate II)	7000	448.8	535.3	375.6	475.0
5 (high)	15000	991.8	1138.9	804.3	1016.2

Clinical observations:

All animals were observed at the beginning and the end of the working day to ensure they were in good health. All animals were observed daily for signs of ill health or overt toxicity. In addition, each animal was given a detailed physical examination including palpation for tissue masses at weekly intervals.

Body weight, food consumption and compound consumption:

Individual body weights were recorded on Day -7, once weekly from Day 1 (before dosing) to Week 16, once every 4 weeks thereafter and on the day of (prior to) necropsy. The amount of food consumed by each cage of animals was determined weekly from Week -1 to Week 16 and then on one week in every four thereafter. Consumption was calculated as g/animal/week.

Compound consumption was calculated once weekly from Week 1 to Week 16 and on one week in every four thereafter. Weekly compound consumption was calculated as mg/kg bw/day and also as an average consumption over the entire treatment period (mg/kg bw/day).

Functional observation battery:

All animals of the chronic toxicity cohorts were subjected to a battery of behavioural tests and observations before initiation of treatment and at once weekly intervals thereafter. In Week 51, an assessment was made of sensory reactivity to stimuli, grip strength and motor activity.

Where possible, the observations were performed at the same time on each occasion. All animals were observed for the following parameters:

- Home cage observations: Posture, activity, gait, tremor, convulsions, excessive vocalisation, arousal upon opening cage.
- Handling observations: Ease of removal from cage, ease of handling, excessive vocalisation, tremor, convulsions, palpebral closure, exophthalmus, lacrimation, lacrimation type, salivation, respiration, piloerection, fur appearance, other.
- Open field observations: Latency to first step, posture, arousal, circling, gait type, gait type severity, stereotypy, tremor, convulsions, other.

And additionally in Week 51 only: Approach response, touch response, tail pinch, air righting ability, pupillary response, corneal tactile reflex test, auditory startle response, hindlimb foot splay, forelimb and hindlimb grip strength.

Ophthalmoscopy:

Investigations were performed on all animals of the chronic toxicity cohort pre-treatment and on the control and high dose group of the chronic toxicity cohort in Week 50. A mydriatic agent was instilled into the eyes before examinations.

Haematology, clinical chemistry and urinalysis:

Haematology:

Blood samples were withdrawn from the chronic toxicity cohort animals in Weeks 13, 26 and 52 and from the carcinogenicity cohort animals in Weeks 78 and 104. Samples were collected from the lateral caudal vein after an overnight period without food (fasting performed only for chronic toxicity cohort). Blood samples from decedents were taken from the abdominal aorta at necropsy, where possible.

For the chronic toxicity cohort, the following parameters were determined on blood taken into EDTA anticoagulant: haemoglobin concentration (Hb), red blood cell count, packed cell volume (PCV), absolute reticulocytes, mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), platelet count, total and differential white blood cell count.

For the carcinogenicity cohort, the following parameters were determined on blood taken into EDTA anticoagulant: total and differential white cell count.

In addition, the following parameters were determined for the chronic toxicity cohort on plasma derived from whole blood taken into trisodium citrate anticoagulant: prothrombin time (PT) and activated partial thromboplastin time (APTT).

A blood smear was routinely prepared but only examined where an assessment of cell morphology was useful to support or clarify abnormalities identified by the automated analyser. Bone marrow smears were prepared at necropsy. They were fixed in methanol but not examined.

Clinical chemistry:

The following parameters were determined for the chronic toxicity cohort on plasma derived from whole blood collected into lithium heparin anticoagulant: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyltransferase (GGT), sodium (Na), calcium (Ca), potassium (K), chloride (Cl), inorganic phosphorus (P), total protein, albumin, globulin, albumin/globulin ratio, triglycerides, total cholesterol, total bilirubin, glucose, urea, creatinine.

Urinalysis:

Urine samples were collected during a six-hour day time period from the chronic toxicity cohort in Week 12, 25 and 51. Food and water were removed during collection.

The following parameters were determined:

Volume (measured by weight, reported in mL; 1 g considered to be equivalent to 1 mL), colour, turbidity, specific gravity, pH*, protein*, glucose*, ketones*, urobilinogen*, bilirubin*, blood*, microscopy of sediment.

*determined semi-quantitatively

Sacrifice and pathology:

All animals, including the decedents, were subjected to necropsy.

The scheduled necropsies were performed after an overnight period without food. Where possible, they were carried out in replicate order. Each animal was given an intraperitoneal overdose of sodium pentobarbitone. Once a suitable deep plane of anaesthesia was established, the animal was exsanguinated by the severing of major blood vessels. A full macroscopic examination was performed under the general supervision of a pathologist and all lesions were recorded.

The following tissues from all animals were preserved in the appropriate fixative.

Table B.6.5.1-2: Tissues preserved in appropriate fixative*; W = weighed; E = processed and examined microscopically

Adrenals	W	E	Oesophagus		E
Animal identification			Optic nerves		E
Aorta		E	Ovaries	W	E
Bone marrow smear (femur) (a) (d)			Pancreas		E
Brain	W	E	Pituitary		E
Caecum		E	Prostate		E
Colon		E	Rectum		E
Duodenum		E	Salivary glands – mandibular, sublingual, parotid		E
Eyes (b)		E	Sciatic nerves		E
Femur with bone marrow and articular surface		E	Seminal vesicles		E
Gross lesions		E	Skin and subcutaneous tissue		E
Harderian glands		E	Spinal cord – cervical		E
Head			Spinal cord – lumbar		E
Heart	W	E	Spinal cord thoracic		E
Ileum		E	Spleen	W	E
Jejunum		E	Sternum with bone marrow		E
Kidney	W	E	Stomach		E
Lacrimal glands			Testes and epididymides (c)	W	E
Larynx		E	Thymus		E
Liver	W	E	Thyroids and parathyroids		E
Lungs with mainstem bronch and bronchioles		E	Tissue masses		E
Mammary		E	Tongue		
Mandibular lymph nodes		E	Trachea		E
Mesenteric lymph nodes		E	Trachea bifurcation		
Muscle (quadriceps)		E	Urinary bladder		E
Nares (e)		E	Uterus including cervix #	W	E
Nasal cavity (e)		E	Vagina		E
Nasopharynx (e)		E			

* Fixative = neutral-buffered 10% formalin except where indicated by:

a – methanol, b - Davidson's fluid, c - Bouin's fixative

d - Bone marrow smears were prepared at necropsy. They were fixed in methanol but not examined.

e – preserved with the head *in situ*

Bone designated for histopathological examination was decalcified using Kristenson's fluid.

Female uterus weights were captured with the oviducts attached to enable more accurate comparison of study data with

background data.

Organ Weights:

Animals were weighed before necropsy. The organs denoted by “W” in the table (tissue list) above were dissected free from fat and other contiguous tissue and weighed before fixation. Left and right organs were weighed together. Organs from all animals of the chronic toxicity cohort and organs from the first 10 animals/sex/group of the carcinogenicity cohort that survived to the scheduled terminal kill were weighed. Due to the presence of lesions, organ weights from animals 58M (400 ppm), 154M (7000 ppm) and 214M (15000 ppm) were excluded and weights were captured from a different animal of the same group and sex. It must be mentioned that the thyroid weight was not recorded as detailed in the updated OECD guideline No. 453 from 07 September 2009. However, pathological examination was conducted. Therefore, this omission is considered not to have affected the outcome or integrity of the study.

Histopathology:

The following tissues were embedded in paraffin wax BP (block stage), sectioned at 5 µm and stained with haematoxylin and eosin:

Group 1 (control) Group 5 (15000 ppm) and decedents (all groups): All tissues denoted by “E” in the table (tissue list) above. The following tissues were examined in Groups 2 (400 ppm), 3 (2000 ppm) and 4 (7000 ppm) of the chronic toxicity cohort: Liver, thyroid, kidney (males only), gross lesions and tissue masses. The following tissues were examined in Groups 2, 3 and 4 of the carcinogenicity cohort: Liver, thyroid, kidney, ovary (females only), gross lesions and tissue masses. Sectioned tissues were examined microscopically by the Study Pathologist.

Statistics:

Analysis of in-life and organ weight parameters:

The control group (Group 1) was taken as the baseline group with which the treated groups (Groups 2, 3, 4, 5) were compared. Body weights, body weight gains, food consumption (carcinogenicity cohort), necropsy body weights, organ weights, organ to necropsy body weight ratios, functional observational battery, locomotor activity, haematology, blood biochemistry and urine analysis variables were analysed using one-way analysis of variance (ANOVA) separately for each sex. Levene's test for equality of variances among the groups was performed. Where this showed no evidence of heterogeneity ($p \geq 0.01$), pairwise comparisons with the control were made using Dunnett's test. A linear contrast was used to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was reported only where none of the pairwise comparisons was significant.

Where Levene's test showed evidence of heterogeneity ($p < 0.01$), the data were analysed either using the same methods after applying a log-transformation or using non-parametric methods. The non-parametric methods employed were the Kruskal-Wallis ANOVA, the Terpstra-Jonckheere test for a dose related trend and the Wilcoxon rank sum test for pairwise comparisons. Where the Kruskal-Wallis ANOVA was not significant, the pairwise comparisons were not reported in order to protect the Type I error.

Selected haematology variables (monocytes, eosinophils, basophils and large unstained cells), clinical pathology parameters with values above or below the limit of the assay and functional observational battery variables with less than five distinct values were analysed using the non-parametric methods described above.

Food consumption (chronic toxicity cohort) was analysed using two-way analysis of variance (ANOVA).

Levene's test for equality of variances across groups, between sexes and for any interaction was performed and where these tests showed no evidence of heterogeneity ($p \geq 0.01$ for all 3 tests), pairwise comparisons with control were made, for each sex separately, using Dunnett's test. For each sex, a linear contrast was

used to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was only reported where none of the pairwise comparisons was significant.

Analysis of macroscopic findings:

All macroscopic finding data for all tissues examined were supplied for analysis. Male and female data were analysed separately. All tests were performed with a two-sided risk. Separate analyses were performed for the following: (i) Terminal kill toxicity animals, (ii) Decedent carcinogenicity animals, (iii) Terminal kill carcinogenicity animals and (iv) All carcinogenicity animals.

For each macroscopic finding, comparisons were made between the incidence in each of the treated groups and that in the control group, using Fisher's Exact test.

Analysis of microscopic findings:

All microscopic finding data for all tissues examined were supplied for analysis. Male and female data were analysed separately. All tests were performed with a two-sided risk. Separate analyses were performed for the following: (i) Terminal kill toxicity animals, (ii) Decedent carcinogenicity animals, (iii) Terminal kill carcinogenicity animals and (iv) All carcinogenicity animals.

For each microscopic finding, comparisons were made between the incidence in each of the treated groups and that in the control group, using Fisher's Exact test. For each microscopic finding of interest, comparisons were made between the incidence in the treated group and that in the control group using the Wilcoxon-Mann-Whitney rank sum test.

Analysis of tumour data:

All tumour data for all tissues examined were supplied for analysis. Male and female data were analysed separately. For groups where all tissues were protocolled to be examined, the numbers of tumour bearing animals were analysed for tumour types found in at least three animals of the given sex. Tumours of similar histogenic origin were merged. Permutational tests for both an increasing and a decreasing dose response were performed across the groups using the dose levels as weighting coefficients, in accordance with the IARC annex. One directional pairwise tests of the treated groups against the control group were also performed.

Non-fatal tumours were analysed using fixed intervals of 1 to 52 weeks, 53 to 78 weeks, 79 to 105 weeks, the interim kill phase and the terminal kill phase. The fatal and non-fatal results were combined in accordance with the IARC annex. At the request of the Study Director, two separate analyses were performed; all animals and carcinogenicity animals only.

Findings:

Mortality and clinical observations:

No treatment-related effect on mortality was detected. The numbers of decedents per group and the percentage of survival are detailed in Tables 6.5.1-3 and 6.5.1-4.

There were no clinical signs in the animals of chronic toxicity cohort or the carcinogenicity cohort that could be attributed to S-2200 TG.

Body weight, body weight gain and food consumption:

Chronic toxicity cohort:

Males offered 15000 ppm had significantly lower body weights than controls at all measured timepoints during the first 24 weeks of the study, which reflected the significant reduction in body weight gain from start to Week 13 (15.0%). Suppression of body weight was also statistically significant at Week 40 (8.1%). These changes are considered to be toxicologically significant.

For females offered 15000 ppm, body weights were lower than control at all measurement intervals, although attaining statistical significance only at Week 52. When compared with control, significant decreases in bw gain were noted from start to Week 13 (9.8%), from Week 28 to 52 (52.6%) and from start to Week 52 (16.4%). In consideration of the findings in the carcinogenicity cohort, the lower body weights in females dosed with 15000 ppm are considered to be toxicologically significant.

In males offered 7000 ppm and 15000 ppm, food consumption was lower than control (4.5% and 4.3%, respectively) over the 52 weeks of treatment, although the reduced intakes were not statistically significant. The lower consumption for males offered 7000 ppm is considered to be not toxicologically relevant based on the lack of a similar effect in the carcinogenicity cohort over this duration and on the lack of a clear alteration in body weight. Females offered 15000 ppm consumed 7.5% less diet than control between Weeks 1 and 52 which was statistically significant in the pairwise comparisons at the $p < 0.05$ level.

All other changes were not considered to be toxicologically relevant based on the lack of temporal consistency and/or dose response relationship. Statistically significant increases in body weight, body weight gain and food consumption in males offered 400 ppm are considered to be incidental as there were no similar findings in the carcinogenicity cohort and in females of both cohorts.

Carcinogenicity cohort:

Significant suppression of body weights was seen from Week 2 onwards for males offered 15000 ppm. A decrease in body weight gain was significant at most of the selected periods culminating in an overall (start to Week 104) mean reduction of 17.6%, when compared with control.

With increasing dose, significant differences from control in female body weights were observed more rapidly. Females offered 2000 ppm had significantly decreased body weights at Week 68 and at all measurement intervals from Week 76 onwards. Differences were significant for females offered 7000 ppm at Week 24 and at all measurement intervals from Week 40 onwards, and for females offered 15000 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 offered 2000, 7000 and 15000 ppm (18.1%, 18.2% and 32.8%, respectively), when compared with control.

No statistically significant differences in food consumption were observed in the carcinogenicity cohort, when compared pairwise with control. However, for animals offered 15000 ppm intakes were consistently lower resulting in overall decreases of 4.4% and 4.8% for males and females, respectively.

Functional observational battery:

There were no findings from any assessment indicative of any neurotoxicological effect of the test formulations.

Ophthalmoscopy:

Ophthalmoscopic examinations performed in Week 50 in the control and high dose groups of the chronic toxicity cohort did not reveal any treatment related effects.

Haematology, clinical chemistry and urine analysis:

Haematology – chronic toxicity cohort:

The decreased haemoglobin concentrations observed in animals offered 15000 ppm were considered to be treatment-related based on temporal consistency. However, these alterations from control were generally small and values were within historical control ranges, where available (historic control data are only available for Week 13 and 26, not for Week 52). In addition, no findings were observed in related endpoints such as red blood cells, reticulocytes or morphological changes in blood forming organs. Therefore, these decreases are considered not to be of toxicological significance.

All other statistically significant differences between control 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.

All statistically significant haematological findings observed in Week 52 are detailed in Table 6.5.1-3.

Haematology – carcinogenicity cohort:

In the carcinogenicity cohort only a total and differential white blood cell count was performed after 78 and 104 weeks of treatment. There were no inter-group differences in the haematological composition of the blood samples that could be attributed to treatment. All statistically significant differences between control and treated groups were small, inconsistent between the 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 neutrophils and/or lymphocytes, which generally correlated microscopically with the presence of haemolymphoreticular tumour.

All statistically significant haematological findings observed in Week 104 are detailed in Table 6.5.1-4.

Clinical chemistry: (chronic toxicity cohort only)

Gamma-glutamyltransferase was statistically significantly increased in both sexes offered 15000 ppm at Week 13 (M: 1.5-fold, F: 2-fold), at Week 26 (M: 2.5-fold, F: 1.5-fold) and at Week 52 (M: 4-fold, F: 3-fold) when compared with controls.

Total cholesterol was higher at Week 13 in animals offered 7000 ppm (M: 29%, F: 38%) and 15000 ppm (M: 47%, F: 56%), at Week 26 in females offered 7000 ppm and 15000 ppm (50% and 65%, respectively) and at Week 52 in females offered 7000 ppm (55%) and in both sexes offered 15000 ppm (M: 26%, F: 50%), when compared with controls. Therefore, the consistent increases seen in females offered 7000 ppm and in both sexes offered 15000 ppm were considered to be toxicologically relevant. As no disturbances were observed on subsequent occasions, the change in males offered 7000 ppm at Week 13 was considered not to be toxicologically significant.

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

All statistically significant findings observed in Week 52 are detailed in Table 6.5.1-3.

Urinalysis: (chronic toxicity cohort only)

There were no obvious differences in urine analysis test results to indicate an effect of treatment.

The mean volume of urine voided by males offered 400 ppm at Week 12 and 51 and by males offered 15000 ppm at Week 51 was statistically increased when compared with control. Quantitative measurements indicated that excretion was approximately 64% to 71% higher. However, as there were no similar effects in females or overt differences in Week 25, these changes are considered to be incidental and not related to treatment. Increased urine output correlated with a decrease in specific gravity.

Sacrifice and pathology - organ weights:

Chronic toxicity cohort:

There were treatment-related changes in liver, kidney and brain weights, when compared with controls.

Increased liver weights were notable in males offered ≥ 400 ppm and in females offered ≥ 2000 ppm. In view of the associated changes in the clinical chemistry and pathological investigations, the statistically significant alterations observed in both sexes offered 7000 ppm and 15000 ppm were considered to be toxicologically significant.

A statistically significant increase in the relative kidney weights was seen in males offered 15000 ppm. A statistically significant decrease in unadjusted brain weight was seen for males offered 15000 ppm (-5%). The relative brain weight was increased for females offered 15000 ppm. Changes in kidney and brain weight were considered not to be toxicologically significant as there were no macroscopic or microscopic correlates, and these changes may be related to body weight suppression.

When compared with control, mean relative ovary weights were statistically significantly increased (+26%) in females offered 15000 ppm. This finding is considered to be incidental as there was no similar difference seen for the carcinogenicity cohort at terminal kill and it was not associated with any pathological correlates. All other organ weight (and/or organ weight ratio) changes were considered not to be biologically significant as they were either small in magnitude, not dose-dependent, inconsistent between the sexes, spontaneous background changes due to normal inter-animal variability and/or lacked a histopathological correlate.

Carcinogenicity cohort:

Increased liver weights were notable in males offered ≥ 7000 ppm and in females offered ≥ 2000 ppm. In view of the associated changes in the clinical chemistry and pathological investigations, the alterations observed in both sexes offered 7000 ppm and 15000 ppm were considered to be toxicologically significant. All other organ weight (and/or organ weight ratio) changes were considered not to be biologically relevant as they were either small in magnitude, not dose-dependent, inconsistent between the sexes, spontaneous background changes due to normal inter-animal variability and/or lacked a histopathological correlate.

Sacrifice and pathology – macroscopic findings:

Chronic toxicity cohort:

Most tissues were macroscopically unremarkable and the findings seen were generally consistent with the usual pattern of findings in animals of this strain and age. However, large liver was recorded in males offered 15000 ppm with a statistically significant incidence. Other statistically significant differences between groups for the macroscopic findings such as mottled liver and red mandibular lymph node, which correlated microscopically with agonal congestion/haemorrhage, were sporadic and considered to not be related to dose or test article toxicity.

Carcinogenicity cohort:

Most tissues were macroscopically unremarkable and the findings seen were generally consistent with the usual pattern of findings in animals of this strain and age. Large liver was variably recorded in males offered 7000 or 15000 ppm, although not statistically significant, which generally correlated with findings seen microscopically. Statistically significant differences between groups for the macroscopic findings noted in decedents and at the terminal kill, such as dark liver which correlated with agonal congestion/haemorrhage, were considered to be secondary and not related to test article toxicity.

Sacrifice and pathology – microscopic findings:

Chronic toxicity cohort:

Microscopic findings were generally infrequent, of a minor nature and consistent with the usual pattern of findings in animals of this strain and age. However, there were findings in the liver and thyroid in males and females, associated with treatment with the test article.

In the liver, there was a statistically significant increase ($p < 0.0001$) in the incidence and severity of hepatocellular eosinophilia/hypertrophy in animals offered 7000 or 15000 ppm, characterised by enlarged hepatocytes with increased amounts of eosinophilic cytoplasm. This was considered to be an adaptive change associated with test article metabolism. There was also a minor increase in hepatocyte vacuolation in males offered 15000 ppm (not statistically significant), characterised by variable numbers of small to large cytoplasmic vacuoles within scattered hepatocytes. The toxicological significance of this change in this study is not clear.

In the thyroid, there was a statistically significant increase in the incidence and in the severity of follicular cell hypertrophy in animals offered 7000 or 15000 ppm. Follicular cell hypertrophy was characterised 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 kidney, hyaline droplets were observed in males offered ≥ 400 ppm, but the incidences were not notably different from that of control males. Hyaline droplets were characterised by small, eosinophilic cytoplasmic inclusions within proximal convoluted tubule epithelium. Hyaline ($\alpha_2\mu$ globulin) droplets in the proximal tubular epithelial cells present a common response of the male rat to xenobiotics by a mechanism that is not relevant to humans.

Other statistically significant differences between dose groups were considered to be incidental and not related to test article toxicity, as they represented recognised background, age-related, non-specific or single-sex findings, decreases in finding incidences, or random occurrences in intermediate dose groups, without association with treatment.

Carcinogenicity cohort – non-neoplastic findings:

Microscopic non-neoplastic findings were generally infrequent, of a minor nature and considered to be within normal background levels in animals of this strain and age. However, in treated animals, there were treatment-related findings in the liver, thyroid gland, kidney and ovary.

In the liver, there was an increase in hepatocyte eosinophilia/hypertrophy in animals offered 2000 ppm and above, characterised by enlarged hepatocytes with increased amounts of pale eosinophilic cytoplasm. Increases in hepatocyte eosinophilia/hypertrophy were statistically significant in the liver of males offered 7000 or 15000 ppm and in females offered 2000 ppm and above. This was considered to be an adaptive change associated with test article metabolism. There was no associated increase in hepatic tumours noted in the study.

There was also a statistically significant increase in hepatocyte vacuolation in treated animals offered 7000 or 15000 ppm, characterised by variable numbers of small to large cytoplasmic vacuoles within scattered hepatocytes. However, the toxicological significance of this change in this study is unknown.

In treated females, and to a lesser extent in males, there was an increase in bile duct hyperplasia. 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 nor progression to bile duct neoplasia.

In the thyroid gland, there was an increase in follicular cell hypertrophy in males, and to a lesser extent in females, offered 15000 ppm; males and females in other dose groups were similar to controls. Thyroid follicular cell hypertrophy showed a statistically significant increase in males offered 15000 ppm. Follicular cell hypertrophy was characterised by follicles with columnar epithelium with increased amounts of apical cytoplasm, with or without a decrease in follicular colloid. This increase in follicular cell hypertrophy was not accompanied by an increase in thyroid follicular cell tumours and is known to be associated with liver hepatocellular hypertrophy. This is generally considered to be an adaptive change due to increased thyroid hormone metabolism in the liver.

In the kidney, there was an increase in papillary and cortico-medullary mineralisation in females offered 2000 ppm and above, and in pelvic mineralisation in females offered 7000 or 15000 ppm, though not necessarily statistically significant. Treated males were similar to controls. Papillary mineralisation was characterised by small amounts of dark basophilic mineral within the renal papillary tubules. Pelvic mineralisation was characterised by similar material within the renal pelvis or pelvic epithelium. Cortico-medullary mineralisation was characterised by small amounts of dark, basophilic material within the tubules and tubular epithelium at the cortico-medullary junction. This overall increase in mineralisation in some treated animals was considered to be associated with renal excretion of the test article and its metabolites, and not direct test article toxicity.

In the ovary, there was a minor increase in sex-cord stromal hyperplasia in females offered the test material although not in a dose-dependent manner and also without statistical significance when compared to controls. Sex-cord stromal hyperplasia was characterised by a hyperplastic lesion composed of any mixture of granulosa, theca, luteal or Sertoli cells within the ovarian parenchyma with minimal involvement of the surface epithelium and was considered to be part of a continuum of change, associated with the

development of sex-cord stromal tumours. The incidence in treated animals was within current historical background data (range: 2-48%). In the background data, sex-cord stromal hyperplasia may be observed frequently in aged animals.

There was a statistically significant increase in oligospermia in the epididymis of males offered 15000 ppm; however, these findings were primarily noted unilaterally. They were considered to be age-related changes due to their predominantly unilateral nature and in the absence of other male reproductive tract findings and therefore not associated with generalised hormonal disruption or test-article toxicity.

There were no other microscopic non-neoplastic findings considered to be related to treatment with S-2200 TG, since these were generally infrequent, of a minor nature and considered to be within normal background levels in animals of this strain and age.

Carcinogenicity cohort – neoplastic findings:

Microscopic neoplastic findings in controls and treated animals were generally consistent with the usual pattern of neoplasms in rats of this strain and age. However, there was an increase in sex-cord stromal tumours in the ovary of females offered 7000 or 15000 ppm. This was not statistically significant when compared with controls by pair-wise analysis. However, for tests of increasing dose response, benign sex cord stromal tumours in the ovary of females were statistically significant in the carcinogenicity cohort when compared to control animals ($p = 0.005$).

The increased incidence of sex-cord stromal tumour in the ovary exceeded the [REDACTED] historical control range (0-3.1%) for this strain of rat. However, it must be highlighted that also the current control animals (incidence $2/50 = 4\%$) exceeded the historical control range for sex-cord stromal tumours. The tumours occurred at dose levels where body weight gain was reduced by more than 20%, indicating that the maximum tolerated dose was exceeded. Sex-cord stromal hyperplasia does occur in aged Wistar rats, and the animals used in this study appear to be derived from a susceptible batch. The incidences of sex-cord stromal proliferative lesions were within historical controls for all groups, and there was no statistically significant difference between groups for hyperplasia or tumours. Higher survival rates in animals offered 7000 and 15000 ppm may have contributed to the higher numbers of tumours. Since there is no interaction of the test article with the oestrogen receptor and steroidogenesis as evidenced by *in vitro* assays (see mechanistic studies, B.6.8.1.2 Ovary issues, *In vitro* Steroidogenesis Assay of S-2200TG in H295R Cells (Kubo, H.; 2012) and Evaluation of Effects of S-2200 TG and its Metabolites on Human Estrogen Receptor alpha and Human Androgen Receptor Using *in vitro* Reporter Gene Assays (Suzuki, N.; 2012)), no direct ovarian toxicity and no reproductive abnormalities, a mode of action via endocrine imbalance is considered to be unlikely. Furthermore, there was no accumulation or persistence of S-2200 TG or its metabolites in the ovary. In the corresponding mouse carcinogenicity bioassay (see chapter B.5.2), the number of tumours in any tissue was not increased by exposure to S-2200 TG. Thus, based on this evidence, the sex-cord stromal lesions are considered unlikely to be toxicologically relevant.

There was a reduction in the incidence of benign mammary fibroadenomas in females offered 15000 ppm and also a statistically significant reduction in the incidence of pituitary tumours (adenoma and carcinoma) in females offered 15000 ppm. This correlates with the reduction in fatal tumours observed in female decedents offered 15000 ppm.

There was also a minor increase in interstitial cell adenomas in the testis of males offered 15000 ppm. However, this was within historical control data at this time (range 0-6%), so it was considered to be a chance effect in the absence of any increase in hyperplastic lesions in this cell type or other evidence of hormonal effects within the male reproductive tract and not due to test article toxicity.

Table B.6.5.1-3: Key results for the combined chronic toxicity/carcinogenicity study in rats – Chronic toxicity cohort

	Males					Females				
Diet concentration (ppm)	0	400	2000	7000	15000	0	400	2000	7000	15000
S-2200 intake (mg/kg bw/day)										
	0	25.5	130.3	448.8	991.8	0	31.3	151.4	535.3	1138.9
Mortality [#]										
	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)										
Week 0 – 28	251.4	285.2 [*]	249.8	239.6	225.0	110.6	105.2	105.5	106.6	101.4
Week 0 – 52	302.5	343.8 [*]	302.7	290.6	271.5	135.6	124.8	129.4	128.3	113.3 ^{**}
Food consumption (g/animal/week)										
Week 1 – 28	155.7	166.2 [*]	157.0	149.0	148.3	119.9	115.4	114.9	115.5	111.3
Week 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 ^{**}	15.6	15.4	15.2	14.9 ^{***}	14.7 ^{***}
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 ^{***}
Ca (mmol/L)	2.64	2.62	2.63	2.66	2.63	2.69	2.62 ^{**}	2.69	2.74 [*]	2.78 ^{***}
Cl (mmol/L)	105	104 [*]	103 ^{***}	102 ^{***}	102 ^{***}	103	103	103	103	102
Inorganic P (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
Globulin (g/L)	24	24	23	23	23	19	20	20	21 [*]	20
A/G ratio	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 ^{**}
γ-glutamyl transferase (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										

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

[illegible]

Terminal body weight (g)	595.3	598.6	561.4	569.9	531.1**	348.6	339.3	309.8**	309.7**	278.8***
Liver (g)	11.512	11.873	11.171	13.104	11.848	6.989	7.262	7.340	7.226	6.748
Relative weight (Ratio %)	2.051	2.029	2.042	2.215	2.334*	2.153	2.038	2.472*	2.446*	2.753***
Macroscopic findings										
Liver (No. examined per group: 50)										
Large	2	4	4	7	9	0	2	2	2	3
Non-neoplastic Microscopic findings										
Liver										
No. examined	32	42	32	41	36	33	35	36	40	41
Hepatocellular eosinophilia/hypertrophy	6	10	12	28***	35***	15	18	30**	36***	37***
Hepatocyte vacuolation	22	37	26	35	33*	15	10	18	29*	34**
Bile duct hyperplasia	2	5	7	4	4	10	17	18	26**	18
Thyroid										
No. examined	32	42	32	40	36	33	35	36	40	41
Follicular cell hypertrophy	0	2	0	0	10**	0	0	0	0	3
Kidney										
No. examined	32	42	32	41	36	33	35	36	40	41
Papillary mineralisation	7	8	7	10	13	17	13	25	30*	26
Pelvic mineralisation	22	30	19	30	26	30	29	34	36	36
corticomedullary mineralisation	0	1	0	0	0	6	7	14*	16*	14
Ovary										
No. examined	-	-	-	-	-	50	50	50	50	50
Sex-cord stromal hyperplasia	-	-	-	-	-	3	8	5	6	5
Neoplastic Microscopic findings										
Benign ovary sex-cord stromal tumours	-	-	-	-	-	2/50 exceed HCD	0/50	1/50	4/50 exceed HCD	6/50 exceed HCD
Benign mammary fibroadenomas	-	-	-	-	-	12/48	2/20	5/21	4/18	5/50
Benign interstitial cell adenomas in testis	0/50	0/13	0/22	2/15	3/50 within HCD	-	-	-	-	-

Pituitary adenomas + carcinomas	9/50	4/12	16/28	8/18	11/50	28/50	20/35	18/31	19/29	18/49
---------------------------------	------	------	-------	------	-------	-------	-------	-------	-------	-------

* p<0.05; **p<0.01; ***p<0.001

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

Conclusion:

Due to the magnitudes of the decreased body weight and body weight gain (at 15000 ppm) and toxicological alterations in the liver including increased liver weights in combination with a higher degree of hepatocellular hypertrophy and hepatocyte vacuolation (both at ≥ 7000 ppm) and/or increased blood biochemistry parameters (total cholesterol and gamma glutamyltransferase in males offered 15000 ppm), the No Observed Adverse Effect Level (NOAEL) for males was considered to be 2000 ppm (105.1 mg/kg bw/day).

For females, body weight and body weight gain was significantly decreased at ≥ 2000 ppm following 104 weeks of treatment. Toxicological alterations in the liver in females included increased liver weights (at ≥ 2000 ppm), a higher degree of hepatocellular hypertrophy and hepatocyte vacuolation (at ≥ 2000 ppm and at ≥ 7000 ppm, respectively) and/or increased blood biochemistry parameters (increased total cholesterol and increased gamma glutamyltransferase at ≥ 7000 ppm and at ≥ 15000 ppm, respectively). Therefore, the NOAEL for females was considered to be 400 ppm (26.7 mg/kg bw/day) for this study following 104 weeks of treatment.

Regarding the carcinogenic potential of S-2200 TG, no increase of neoplastic findings exceeding the historical control range was observed in any organ of treated animals, with exception of benign sex-cord stromal tumours in the ovary. Four and six cases occurred in female rats of the carcinogenicity cohort dosed with 7000 ppm and 15000 ppm, respectively, at dose levels where body weight gain was reduced by more than 20%, indicating that the maximum tolerated dose was exceeded. S-2200 TG is not genotoxic. The incidence of only one benign tumour type was increased. Sex-cord stromal hyperplasia does occur in aged Wistar rats, and the animals used in this study appear to be derived from a susceptible batch. The incidences of sex-cord stromal proliferative lesions were within historical controls for all groups, and there was no statistically significant difference between groups for hyperplasia or tumours. Higher survival rates in animals offered 7000 and 15000 ppm may have contributed to the higher numbers of tumours. The increased incidence of sex-cord stromal tumour in the ovary at 7000 ppm and 15000 ppm exceeded the Covance, Harrogate historical control range (0-3.1%) for this strain of rat. However, it must be highlighted that also the concurrent control animals (incidence 2/50 = 4%) exceeded the historical control range for sex-cord stromal tumours.

Since there is no interaction of the test article with the oestrogen receptor and steroidogenesis as evidenced by *in vitro* assays, no direct ovarian toxicity and no reproductive abnormalities, a mode of action via endocrine imbalance is considered to be unlikely. Furthermore, there was no accumulation or persistence of S-2200 TG or its metabolites in the ovary. In the corresponding mouse carcinogenicity bioassay, the number of tumours in any tissue was not increased by exposure to S-2200 TG. Thus, based on this evidence, the sex-cord stromal lesions are considered unlikely to be toxicologically relevant.

Overall, according to Regulation (EC) No. 1272/2008 and in consideration of the *Guidance on the Application of CLP Criteria, Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, version 2.0 April 2012*, no classification and labelling as carcinogenic substance is proposed for the active substance mandestrobin.

B.6.5.2 Carcinogenicity study in the mouse

Reference:	S-2200 Technical Grade: 78 Week Oral (Dietary) Administration Carcinogenicity Study in the Mouse
Author(s), year:	██████████ 2012c
Report/Doc. number:	Sumitomo Chemical Co. Ltd. Report No. ROT-0073
Guideline(s):	OECD 451 (1981), Reg. (EC) No. 440/2008, B32 (2008), EPA OPPTS 870.4200 (1998), Japanese MAFF 12 Nohsan 8147 (2-1-15) (2001)
GLP:	Yes (laboratory certified by National Authority)
Deviations:	None
Validity:	Yes

Material and methods:

<i>Test Material:</i>	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G
Purity:	93.4%
Stability of test compound:	Expiry date: 21 November 2011 (after completion of treatment)
Vehicle:	None. Test material was mixed directly into diet.
<i>Test animals:</i>	
Species:	Mouse
Strain:	██████████:CD1(ICR)
Age:	6 to 7 weeks old at the start of treatment
Weight at dosing:	29.4 to 40.1 g for males and 20.5 to 30.6 g for females
Source:	██
Diet:	Finely ground SQC Rat and Mouse Maintenance Diet No 1 (Special Diets Services Ltd, Witham, UK) <i>ad libitum</i>

The purpose of this study was to assess the carcinogenic potential of S-2200 TG after dietary administration to mice for 52 weeks (satellite groups; 12 animals per sex and dose group) and 78 weeks (main groups; 51 animals per sex and dose group).

Animal assignment and treatment:

63 animals per sex were assigned to each treatment group using a total randomisation procedure. 12 animals per sex (i.e. satellite group) in each treatment group were used for interim sacrifice and examination at 52 weeks. Following the first full weighing (Day -7), group mean body weights and standard deviations were calculated and inspected to ensure there were no unacceptable differences between the groups. The mice received S-2200 TG in the diet at concentrations of 0 (control), 700, 2000 and 7000 ppm.

Diet preparation and analysis:

Formulations were prepared weekly. The test article was formulated as a diet mix in SQC Rat and Mouse Maintenance Diet No 1 (ground fine).

Formulations were analysed for their test article content and homogeneity using High Performance Liquid Chromatography with Ultra Violet detection (HPLC-UV), based on a method supplied by the Sponsor, and was validated in ██████████ Study Number ██████████

The test article was demonstrated to be stable in diet at 100 and 20000 ppm when stored for 15 days at ambient temperature (██████████ Study Number ██████████).

WARNING: This document is part of an application data package and should be read in isolation. No part of this document may be reproduced or transmitted in any form or by any means electronic or mechanical, including photocopying and recording, or by any information storage or retrieval system, without permission in writing from the copyright owner. This document must not be granted on the basis of this document.

Table B.6.5.2-1: Group mean compound intakes (mg/kg bw/d) following 52 (satellite group) and 78 (main group) weeks of treatment

Group	Dose level (ppm)	Mean compound consumption (mg/kg bw/d)			
		Satellite group		Main group	
		Male	Female	Male	Female
1 (control)	0	-	-	-	-
2 (low)	700	88.4	104.0	82.5	99.2
3 (intermediate)	2000	255.0	325.0	238.8	280.3
4 (high)	7000	883.3	1045.1	823.9	994.0

Clinical observations:

All animals were observed at the beginning and the end of the working day to ensure they were in good health. Any animal which showed marked signs of ill health was isolated. All animals were observed daily for signs of ill health or overt toxicity. In addition, each animal was given a detailed physical examination at weekly intervals. An individual record was maintained of the clinical condition of each animal.

Body weight, food consumption and compound consumption:

Individual body weights were recorded on Day -7, once weekly from Day 1 (before dosing) to Week 16, once every 4 weeks thereafter and on the day of (prior to) necropsy. In addition to occasions stated in the protocol, main group body weights were also recorded in Weeks 77 and 78.

The amount of food consumed by each cage of animals was determined weekly from Week -1 to Week 16 and on one week in every four thereafter. In addition to occasions stated in the protocol, main group food consumption was also recorded in Weeks 77 and 78. Consumption was calculated as g/animal/week. Compound consumption was calculated once weekly from Week 1 to Week 16 and on one week in every four thereafter, and in addition also in Weeks 77 and 78. Weekly compound consumption was calculated as mg/kg/day and also as an average consumption over the entire treatment period (mg/kg/day).

Haematology:

Blood samples (nominally 0.5 mL into EDTA anticoagulant) were withdrawn by orbital sinus puncture from satellite group animals in Week 52 and from main group animals in Week 78. Blood samples were also taken from decedents, where possible.

Samples were analysed for total and differential white cell count. A blood film was routinely prepared but only examined where an assessment of cell morphology was useful to support or clarify abnormalities identified by the automated analyser.

Sacrifice and pathology:

All animals, including the decedents, were subjected to necropsy.

Where possible, the scheduled necropsies were performed in replicate order. Each animal was given an intraperitoneal overdose of sodium pentobarbitone. Once a suitable deep plane of anaesthesia was established, the animal was exsanguinated by the severing of major blood vessels. A full macroscopic examination was performed under the general supervision of a pathologist and all lesions were recorded. The following tissues from all animals were preserved in the appropriate fixative.

Table B.6.5.2-2: Tissues preserved in appropriate fixative*; W = weighed; E = processed and examined microscopically

Adrenals	W	E	Oesophagus		E
Animal identification			Optic nerves		E
Aorta		E	Ovaries	W	E
Brain	W	E	Pancreas		E
Caecum		E	Pituitary		E
Colon		E	Prostate		E
Duodenum		E	Rectum		E
Eyes (a)		E	Salivary glands – mandibular, sublingual, parotid		E
Femur with bone marrow and stifle joint		E	Sciatic nerves		E
Gall bladder		E	Seminal vesicles		E
Gross lesions		E	Skin and subcutaneous tissue		E
Harderian glands		E	Spinal cord – cervical		E
Head			Spinal cord – lumbar		E
Heart	W	E	Spinal cord thoracic		E
Ileum		E	Spleen	W	E
Jejunum		E	Sternum with bone marrow		E
Kidney	W	E	Stomach		E
Lacrimal glands			Testes and epididymides (b)	W	E
Larynx		E	Thymus		E
Liver	W	E	Thyroids and parathyroids		E
Lungs with mainstem bronchi and bronchioles		E	Tissue masses		E
Mammary		E	Tongue		
Mandibular lymph nodes		E	Trachea		E
Mesenteric lymph nodes		E	Trachea bifurcation		
Muscle (quadriceps)		E	Urinary bladder		E
Nares		E	Uterus including cervix #	W	E
Nasal cavity		E	Vagina		E
Nasopharynx		E			

* Fixative = neutral-buffered 10% formalin except where indicated by:

a – Davidson's fluid

b – Bouin's fixative

Bone designated for histopathological examination was decalcified using Kristenson's fluid.

Organ Weights:

Animals were weighed before necropsy. The organs denoted by "W" in the table (tissue list) above from all interim group animals, excluding decedents, and generally from the first 10 scheduled main group animals/group/sex were dissected free from fat and other contiguous tissue and weighed before fixation. Left and right organs were weighed together.

In error, organ weights from 11 control females (group 1) were recorded in total; the values for animal 270F (11th animal) were excluded from the calculation of the group means and standard deviations.

It must be mentioned that the thyroid weight was not recorded as detailed in the updated OECD guideline No. 453 from 07 September 2009. However, pathological examination was conducted. Therefore, this omission is considered not to have affected the outcome or integrity of the study.

Histopathology:

The following tissues were embedded in paraffin wax BP (block stage), sectioned at a nominal 5 µm, stained with haematoxylin and eosin (slide stage) and examined microscopically by the Study Pathologist:

Satellite Group:

Group 1 (control) and Group 4 (high dose): Liver, gross lesions and tissue masses

Decedents (all dose groups): All tissues denoted by “E” in the table (tissue list) above

Main group:

Group 1, Group 4 and all decedents: All tissues denoted by “E” in the table (tissue list) above

Group 2 (low dose) and Group 3 (intermediate dose): Liver, gross lesions and tissue masses

Csaba stain was used on selected tissues from animals 33, 442, 459 and 480 to rule in or rule out mast cell tumours.

Statistics:

Analysis of survival data:

Male and female data were analysed separately. Survival probability functions were estimated by the Kaplan Meier technique. Survival curves were compared to the start of the terminal kill phase (during Week 79).

Permutational tests for both an increasing and a decreasing dose response in mortality were performed across all groups using the dose levels as weighting coefficients, in accordance with the IARC annex. One-directional pairwise tests of the treated groups against the control group were also performed.

Analysis of in-life and organ weight parameters:

The control group was taken as the baseline group with which the treated groups were compared. Absolute body weights, body weight gains, necropsy body weights, organ weights, organ to necropsy body weight ratios, food consumption over periods and selected haematology variables (white blood cell counts, neutrophils and lymphocytes) were analysed using one-way analysis of variance (ANOVA), separately for each sex. Levene's test for equality of variances among the groups was performed. Where this showed no evidence of heterogeneity ($p \geq 0.01$), pairwise comparisons with control were made using Dunnett's test. A linear contrast was used to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was reported only where none of the pairwise comparisons was significant.

Where Levene's test showed evidence of heterogeneity ($p < 0.01$), the data were analysed either using the same methods after applying a log-transformation or using non-parametric methods. The non-parametric methods employed were the Kruskal-Wallis ANOVA, the Terpstra-Jonckheere test for a dose related trend and the Wilcoxon rank sum test for pairwise comparisons. Where the Kruskal-Wallis ANOVA was not significant, the pairwise comparisons were not reported in order to protect the Type I error.

Selected haematology variables (monocytes, eosinophils, basophils and large unstained cells) were analysed using the non-parametric methods described above.

Analysis of tumour (neoplastic) data:

All tumour data for all tissues examined were supplied for analysis. Male and female data were analysed separately. The numbers of tumour bearing animals were analysed for tumour types found in at least three animals of the given sex. Tumours of similar histogenic origin were merged, as requested by the Pathologist. Permutational tests for both an increasing and a decreasing dose response were performed across the groups using the dose levels as weighting coefficients, in accordance with the IARC annex. One-directional pairwise tests of the treated groups against the control group were also performed.

Non-fatal tumours were analysed using fixed intervals of 1 to 52 weeks, 53 to 78 weeks, the interim kill phase and the terminal kill phase. The fatal and non-fatal results were combined in accordance with the IARC annex. For each macroscopic finding, comparisons were made between the incidence in each of the treated groups and that in the control group, using Fisher's Exact test.

Analysis of macroscopic/microscopic (non-neoplastic) findings:

All macroscopic and microscopic finding data for all tissues examined were supplied for analysis. Male and female data were analysed separately. All tests were performed with a two-sided risk. At the request of the Study Director, four separate analyses were performed; including all animals, decedents only, terminal kill animals only and interim kill animals only. For each macroscopic or microscopic finding, comparisons were made between the incidence in each of the treated groups and that in the control group, using Fisher's Exact test.

Findings:

Mortality and clinical observations:

There was no effect of treatment on survival. Mortalities are detailed in the summary table below (Table B.6.5.2-3). Survival was acceptable for deriving a conclusion with regard to carcinogenicity. Of the 119 decedents, there were 16 animals where a cause of demise was not determined. In addition to the findings associated with terminal events, the range of macroscopic and microscopic findings in decedents was generally similar to those in animals surviving to terminal kill.

The general ranges of clinical signs observed were considered typical of laboratory maintained mice and were not adversely affected by treatment. Clinical signs seen in moribund animals generally included hunched posture, sluggishness, pale appearance, laboured/rapid respiration, swollen abdomen and/or thin appearance. 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.

Body weight, body weight gain and food consumption:

In the satellite groups, no statistically significant effects on bodyweight or body weight gain were seen in the pairwise comparisons. Satellite group females offered 7000 ppm showed approximately 10% less body weight than control between Week 40 to 52, while females in the main study using more animals did not show these findings over this designated period. Therefore these changes were considered to be not adverse.

In the main study, statistically significant differences from the control for absolute body weight were observed at some of the measurement occasions for males offered 2000 ppm (Week 13) or 7000 ppm (Weeks 5, 9, 20 and 36), which resulted in a significant reduction in body weight gain from Start to Week 28 (17% less) for the high dose males. From Weeks 52 to 78, a statistically significant body weight gain increase was observed for males offered 2000 or 7000 ppm mainly as a result of mean body weight loss in the contemporaneous control group over the same period.

There were no statistically significant effects on absolute body weight of main study females. The overall growth rate of females offered 700 ppm was similar to control. The overall mean body weight gain of females offered 2000 ppm and 7000 ppm was 8% more and 8% less than that of the control group, respectively.

For both the satellite group and the main group, there were no statistically significant differences in food consumption when compared to control.

Haematology:

In samples collected at the Week 52 (interim) or 78 (main study) investigations, there were no statistically significant treatment related differences in the haematological composition of the blood between control and treated animals.

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.

Sacrifice and pathology

Organ weights:

Organ weight increases were noted in the liver of treated males and females (see Table B.6.5.2-3). Increases of relative liver weights were statistically significant in the high dose males of the interim study as well as from the main study and in high dose females from the interim study. In the absence of histopathological findings in the liver, these increases were considered to be an adaptive change related to drug metabolism and not an adverse toxic effect.

The mode of action for the liver weight increase is considered to be due to liver enzyme induction, via activation of the constitutive androstane receptor by S-2200 TG, as evidenced by mode of action investigations (see mechanistic studies chapter B.6.8.1.1). Because a mode of action is ascribable, the increase in liver weights is considered non-adverse, particularly in the absence of any other biochemical or histological indicators of an adverse effect on the liver.

All other changes in absolute or relative organ weights were not statistically significant when compared with controls by pair-wise analysis and were furthermore considered not to be relevant as they were either small in magnitude, not dose-dependent, inconsistent between the sexes, spontaneous background changes due to normal inter-animal variability and/or lacked a histopathological correlate.

Macroscopic findings:

Most tissues were macroscopically unremarkable, and the findings seen were generally consistent with the usual pattern of findings in mice of this strain and age. There were no macroscopic findings that could be discerned as treatment-related.

Microscopic findings:

Non-neoplastic microscopic findings in control and treated animals were generally consistent with the usual pattern of findings in mice of this strain and age. There were no non-neoplastic findings that could be related to treatment with the test article.

Neoplastic microscopic findings in control and treated animals were generally consistent with the usual pattern of neoplasms in mice of this strain and age. There was no statistically significant increase or decrease in tumour incidence. There were no tumours suggestive of test article carcinogenicity.

Table B.6.5.2-3: Key results for the carcinogenicity study in mice

	Males				Females			
Diet concentration (ppm)	0	700	2000	7000	0	700	2000	7000
Mortality								
Mortality [#] (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 [#] (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
Body weight (g)								
Week 0 (satellite groups)	33.7	34.7	33.7	33.4	25.0	24.5	25.1	24.2
Week 52 (satellite groups)	52.0	50.8	49.2	49.3	43.4	42.3	44.1	37.3
Week 0 (main groups)	34.3	34.6	34.5	34.2	24.4	24.5	24.4	24.6
Week 78 (main groups)	51.2	51.3	51.8	50.3	43.1	43.6	44.7	41.6
Body weight gain (g)								
Week 0-28 (satellite groups)	12.4	11.6	12.1	12.1	10.9	10.0	11.3	9.8
Week 28-52 (satellite groups)	6.1	4.5	3.4	4.2	7.4	7.8	8.1	3.3

Week 0-28 (main groups)	13.9	13.9	12.1	11.6*	10.6	11.4	11.0	11.2
Week 28-52 (main groups)	4.1	5.4	4.7	5.0	7.3	6.6	7.9	6.1
Week 52-78 (main groups)	-1.4	-1.5	0.1*	0.1*	0.8	0.8	-0.2	-0.3
Organ weights								
Liver, wk 52 (g)	2.34	2.47	2.43	2.59	1.75	1.80	1.93	1.97
% difference from control		5.6	3.8	10.7		2.9	10.3	12.6
Liver, wk 52 (% bw)	4.42	4.67	4.76	5.10**	4.07	4.19	4.41	5.10**
% difference from control		5.7	7.7	15.4		2.9	8.4	25.3
Liver, wk 78 (g)	2.40	2.55	2.54	2.76	1.93	2.20	2.32	2.09
% difference from control		6.3	5.8	15.0		14.0	20.2	8.3
Liver, wk 78 (% bw)	4.59	4.85	4.81	5.24**	4.42	4.65	4.96	4.90
% difference from control		5.7	4.8	14.2		5.2	12.2	10.9

* p < 0.05 in comparison to controls, ** p<0.01 in comparison to controls.

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

Conclusion:

Treatment with S-2200 Technical Grade was well-tolerated. In the absence of adverse effects, the NOAEL for this study was considered to be 7000 ppm (823.9 mg/kg bw/day for males and 994.0 mg/kg bw/day for females), the top dose tested, following 78 weeks of treatment. There were no effects on survival/mortality or on the incidence or morphology of tumours to indicate any oncogenic potential.

B.6.5.3 Summary of long term toxicity/carcinogenicity studies

Groups of 70 male and 70 female Wistar rats were offered S-2200 TG in the diet at concentrations of 0 (control), 400, 2000, 7000, 15000 ppm. After 52 weeks, satellite groups of 20 males and 20 females were used for interim sacrifice and the remaining survivors sacrificed after 104 weeks of treatment.

Due to the magnitudes of the decreased body weight and body weight gain (at 15000 ppm) and toxicological alterations in the liver including increased liver weights in combination with a higher degree of hepatocellular hypertrophy and hepatocyte vacuolation (both at ≥ 7000 ppm) and increased blood biochemistry parameters (total cholesterol and gamma-glutamyltransferase in males offered 15000 ppm), the No Observed Adverse Effect Level (NOAEL) for males was considered to be 2000 ppm (105.1 mg/kg bw/day).

For females, body weight and body weight gain was significantly decreased at ≥ 2000 ppm following 104 weeks of treatment. Toxicological alterations in the liver in females included increased liver weights (at ≥ 2000 ppm), a higher degree of hepatocellular hypertrophy and hepatocyte vacuolation (at ≥ 2000 ppm and at ≥ 7000 ppm, respectively) and increased blood biochemistry parameters (increased total cholesterol and increased gamma glutamyltransferase at ≥ 7000 ppm and at ≥ 15000 ppm, respectively). Therefore, the NOAEL for females was considered to be 400 ppm (26.7 mg/kg bw/day) for this study following 104 weeks of treatment.

Regarding the carcinogenic potential of S-2200 TG, no increase of neoplastic findings exceeding the historical control range was observed in any organ of treated animals, with exception of benign sex-cord stromal tumours in the ovary. Four and six cases occurred in female rats of the carcinogenicity cohort dosed with 7000 ppm and 15000 ppm, respectively, at dose levels where body weight gain was reduced by more than 20%, indicating that the maximum tolerated dose was exceeded. S-2200 TG is not genotoxic. The incidence of only one benign tumour type was increased. Sex-cord stromal hyperplasia does occur in aged Wistar rats, and the animals used in this study appear to be derived from a susceptible batch. The

incidences of sex-cord stromal proliferative lesions were within historical controls for all groups, and there was no statistically significant difference between groups for hyperplasia or tumours. Higher survival rates in animals offered 7000 and 15000 ppm may have contributed to the higher numbers of tumours. The increased incidence of sex-cord stromal tumour in the ovary at 7000 ppm and 15000 ppm exceeded the Covance, Harrogate historical control range (0-3.1%) for this strain of rat. However, it must be highlighted that also the concurrent control animals (incidence 2/50 = 4%) exceeded the historical control range for sex-cord stromal tumours.

Since there is no interaction of the test article with the oestrogen receptor and steroidogenesis as evidenced by *in vitro* assays, no direct ovarian toxicity and no reproductive abnormalities, a mode of action via endocrine imbalance is considered to be unlikely. Furthermore, there was no accumulation or persistence of S-2200 TG or its metabolites in the ovary. In the corresponding mouse carcinogenicity bioassay, the number of tumours in any tissue was not increased by exposure to S-2200 TG. Thus, based on this evidence, the sex-cord stromal lesions are considered unlikely to be toxicologically relevant.

Overall, according to Regulation (EC) No. 1272/2008 and in consideration of the *Guidance on the Application of CLP Criteria, Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, version 2.0 April 2012*, no classification and labelling as carcinogenic substance is proposed for the active substance mandestrobin.

Groups of 51 male and 51 female CD-1 mice were given S-2200 TG in the diet at concentrations of 0 (controls), 700, 2000, and 7000 ppm for 78 weeks. Satellite groups of 12 mice per sex per dose were reared up to 52 weeks for interim sacrifice.

Treatment with S-2200 TG was well-tolerated. In the absence of adverse effects, the NOAEL for this study was considered to be 7000 ppm (823.9 mg/kg bw/day for males and 994.0 mg/kg bw/day for females), the top dose tested, following 78 weeks of treatment. There were no effects on survival/mortality or on the incidence or morphology of tumours to indicate any oncogenic potential.

Table B.6.5.3-1: Summary of combined chronic toxicity and carcinogenicity studies

Study	Dose Levels	NOAEL mg/kg bw/day	Effects/target organ systems
Rat oral via diet, 104 weeks [REDACTED] 2012b)	0, 400, 2000, 7000, 15000 ppm equivalent to 0, 21.8, 105.1, 375.6 and 804.3 mg/kg bw/day (males) and 0, 26.7, 135.2, 475.0 and 1016.2 mg/kg bw/d (females)	♂ 105.1 ♀ 26.7	↓ body weight and bw gain (♂ at 15000 ppm, ♀ at ≥ 2000 ppm) ↑ liver weight (♂ at ≥ 7000 ppm, ♀ at ≥ 2000 ppm) ↑ hepatocellular hypertrophy (♂ at ≥ 7000 ppm, ♀ at ≥ 2000 ppm) ↑ hepatocyte vacuolation (≥ 7000 ppm) ↑ total cholesterol (♂ at 15000 ppm, ♀ at ≥ 7000 ppm) ↑ GGT (at 15000 ppm)
Mouse oral via diet, 78 weeks [REDACTED] 2012c)	0, 700, 2000, 7000 ppm equivalent to 0, 82.5, 238.8 and 823.9 mg/kg bw/d in males and 0, 99.2, 280.3 and 994.0 mg/kg bw/d in females	♂ 823.9 ♀ 994.0	No adverse effects of treatment at the highest dose tested

B.6.6 Reproductive toxicity (Annex IIA 5.6)

B.6.6.1 Multi-generation reproductive toxicity in the rat

Reference:	Dose Range-Finding Study for Two-Generation Reproduction Study of S-2200 TG in Rats
Author(s), year:	██████████ 2010
Report/Doc. number:	Sumitomo Chemical Co. Ltd. Report No. ROT-0018
Guideline(s):	None
GLP:	No
Deviations:	One female of the low dose group was judged as non-copulation by vaginal smear examination during the mating period; however this female was proved to be pregnant. Consequently, several parameters during gestation period were not obtained (chemical intake, bw, food consumption). Data of lactation period was normally collected; no effect on evaluation of study results.
Validity:	Yes; supporting study

Material and methods:

<i>Test Material:</i>	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G
Purity:	93.4%
<i>Test animals:</i>	
Species:	Rat
Strain:	Wistar, B6Han: WIST@Jcl (GALAS)
Age:	8 weeks old at start of administration
Weight at start:	Males 205 – 228 g, females 149 – 168 g
Source:	██████████
Diet:	CRF-1 (Oriental Yeast Co., Ltd.) sterilized by gamma rays <i>ad libitum</i>

The purpose of this dose range-finding study was to obtain information for selecting dose levels for the definitive study aimed at assessing effects of S-2200 TG on the reproductive system, including mating, gestation, parturition and lactation of the parental animals by dietary administration from 4 weeks before mating to Day 21 of lactation in rats.

Animal assignment and treatment

Groups of 10 male and 10 female young adult rats were offered S-2200 TG in diet at dietary concentrations of 0 (control), 5000, 10000 or 20000 ppm. The dose levels were selected based on the results of a thirteen-week dietary toxicity study of S-2200 TG in rats (Study No. 0333/290, dose levels: 0, 800, 4000, 10000, and 20000 ppm). A dose level of 20000 ppm was selected as the highest dose for this study, at which some toxic signs occurred in the thirteen week study (decreased body weight and suppressed body weight gain in both sexes). The intermediate and low doses were set at 10000 and 5000 ppm, respectively, by decreasing geometrically based on a factor of 2.

Males were dosed daily for 4 weeks before mating and through the mating period until the day of necropsy. Females were dosed daily for 4 weeks before mating and through the mating period until weaning of F1 offspring (Day 21 of lactation).

Diet preparation and analysis:

The mixed diet with the test substance was prepared once within 14 days. For each dose, a prescribed amount of the test substance and the primary diet were weighed. Then, the test substance and the proper primary diet were ground and mixed with a compact mill, and further primary diet was added in a stepwise manner to achieve the prescribed concentrations of the test substance in diet. The mixed diet with the test substance was subdivided into portions for one week in plastic bags. The primary diet for the control group was also subdivided into portions in plastic bag in the same manner without any preparation. The prepared diet was refrigerated and stored in a dark place until use. No analysis of concentration, stability or homogeneity in the diet was made.

Mean test substance intakes (mg/kg bw/d) are given in Table B.6.6.1-1.

Table B.6.6.1-1: Mean test substance intakes (mg/kg bw/d) – F0 animals only

Sex	Doses Period of treatment	5000 ppm (Group 2)	10000 ppm (Group 3)	20000 ppm (Group 4)
males	Before mating	317.9	636.0	1253.0
	After mating	244.1	498.5	1032.5
females	Before mating	334.6	667.1	1326.0
	Gestation	316.8	667.8	1229.0
	Lactation	781.7	1429.2	2441.0

Observation and examination of parental animals (F0)

Clinical observations:

The animals were observed by cage-side once daily and were observed by palpation once weekly.

Body weight:

Body weights were measured once a week from the first day of dosing to necropsy for males and to copulation for females. The mated females were weighed on Days 0 (day of copulation), 7, 14, and 20 of gestation and on Days 0 (day of parturition), 4, 7, 14, and 21 of lactation. Males and copulated females were weighed on the day of necropsy. Body weight gain was calculated on the basis of the body weight on the first day of dosing during the pre-mating period, Day 0 of gestation during the gestation period, and Day 0 of lactation during the lactation period.

Food consumption:

Gross weight of the diet for each cage was measured once a week from the first day of dosing to 4 weeks after the start of dosing for males and females during the pre-mating period. For mated females, gross weight of the diet was individually measured on Days 0, 7, 14, and 20 of gestation and Days 0, 7, 14, and 21 of lactation. For mated males, gross weight of the diet was weighed in the same interval re-starting from the nearest measurement day to the day of necropsy. Daily food consumption of each animal was calculated by dividing the difference of the measured gross weights by the number of days between each measurement.

Reproductive function:

Males and females were cohabited day and night on one-to-one basis within the same group for up to 14 days (first mating). From the day after the start of mating, vaginal smears were collected every day in the morning, while observing for the presence of vaginal plugs, and the smears were examined microscopically to determine oestrous cycles and the presence of sperm. Copulation was confirmed by the presence of a

vaginal plug or sperm in the vaginal smears, and the day on which the evidence of copulation was found was designated as day 0 of gestation. As a result of the first mating, all females of each group showed evidence of copulation by 14 days after the start of mating. Therefore, the test substance was judged to have no effect on the mating ability, and mating was terminated. Based on the above results, the following indices were calculated:

- Days until copulation: Number of days from the start of mating to the detection of copulation
- Number of oestrus stages without copulation
- Copulation index (%): $(\text{Number of animals with successful copulation} / \text{Number of paired animals}) \times 100$
- Fertility index (%): $(\text{Number of pregnant animals} / \text{Number of animals with successful copulation}) \times 100$

Observation of parturition and nursing:

For parturition, the dams were observed twice a day from Day 21 through Day 23 of gestation. The animals that delivered their litter completely by 4:00 p.m. were judged as dams giving birth on that day. For nursing, the dams were observed once a day for maternal behaviour, including lactation, nest building, and cannibalism, until Day 21 of lactation. At the necropsy on Day 21 of lactation, the uterus was removed and examined for the number of implantations. The following indices were calculated based on these results:

- Gestation length: Number of days from Day 0 of gestation to the day of parturition
- Gestation index (%): $(\text{Number of females with live offspring} / \text{Number of pregnant females}) \times 100$
- Birth index (%): $(\text{Number of offspring born alive} / \text{Number of implantations}) \times 100$

Necropsy:

Four days after delivery, males were euthanized by exsanguination from the abdominal aorta under anaesthesia, and the thoracic and abdominal organs/tissues were examined macroscopically. Two males (#00109 and 00309) that displayed delayed copulation compared to other mated males were necropsied before Day 4 of lactation of the mating pair, because the no effects of test substance on gestation index or the number of live offspring at the delivery had already been concluded. The females were necropsied on Day 21 of lactation in the same manner as the males.

The thyroid, liver, testis, epididymis, prostate (ventral, lateral, and dorsal lobe), seminal vesicle (including coagulating gland), ovary, uterus, and mammary gland were fixed and preserved in a 10 vol% neutral phosphate-buffered formalin. The testes were fixed in Bouin's fluid and preserved in 10 vol% neutral phosphate-buffered formalin. Stomachs showing abnormal signs were also fixed and preserved in 10 vol% neutral phosphate-buffered formalin. The stomachs of 3 females in the control group was also fixed and preserved in the same manner as comparative samples.

Organ weights:

At necropsy, body weights and the following organs were weighed:

Thyroid, liver, testis, epididymis, prostate (ventral lobe), seminal vesicle (including coagulating gland), ovary, uterus (including cervical region).

Relative organ weights (body weight ratio) were calculated based on each body weight at necropsy. The thyroid was removed and weighed after fixation in 10 vol% neutral phosphate-buffered formalin. The bilateral organs were weighed individually and calculated as a sum.

Histopathology:

The liver and thyroid from all animals and the ovaries and uterus from all females were processed by the standard method to prepare haematoxylin- and eosin-stained (HE) sections for microscopic examination.

Observation and examination of offspring (F1)

Litter examinations:

The birthday was designated as postnatal Day 0.

On postnatal Day 0, the newborns were examined for the number of offspring (live or stillborn), sex, and presence of external anomalies. After that, the pups were observed daily for clinical signs and mortality until postnatal Day 21. On postnatal Day 4, litter size was reduced randomly to 8 pups (equal sex ratio, in principle). The litter with less than 8 pups was not subjected to adjustment. The offspring culled at the litter size adjustment were euthanized by inhalation of carbon dioxide and preserved in a 10 vol% neutral phosphate-buffered formalin. From the numbers of live offspring on postnatal Days 0, 4, 7, 14, and 21 (weaning day), the following indices were calculated:

- Live birth index (%) = viability index on Day 0 (%): (Number of live offspring born alive/Number of offspring born) × 100
- Viability index on Day 4 (%): (Number of offspring alive on Day 4/Number of offspring born alive) × 100
- Viability index on Day 7 (%): (Number of offspring alive on Day 7/Number of live offspring after culling) × 100
- Viability index on Day 14 (%): (Number of offspring alive on Day 14/Number of live offspring after culling) × 100
- Weaning index (%): (Number of live offspring at weaning/Number of live offspring after culling) × 100

Body weights:

The offspring were weighed individually on postnatal Days 0, 4, 7, 14, and 21. The body weight gain was calculated by each litter unit on the basis of the body weight at birth before the litter size adjustment, and by each offspring on the basis of the body weight on postnatal Day 4 thereafter.

Necropsy:

On postnatal Day 21, all F1 offspring were euthanized by exsanguination from the abdominal aorta under anaesthesia, and the head, thoracic, and abdominal organs/tissues were examined macroscopically. The eyeball showing abnormal signs and brain, thymus, spleen, and uterus weighed on postnatal Day 21 were fixed and preserved in 10 vol% neutral phosphate-buffered formalin. The same organs of 3 females in the control group were also fixed and preserved in the same manner as the comparative samples. Dead pups (before the litter size adjustment) were examined for the presence of external anomalies and the whole bodies were preserved in 10 vol% neutral phosphate-buffered formalin. Cannibalized pups were stored in the same manner.

Organ weights:

At necropsy on postnatal Day 21, one male and one female were selected from each litter (in numerical order of the offspring number in each litter) and weighed for the following organs: Brain, thymus, spleen, and uterus.

Relative organ weights (body weight ratio) were calculated based on individual body weight at necropsy.

Statistics

The data of offspring obtained before weaning were analysed on the basis of litter mean values.

The metrical data were analysed by multiple comparison tests for statistical significance. The homogeneity of variance was tested first by Bartlett's test. When the variance was homogeneous, the one-way analysis of variance was performed for statistical comparison. When it was heterogeneous, the Kruskal-Wallis test was used. When a significant inter-group difference was found, Dunnett's or the Dunnett-type multiple comparison test was used. For some of the data, the Kruskal-Wallis test was applied first, and when a

significant inter-group difference was found, the Dunnett-type multiple comparison test was used. The numerical data were analysed by Fisher's exact probability test. The significance level of 5% was set for all statistical analyses. The statistical analyses were performed on the items listed below. The analyses were not performed on clinical signs and necropsy findings.

- Multiple comparison test: Body weight, body weight gain, food consumption, organ weights, number of implantations, number of offspring, number of live offspring
- Kruskal-Wallis test and Dunnett-type multiple comparison test: Days until copulation, number of oestrus stages without copulation, gestation length, birth index, live birth index, viability index on Days 4, 7, and 14, weaning index, incidence of offspring with external anomalies
- Fisher's exact probability test: Copulation index, fertility index, gestation index, sex ratio (male/female), dams with external abnormal offspring.

Findings:

Effects on parental animals (F0)

Clinical signs:

No abnormal clinical signs were noted in parental males or females of any group, including the control group.

Body weight:

In males, statistically significant decreases in body weights and body weight gain were noted in the 20000 ppm group during the dosing period. No treatment-related changes were noted in the 5000 or 10000 ppm group.

In females, statistically significant decreases in body weight or body weight gain were noted in the 20000 ppm group from Day 28 of dosing through the gestation period to the lactation period. In females of the 10000 ppm group, statistically significant decreases in body weight or body weight gain were noted on Day 28 of dosing and Day 20 of gestation. No treatment-related changes were noted in the 5000 ppm group.

Food consumption:

In males, a statistically significant decrease in food consumption was noted in the high dose group (20000 ppm) on Day 7 of dosing; however, this change was considered to be not relevant, because it was transient and food consumption recovered by Day 14 of dosing. No treatment-related changes were noted in males at 5000 or 10000 ppm.

In females, statistically significant decreases in food consumption were noted in the high dose group (20000 ppm) on Day 7 of dosing and through the gestation period to the lactation period. In the mid dose group (10000 ppm), statistically significant decreases in food consumption were noted on Days 7 and 28 of dosing and during the lactation period. In the low dose group (5000 ppm), statistically significant decreases in food consumption were noted on Days 7 and 14 of dosing; however, these changes were considered to be not relevant, because they were transient and the food consumption recovered by Day 21 of dosing.

Reproductive performance, parturition and nursing:

All the mating pairs of each group copulated during the first oestrus stage after the start of mating, and no significant differences were noted in the number of days from the mating to the copulation. The copulation and fertility indices were 100.0% in all groups.

All dams in each group delivered normally between Days 21 and 23 of gestation and no significant differences were noted in the gestation length, number of implantations, birth index, or gestation index between the control and treated groups. In the observation of nursing, there were no abnormalities in maternal behaviour in dams of any group. The number of offspring born alive decreased in all treated groups, statistically significant in the high dose group.

Necropsy:

Dark brownish change in the liver was noted in one female in the mid dose group (10000 ppm), and in 10 males and 8 females in the high dose group (20000 ppm). Enlargement of the liver was noted in 5 males and 1 females at 10000 ppm, and in 10 males and 7 females at 20000 ppm. Enlargement of the thyroid was noted in 1, 1, and 4 males and in 0, 1, and 1 females in the 5000, 10000, and 20000 ppm groups, respectively.

Occasional other findings including abnormal lobation and reddish patch in the liver, mucosal reddish patch in the glandular stomach, and unilateral nodule in the epididymis were noted. However, these changes were considered to be incidental, because of the lack of dose-dependency and their pathological nature.

Organ weights:

Statistically significantly increased absolute and/or relative weights of the thyroid and liver were noted in males in all treated groups. In females, statistically significantly increased absolute and relative weights of the liver were noted, and decreased weights of the uterus were noted in the 10000 and 20000 ppm groups. In addition, decreased ovary weights were noted in the 20000 ppm group. A statistically significant increase in relative weight of testis was noted in males in the 20000 ppm group; however, there was no significant difference in the absolute weight, as compared with the control group. Therefore, this change was considered attributable to the significantly lower body weight at necropsy, and was judged to be toxicologically insignificant.

Histopathology:

Liver: Brown pigment in the bile ducts was noted in 1 male and 5 females in the 10000 ppm group, and in 6 males and 9 females in the 20000 ppm group. Brown pigment deposition in the perilobular hepatocytes was noted in 2 females in the 10000 ppm group and in 3 males and 6 females in the 20000 ppm group. Focal periductular inflammatory cell infiltration was noted in 1 male and 3 females in the 10000 ppm group, and in 4 males and 8 females in the 20000 ppm group. Proliferation of the bile ducts was noted in 3 females in the 10000 ppm group and in 1 male and 5 females in the 20000 ppm group. Eosinophilic focus of the altered hepatocytes was noted in 1 male and 1 female in the 20000 ppm group. Centrilobular hypertrophy of the hepatocytes was noted in 4, 10, and 10 males and 6, 10, and 10 females in the 5000, 10000, and 20000 ppm groups, respectively.

The finding of focal necrosis of hepatocytes noted in 2 females in the 10000 ppm group was considered to be not relevant due to the lack of dose-response.

Thyroid: Diffuse hypertrophy of the follicular cells was noted in 2 males in the 10000 ppm group and in 5 males in the 20000 ppm group. Among 8 animals with enlarged thyroid at necropsy, diffuse hypertrophy of the follicular cells was noted in 2 males in the 20000 ppm group. In the other 6 animals, vacuolation of the follicular cells was noted. It has been reported that the vacuolation of thyroid follicular cells is noted as a spontaneous lesion in Br/Han:WIST@Jcl (GALAS) rats (Shimoi, A. et al., 2001, J Tox Pathol, 2001; 14:253-7). In the present study, this change was also noted in 1 female in the control group. Therefore, the vacuolation of the follicular cells was considered to be incidental.

Ovary: Decrease of vacuolation in the interstitial gland was noted in 1 female in the 10000 ppm group and in 7 females in the 20000 ppm group.

Uterus: Atrophy was noted in 2 females in the 10000 ppm group and in 10 females in the 20000 ppm group.

Effects on offspring (F1)

Litter examinations:

A statistically significant decrease in the viability index on Day 0 was noted in the 20000 ppm group. Furthermore, a decrease of weaning index (not statistically significant) was noted in the 20000 ppm group, and this change was induced by the death of 4 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 20000 ppm group. However, the number of live offspring at birth in the control group was above the background range of the test facility, and that in the 20000 ppm group was slightly below the background range. Accordingly, it was considered that the decrease in the number of live offspring at birth in the high dose group was an incidental change attributable to the increased number of live offspring at birth in the control group, and was judged to be toxicologically insignificant.

A statistically significant decrease in the total number of male pups at birth (and in consequence, in the number of live males at birth and in the number live males on Day 4 before culling) was observed at 5000 ppm and at 10000 ppm. However, these changes were not considered to be treatment related because of the lack of dose-dependency.

External examination and clinical signs:

There were no external anomalies in the offspring of any group.

No abnormal clinical signs were noted in males or females or F1 pups of any group, including the control group.

Body weights:

Body weights of offspring were statistically significantly decreased in the mid dose group (10000 ppm) on postnatal Day 21 in both sexes and in the high dose group (20000 ppm) from postnatal Days 7 and 4 onwards in males and females, respectively. Body weight gain was statistically significantly decreased in the mid dose group (10000 ppm) from postnatal Days 7 and 14 onwards in males and females, respectively, and in the high dose group (20000 ppm) in both sexes at every measurement (starting before culling at postnatal Day 4 up to weaning).

Necropsy:

No abnormal changes attributable to the test substance were noted in either sex. In one female of the low dose group (5000 ppm), a unilateral small eyeball was noted. This isolated finding was considered to be incidental, as there was no dose-response.

Organ weights:

At the dose level of 20000 ppm, statistically significant decreases in absolute organ weights were observed in both sexes for brain, thymus and spleen, and in females for the uterus. In addition, absolute spleen weights were also statistically significantly decreased in the mid dose male pups (10000 ppm). Furthermore, statistically significant decreases in relative weights of thymus and spleen were noted in both sexes at 20000 ppm.

Statistically significant increases of relative brain weights (in mid and high dose males as well as in high dose females) and of relative uterus weights (in high dose females) were considered to result from excessive lower body weight at necropsy.

The results of the range finding study for reproduction toxicity study are given in Table B.6.6.1-2.

Table B.6.6.1-2: Results of the preliminary reproduction range finding study in rats

	Males				Females			
Diet concentration (ppm)	0	5000	10000	20000	0	5000	10000	20000
Parental animals (F0)	Body weight (g)							
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**
	Food consumption (g/animal/day)							
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**
	Necropsy findings							
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
	Organ weights							
Terminal body weight (g)	379	383	374	347*	268	277	268	244**
Liver (g)	10.8	13.4**	14.7**	16.3**	12.0	14.1	16.4**	19.8**
Thyroid (mg)	22.4	29.9*	30.1*	33.4**	18.2	18.3	20.8	19.6
Ovary (mg)					93.9	84.7	82.6	64.8**
Uterus (g)					0.503	0.490	0.301**	0.229**
	Histopathology							
Number of animals examined	10	10	10	10	10	10	10	10
Liver: brown pigment in bile duct, focal (Grade 1-3)	0	0	1	6	0	0	5	9
Liver: brown pigment deposition in perilobular hepatocytes (Grade 1)	0	0	0	3	0	0	2	6
Liver: periductular inflammatory cell infiltration, focal (Grade 1)	0	0	1	4	0	0	3	8
Liver: eosinophilic foci of altered hepatocytes (Grade 1)	0	0	0	1	0	0	0	1
Liver: centrilobular hypertrophy, hepatocyte (Grade 1-3)	0	4 (Grade 1)	10 (Gr. 1-3)	10 (Gr. 1-3)	0	6 (Gr. 1-2)	10 (Gr. 1-2)	10 (Gr. 2-3)
Liver: Bile duct proliferation (Grade 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: diffuse hypertrophy of follicular cells (Grade 1)	0	0	2	5	0	0	0	0
	Reproductive performance							
Copulation Index (%)	100	100	100	100	100	100	100	100
Fertility Index (%)	100	100	100	100	100	100	100	100
Litter examination	Pup Viability							
Implantation sites per dam					13.5	12.1	11.5	11.0
Offspring at birth per dam					13.0	10.4	10.0	10.5
Live offspring at birth per dam					13.0	10.4	10.0	10.1*
Viability index on Day 0					100	100	100	96.50*
Weaning index					100	100	100	95
Offspring (F1)	Pup body weight (g)							
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**
	Pup organ weights on weaning							
Terminal body weight (g)	53.1	49.9	44.9**	26.3**	50.5	48.8	44.3*	25.8**
Brain (g)	1.459	1.437	1.438	1.258**	1.424	1.417	1.390	1.228**
Thymus (mg)	202.2	194.1	172.2	75.6**	208.2	206.4	174.0	80.1**
Spleen (g)	0.248	0.210	0.194*	0.091**	0.228	0.210	0.189	0.088**
Uterus (mg)	-	-	-	-	36.91	37.98	31.38	25.18**

Grade: 1 Minimal; 2 Mild; 3 Moderate

* p < 0.05 in comparison to controls, ** p < 0.01 in comparison to controls

Conclusion:

In a dose range finding reproduction study, groups of 10 male and 10 female Han Wistar rats (F0 generation) were exposed to S-2200 TG at dietary concentrations of 0 (control), 5000, 10000 or 20000 ppm for 4 weeks before mating, and throughout mating, gestation, and lactation periods.

Regarding the general toxicological effects on parental animals, suppression of body weight and body weight gain was noted in the males of the high dose group (20000 ppm). In females, body weight, body weight gain and food consumption were significantly decreased at 10000 ppm and above. Pathological examination revealed brown pigment in the bile duct and in perilobular hepatocytes, followed by inflammatory cell infiltration into the periductular region and proliferation of bile ducts in the 10000 ppm group and above. Dark brownish change and enlargement of the liver was noted at the necropsy in the 10000 ppm group and above. Significant liver weight increase was observed in males at ≥ 5000 ppm and in females at ≥ 10000 ppm. These liver changes were considered to be toxicologically relevant.

In females, decreased vacuolation in the interstitial gland in the ovary and atrophy of the uterus were noted in the 10000 ppm group and above. Ovary weights were decreased at 20000 ppm, and uterus weight decreased in the 10000 ppm group and above. The changes in the ovary and uterus were considered to be test substance-related changes; however, there were no treatment-related changes in reproductive function such as mating ability, fertility, pregnancy, parturition, or nursing behaviour in females.

Regarding the effects on offspring, the viability index on Day 0 decreased in the 20000 ppm group. Suppressed body weight and body weight gain was noted in both sexes in the 10000 ppm group at weaning and at 20000 ppm throughout the lactation period. Pathological examination revealed reduced spleen weights at ≥ 10000 ppm and reduced organ weights of brain, thymus and uterus in the 20000 ppm group.

The no-observed-adverse-effect-level (NOAEL) for parental systemic toxicity is set at 5000 ppm for females (equivalent to 317 mg/kg bw/d; mean test substance intake during gestation) based on decreased body weight and food consumption and on liver effects at 10000 ppm. The dose level of 5000 ppm (244 mg/kg bw/day as the lowest substance intake after mating) is considered to be a lowest-observed-adverse-effect-level (LOAEL) for males, as liver weight increase greater than 20% was observed at this dose level. A reproductive NOAEL of 20000 ppm (equivalent to 1229 mg/kg bw/d; mean test substance intake in females during gestation) was derived, due to the absence of effects on reproduction at the highest dose level tested.

The NOAEL for offspring systemic toxicity is proposed to be set at 5000 ppm (equivalent to 317 mg/kg bw/d; mean test substance intake during gestation) based on significantly lower body weight of pups at weaning and reduced spleen weights at 10000 ppm.

A dose of 10000 ppm was concluded to be appropriate as the high dose for the two-generation reproduction study of S-2200 TG in rats, at which some toxic signs should occur in at least F0 dams, but is also expected to ensure sufficient survival over two generations.

Reference:	Two-Generation Reproduction Toxicity Study of S-2200 TG in Rats
Author(s), year:	██████████ 2012
Report/Doc. number:	Sumitomo Chemical Co., Ltd., Report No. ROT-0064
Guideline(s):	Japanese MAFF 12-Nousan-8147 (2000), US EPA OPPTS 870.3800 (1998), OECD TG 416 (2001)
GLP:	Yes (laboratory certified by National Authority)
Deviations:	Due to the Great East Japan Earthquake occurring at 14:46 on March 11, 2011, the utilities (electricity, steam, water) were shut down, which caused deviations from the protocol. However, there were no related abnormalities in any observation or investigation, including reproductive function and pathological findings, and all animals were judged to be healthy. Therefore, it was judged to have had no adverse effect on the reliability of the study. In addition, a number of further minor deviations from the protocol occurred, which were all considered as not relevant for the validity of the study.
Validity:	Yes

Material and methods:

Test Material:	S-2200 Technical Grade (S-2200TG)
Lot/Batch:	ST-0811G
Purity:	93.4% (by Certificate of Analysis)
Test animals:	
Species:	Rat
Strain:	Wistar, BrlHan: WIST@Jcl (GALAS)
Age:	6 weeks at start of administration
Weight at start:	Males 174-199 g, females 122-147 g
Source:	██████████
Diet:	CRF-1 (Oriental Yeast Co., Ltd.) sterilized by gamma rays <i>ad libitum</i>

This study was conducted to assess the effects of S-2200 TG on the reproductive functions, such as oestrus cycle, mating behaviour, conception, gestation, parturition, lactation, and weaning, and on the growth and development of the offspring when administering S-2200 TG orally via diet over two generations in rats.

Animal assignment and treatment:

The dose levels were selected based on the results of a dose range-finding study (dietary dose levels: 0, 5000, 10000, 20000 ppm, study No. B091037). A dose of 10000 ppm was selected as the highest dose for the main study, at which some toxic signs occurred in parental animals. The intermediate and low doses were set at 3000 and 1000 ppm, respectively, with a common ratio of about 3.

Table B.6.6.1-3: Study Design for the 2-generation reproduction study

Group	Diet concentration (ppm)	Number of F0 animals* ¹		Number of F1 animals* ²	
		Male	Female	Male	Female
1	0	26	26	25	25
2	1000	26	26	26	26
3	3000	26	26	26	26
4	10000	26	26	24	24

*¹: Number of treated animals

*²: For F1 animals, 1 male and 1 female were selected at weaning (postnatal Day 21) from each litter in numerical order of the animal number as animals for post-weaning examination (F1 parental animals) and subjected to administration.

Groups of 26 male and 26 female young adult rats were allocated to treatment groups (F0 generation). These rats were treated every day for 10 weeks before mating for both sexes, through the mating period until the day of necropsy in males, and through the mating, gestation, and lactation periods until the day of weaning of F1 offspring (Day 21 of lactation) in females (total administration period: 17 weeks for males and 16-18 weeks for females). F1 animals were treated every day from weaning (postnatal Day 21) for 10 to 12 weeks before mating for both sexes, and thereafter, in the same way as F0 animals (total administration period: 17-18 weeks for males and 16-18 weeks for females). Non-delivering females, however, were treated until 26 days after copulation (the day of necropsy).

Diet preparation and analysis:

Based on the results of analysis of storage stability of the test compound in the diet, the diet was prepared 9 times at a frequency of once within every 5 weeks (expiration period: 8 weeks). Slightly more than the required amount of the test compound was ground using a porcelain mortar and pestle. The test compound and an appropriate amount of the weighed basal diet were mixed and ground with a compact mill as the primary mixed diet. Then, the primary mixed diet was added with a portion of the weighed basal diet at a total amount of approximately 2 kg, and mixed with a table top universal mixer to make a secondary mixed diet. Finally, the secondary mixed diet and the remaining weighed basal diet were mixed with a V or W type mixer, according to the amount of the preparation, for about 30 min. The prepared diet was subdivided into portions for about a week in plastic bags. For the control group, the basal diet was packed and sealed in a plastic bag by the supplier and was used without any preparation.

The mixed diet for the analysis of storage stability of the test compound in the diet was prepared as follows. A primary mixed diet prepared by the same method described above was added with a portion of the weighed basal diet, and it was mixed by shaking in a plastic bag for 1 min to make a secondary mixed diet. The secondary mixed diet and the remainder of the weighed basal diet were mixed and shaken in a plastic bag in the same manner. In order to confirm the stability of the test compound in the diet, the concentration of the test compound was analysed for the 1000 and 10000 ppm dietary formulations after storage for 2, 4,

and 8 weeks under refrigeration (5.2 to 6.4°C) in a dark place, and for 2 weeks under the animal room conditions (21.9 to 22.9°C). Analytical samples (n=1) were collected from 3 points (top, middle, and bottom layers, one sample from each layer) of each of the dietary formulations, and analysed. As a result, the 1000 and 10000 ppm dietary formulations were confirmed to be stable under the tested conditions. With respect to the homogeneity of the test substance in the mixed diet, samples from the top, middle, and bottom layers (n=2) were analysed for each dose level at the first preparation, and the coefficient of variation (C.V. value) of the mean concentration was confirmed to be ≤ 10%.

Observation and examination of parental animals (F0 and F1)

Clinical observations:

Cage-side observations were carried out once a day, and animals were palpated at least once a week (the day of body weight measurement).

Body weight:

Body weights were measured on the first day of dosing, and once a week thereafter until necropsy including the day of the necropsy for males, and until copulation for females. Copulated females were weighed on Days 0 (day of copulation), 7, 14, and 20 of gestation and on Days 0 (day of parturition), 4, 7, 14, and 21 of lactation (Day 21 = day of the necropsy). Body weight gain was calculated on the basis of the body weight on the first day of dosing during the pre-mating and mating periods, Day 0 of gestation during the gestation period, and Day 0 of lactation during the lactation period.

Food consumption

Food consumption was measured once a week by cage unit from the first day of dosing to the last week before the necropsy for males, except for the period of cohabitation with a female, and to 10 weeks after the start of dosing for females. For copulated females, food consumption was individually measured on Days 0, 7, 14, and 20 of gestation and days 0, 4, 7, 14, and 21 of lactation.

Chemical intake was calculated based on the concentrations of the test compound in diet (nominal concentration) and body weight and food consumption of each animal.

Oestrus cycle:

The oestrous cycle was examined by collecting vaginal smears every day in the morning for 3 weeks from 7 weeks after the start of dosing in the pre-mating period (F0 female: 13 weeks of age; F1 female: 10 weeks of age) and from the day after the start of mating, until copulation was confirmed in the mating period. From the results of the examination in the pre-mating period, the mean oestrous cycle length for each female and the incidence of females with irregular oestrous cycles were calculated. Females that had an oestrous cycle length other than 4 to 6 days were judged as having irregular oestrous cycles.

Reproductive function:

After the pre-mating administration period (F0 animals: 10 weeks, F1 animals: 10 to 12 weeks), males and females of the same group were cohabited day and night on one-to-one basis avoiding siblings in F1 animals for up to 14 days (first mating). From the day after the start of mating, vaginal smears were collected every day in the morning, while observing for the presence of vaginal plugs, and the smears were examined microscopically to determine oestrous cycle stage and the presence of sperm. Copulation was confirmed by the presence of a vaginal plug or sperm in the vaginal smears, and the day on which the evidence of copulation was found was designated as Day 0 of gestation.

The following indices were calculated:

- Days until copulation: Number of days from the start of mating to detection of copulation
- Number of oestrus stages without copulation

- Copulation index (%): (Number of animals with successful copulation/Number of animals paired) × 100
- Fertility index (%): (Number of pregnant animals/Number of animals with successful copulation) × 100

Sperm analysis:

At the necropsy of adult males, the right testis and cauda epididymis were removed, weighed, and examined for the following parameters:

Sperm motility

For all males of each group, a part of the cauda epididymis was incised to collect sperm. The sample was suspended in Medium 199 supplemented with 1% bovine serum albumin (BSA). The percentage of motile sperm was determined by analysing approximately 200 sperm cells per animal with an automatic sperm analyser.

Sperm morphology

Sperm smears stained with eosin Y were prepared from the sperm suspensions prepared for the sperm motility analysis. The incidence of abnormal sperm in the control and the high dose (10000 ppm) groups was determined by counting the morphologically abnormal sperm out of 200 complete sperm with head and a tail. The abnormal sperm was morphologically classified as sperm without hook, sperm with banana-like head, amorphous head, or folded midpiece, or others. The incidence of tailless sperm was determined by counting the sperm separated into the head and tail out of 100 sperm. The lower dose groups in both generations were not examined, because no effects of the test compound were detected in F0 or F1 males in the high dose group (10000 ppm).

Spermatid count and sperm count

After the sperm motility analysis, the testis and cauda epididymis were stored frozen at -20°C or below (-27.9 to -21.6°C). Before the analysis, the organs of F0 and F1 males in the control and 10000 ppm groups were thawed at room temperature and homogenized in purified water to make stock solutions for the determination of spermatid and sperm counts. The sperm heads in the homogenates were counted with the automatic sperm analyser to determine the number of spermatids per gram of testis (homogenization-resistant spermatid) and the number of sperm per gram of cauda epididymis. The lower dose groups in both generations were not examined, because no effects of the test compound were detected in F0 or F1 males in the high dose group (10000 ppm).

Observation of parturition and nursing:

For parturition, the dams were observed twice a day from Day 21 to Day 25 of gestation. The females that delivered their litter completely by 16:00 were judged as dams giving birth on that day. The females that did not deliver by 25 days after copulation were judged as "non-delivery." For nursing, the dams were observed once a day for maternal behaviour, including lactation, nest building, and cannibalism, until Day 21 of lactation. At necropsy on Day 21 of lactation, the uterus was removed and examined for the number of implantations. The animals judged "non-delivery" were examined 26 days after copulation in the same way. The uterus without visible implantation sites was immersed in a 10% ammonium sulfide solution to detect the presence of implantation sites. The following indices were calculated based on these results:

- Gestation length: Number of days from Day 0 of gestation to the day of parturition
- Gestation index (%): (Number of females with live offspring/Number of pregnant females) × 100
- Birth index (%): (Number of offspring born alive/Number of implantations) × 100

Pathological examination:

Necropsy:

Upon completion, or near completion, of the scheduled necropsy of females on Day 21 of lactation, males were euthanized by exsanguination from the abdominal aorta under anaesthesia, and the head, thoracic, and abdominal organs/tissues were examined macroscopically (F0 males: 5 weeks after the mating period, F1 males: 6 weeks after the mating period). Females were necropsied in the same manner as males at the following time: on Day 21 of lactation for dams delivered, 26 days after copulation for non-delivery females (2 F0 females and 3 F1 females), and 14 days after the mating period for the non-copulated female (1 F0 female only). For dams sacrificed on Day 21 of lactation, vaginal smears were collected in the morning on the day of the necropsy and examined for the oestrous cycle stage. The age at necropsy was 23 to 24 weeks for F0 males, 20 to 24 weeks for F0 females, 20 to 21 weeks for F1 males, and 18 to 21 weeks for F1 females.

Organ weight:

At the scheduled necropsy of males and dams after weaning, the following organs were weighed: Brain, pituitary, thyroid, liver, kidney, adrenal, spleen, testis, epididymis (whole and cauda), prostate (ventral lobe), seminal vesicle (including coagulating gland), ovary, uterus (including cervical region).

Relative organ weights (body weight ratio) were calculated based on each body weight at necropsy. The pituitary and thyroid were weighed after fixation in 10 vol% phosphate-buffered formalin. The bilateral organs were weighed individually and calculated as a sum.

Histopathological examination:

The following organs/tissues of all animals were fixed and preserved in 10 vol% phosphate-buffered formalin (apart from the testes which were pre-fixed in Bouin's fluid for more than 2 hours and then transferred to 10 vol% phosphate-buffered formalin): Brain, pituitary, thyroid, thymus, liver, kidney, adrenal, spleen, testis, epididymis, seminal vesicle, coagulating gland, prostate (ventral lobe), ovary, uterine tube, uterus (including cervical region), vagina, mammary gland (female), gross lesions.

For the histopathological examination, the following organs/tissues of all males and females in the control and high dose (10000 ppm) groups were stained with haematoxylin and eosin (H.E.) by standard methods, and were examined microscopically: Pituitary, adrenal (bilateral), testis (unilateral), epididymis (unilateral), seminal vesicle (bilateral), coagulating gland (bilateral), prostate (ventral lobe), ovary (bilateral), uterine tube (bilateral), uterus (including cervical region), and vagina.

In addition, the liver of males and females and the thyroid of males in the F0 and F1 generations, and the kidney of F0 females were examined in the same way, because effects of the test substance were suspected from the results of organ weight measurement.

For the epididymis, longitudinal sections of the unilateral head, body, and caudal portion were prepared and examined. The ovary of F1 females was cut at the maximum diameter, embedded in paraffin (left and right ovaries separately), and sectioned. The paraffin-embedded sections were subjected to immunohistochemical staining for proliferating cell nuclear antigen (PCNA), and the number of small follicles, medium-sized follicles, and large follicles was counted (according to the classification by Pedersen *et al.*).

Since treatment-related changes were seen in liver of top-dose F0 and F1 males and females, in thyroid of top dose F0 and F1 males, and in adrenals of top dose F1 females, these organs of F0 and F1 animals in the 1000 and 3000 ppm groups were also examined in the same manner. In addition, liver sections from 2 males and 2 females each in the control and 10000 ppm groups in the F0 and F1 generations were stained with Schmorl, and Hall and Berlin blue for the differentiation of the brown pigment observed in the liver of the animals in the 10000 ppm group. Liver sections stained with H.E. were also examined using a polarizing lens and a fluorescence microscope to verify whether the brown pigments had birefringent and red autofluorescence or not. For the special stainings of the liver, animals that had severe changes in the 10000 ppm group were selected.

Observation and examination of offspring (F1 and F2)

Litter examinations:

The day of birth was designated as postnatal Day 0.

On postnatal Day 0, the newborns were examined for the number of offspring (live or stillborn), sex, and presence of external anomalies. After that, the pups were observed daily for clinical signs and mortality until postnatal Day 21. On postnatal Day 4, litter size was randomly adjusted to 8 pups (equal sex ratio, in principle). Litters with less than 8 pups were maintained as they were. The pups culled at the litter size adjustment were subjected to necropsy. From the numbers of live offspring on postnatal Days 0, 4, 7, 14, and 21 (weaning day), the following indices were calculated:

- Live birth index (%): $(\text{Number of offspring born alive} / \text{Number of offspring born}) \times 100$
- Viability index on Day 4 (%): $(\text{Number of offspring alive on Day 4} / \text{Number of offspring born alive}) \times 100$
- Viability index on Day 7 (%): $(\text{Number of offspring alive on Day 7} / \text{Number of live offspring after culling}) \times 100$
- Viability index on Day 14 (%): $(\text{Number of offspring alive on Day 14} / \text{Number of live offspring after culling}) \times 100$
- Weaning index [viability index on Day 21] (%): $(\text{Number of live weanlings} / \text{Number of live offspring after culling}) \times 100$

For F1 animals, 1 male and 1 female were randomly selected from each litter (the first offspring number of male and female pups selected randomly at the litter size adjustment) on postnatal Day 21 and became the parental animals for the second generation (F1 parental animals). Other F1 pups and all F2 pups were necropsied on postnatal Day 21.

Body weight:

The offspring were weighed individually on postnatal Days 0, 4, 7, 14, and 21. The body weight gain was calculated by litter unit on the basis of the body weight at birth before the litter size adjustment, and by each offspring on the basis of the body weight on postnatal Day 4 after the litter size adjustment.

Sexual maturation:

The F1 offspring were examined for the day of vaginal opening from postnatal Day 27 for females and for the day of preputial separation (cleavage of the balanopreputial gland) from postnatal Day 35 for males. Body weights were measured on the corresponding days.

Pathological examination

Necropsy:

Pups culled at the litter size adjustment on postnatal Day 4 were euthanized by exsanguination from the abdominal aorta under anaesthesia, and the head, thoracic, and abdominal organs/tissues were examined macroscopically. All of the other pups, except for F1 animals selected as parents for the next generation, were necropsied in the same manner on postnatal Day 21. The following organs of pups subjected to the organ weight measurement (one pup/sex/litter) and organs/tissues showing gross abnormality were fixed in 10 vol% phosphate-buffered formalin: Brain, thymus, spleen, testis, epididymis, seminal vesicle, prostate, ovary, uterus, and vagina.

The pups that died before the litter size adjustment, except for cannibalized ones, were examined for the presence of external anomalies and the whole bodies were preserved in 10 vol% phosphate-buffered formalin. The pups that died after the litter size adjustment could not be necropsied, because all were cannibalized by their dams.

Organ weight:

At necropsy of F1 pups on postnatal Day 21, 1 male and 1 female were selected from each litter (in numerical order of the offspring number in each litter) for organ weight measurement (limited to: brain, thymus, spleen, uterus). Relative organ weights (body weight ratio) were calculated based on individual body weight at necropsy.

Statistics:

Offspring data obtained before weaning were analysed on the basis of litter mean values, except for the sex ratio. The body weights and food consumption data from non-pregnant females after copulation were excluded from the analysis.

The metric data were analysed by multiple comparison tests for statistical significance. The homogeneity of variance was tested first by Bartlett's test. When the variance was homogeneous, the one-way analysis of variance was performed; when heterogeneous, the Kruskal-Wallis test was used. When a significant inter-group difference was found, Dunnett's or the Dunnett-type multiple comparison test was used. For some of the data, the Kruskal-Wallis test was applied first, and when a significant inter-group difference was found, the Dunnett-type multiple comparison test was used. However, for the comparison of spermatid count, sperm count, and ovarian follicle count between the control and 10000 ppm groups, the homogeneity of variance was tested by the F test, and when the variance was homogeneous, Student's t test was performed for the statistical comparison; when heterogeneous, the Aspin-Welch t test was used. In addition, Wilcoxon's rank sum test was used for the comparison of the incidence of abnormal sperm (morphologically abnormal sperm and tailless sperm) between the control and 10000 ppm groups. The count data were analysed by Wilcoxon's rank sum test for histopathological findings and by Fisher's exact probability test for the others. The significance level was set at 5% for all statistical analyses. All statistical analyses were performed with the Toxicological Data Processing System (MiTOX, Mitsui Zosen Systems Research Inc.).

Statistical analyses were performed on the items listed below. The analyses were not conducted on the clinical observations.

- *Multiple comparison test:* Body weight, body weight gain, food consumption, organ weight, number of implantations, number of offspring, number of live offspring, oestrous cycle length
- *Kruskal-Wallis test and Dunnett-type multiple comparison test:* Days until copulation, number of oestrus stages without copulation, gestation length, birth index, live birth index, viability index on Days 4, 7, and 14, weaning index, incidence of offspring with external anomalies, sexual maturation (vaginal opening, preputial separation), sperm motility
- *F test and t test:* Spermatid count, sperm count, ovarian follicle count
- *Wilcoxon's rank sum test:* Incidence of abnormal sperm, histopathological findings, percentage of ovarian follicles
- *Fisher's exact probability test:* Copulation index, fertility index, gestation index, sex ratio (male/female), incidence of females with irregular oestrous cycles, incidence of dams with externally abnormal offspring, necropsy findings

Findings:

Chemical intake:

The mean test substance intakes in the treated groups are shown in the following table (Table B.6.6.1-4). The mean intakes during the pre-mating period in F1 animals were approximately 1.5-fold higher for both sexes compared with those in F0 animals, reflecting the higher intake in the growth period after weaning. The mean intake of males after mating and that of females during both the gestation and lactation periods were approximately the same between F0 and F1 animals for each treated group. The mean intake during the gestation period was similar to that in the pre-mating period, whereas during lactation it was more than 2-fold higher than during gestation in both F0 and F1 females.

Table B.6.6.1-4: Mean test substance intakes (mg/kg bw/d)

Sex	Doses Period of treatment	1000 ppm (Group 2)	3000 ppm (Group 3)	10000 ppm (Group 4)
F0 generation				
Males	Pre-mating period	56.15	166.3	559.1
	After mating period	43.27	132.0	452.1
Females	Pre-mating period	62.48	195.3	628.5
	Gestation period	60.19	186.2	602.8
	Lactation period	162.8	511.0	1634.6
F1 generation				
Males	Pre-mating period	84.73	254.6	881.2
	After mating period	47.77	145.7	511.7
Females	Pre-mating period	90.11	274.9	929.3
	Gestation period	65.68	200.3	672.0
	Lactation period	163.4	504.6	1687.5

Clinical observations:

There was no mortality among parental animals, or among F1 animals selected for rearing.

F0 animals

No abnormal clinical signs were observed in males or females of any group throughout the observation period, including the gestation and lactation periods of pregnant females.

F1 animals

No treatment-related abnormal clinical signs were observed in males or females in any treated group throughout the observation period.

Before weaning, death was observed sporadically in a few pups in all groups, including the control group, mainly up to postnatal Day 4. There was no death after the litter size adjustment on postnatal Day 4, except for 1 female (#60425-6) that died on postnatal Day 13 in the 10000 ppm group. Most of the pups that died were cannibalized by their dams. Trauma or internal haemorrhage was observed in one pup of the 1000 ppm group and in one pup of the 3000 ppm group during the period before postnatal Day 4. Moreover, loss of suckling was seen on postnatal Days 2 and 3 in pups of one dam (#50221) that showed loss of retrieving on Day 3 of lactation in the 1000 ppm group, resulting in body weight loss or remarkably reduced body weight gain in these pups. However, these findings were judged to be not treatment-related because there was no dose-dependency in their incidences or because of their low occurrence.

F2 animals

No treatment-related abnormal clinical signs were observed in males or females in any treated group throughout the observation period.

Death was observed sporadically in a few pups in all dose groups, including the control group, mainly up to postnatal Day 4. There was no death after the litter size adjustment on postnatal Day 4, except for 1 male and 1 female (#20411-43, #70404-58) that died on postnatal Day 5 in the 10000 ppm group. Most of the pups that died were cannibalized by their dams.

Body weight:

F0 animals

In the 10000 ppm group, body weight gain was suppressed in males, with significant differences in body weights and body weight gain throughout the observation period. In females, although there were no

significant differences in body weights or body weight gain during the pre-mating period, the body weights were suppressed in the gestation and lactation periods, and significant differences were noted in body weight gain on Day 14 of gestation and in body weights on Days 7 and 14 of lactation. However, the body weight gain during the lactation period was almost comparable to that in the control group up to Day 14 of lactation, and a significantly higher value was observed on Day 21 of lactation¹.

In the 1000 and 3000 ppm groups, body weights were comparable to those in the control group in males and females throughout the observation period, including the gestation and lactation periods of pregnant females, with no significant differences in body weights or body weight gain.

F1 animals

In the 10000 ppm group, 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 were noted in both sexes in body weight and body weight gain from postnatal Days 7 to 21. After weaning, suppressed body weight gain continued in males until the terminal necropsy, with significant differences in body weight and body weight gain at almost all measurement points. In females, significantly lower body weights also continued after weaning. However, a significant difference in body weight gain was observed only on postnatal Day 28, and the difference in the body weights from the control group diminished with time, with no significant differences observable after postnatal day 63 in the pre-mating period. In the gestation and lactation periods, the body weights were again suppressed, and significant differences were noted in body weights on Day 7 of gestation and on Days 4 to 14 of lactation. However, the body weight gain during the gestation and lactation periods was almost comparable to that in the control group up to Day 14 of lactation, and a significantly higher value was observed on Day 21 of lactation.

In the 1000 and 3000 ppm groups, birth weights, postnatal body weights, and body weight gain in males and females were comparable to those in the control group throughout the observation period, including the gestation and lactation periods. Significantly higher values in body weight gain were observed on postnatal Days 28 and 35 in the 3000 ppm group and in body weights on Day 21 of lactation in the 1000 and 3000 ppm groups; however, they were judged to be incidental because of a transient change with no dose-dependency.

F2 animals

Although birth weights were almost the same as those in the control group, postnatal body weight gain was suppressed in males and females at 10000 ppm, and significant differences were noted in both sexes in body weights and/or body weight gain from postnatal Days 7 to 21.

In the 1000 and 3000 ppm groups, birth weights, postnatal body weights, and body weight gain were comparable to those in the control group in males and females throughout the observation period, with no significant differences in body weight or body weight gain.

Food consumption

F0 animals

In the 10000 ppm group, food consumption decreased significantly in males from Days 28 to 63 of dosing. In females, although there were no significant differences during the pre-mating or gestation period, a significantly lower value was noted on Day 7 of lactation.

In the 1000 and 3000 ppm groups, food consumption was almost comparable to that in the control group in

¹ Regarding the increased body weight gain of F0 and F1 dams on lactation Day 21 at 10000 ppm compared to control: It is well known that the body weights of lactating dams increase due to the development and engorgement of the mammary gland in the early stage of lactation and decrease in the later period, because the pups consume the diet during the last week of lactation which triggers involution of the mammary gland, with precipitous decline after weaning of their pups. In the 10000 ppm group, the body weight gain of the pups was remarkably suppressed, suggesting prolongation of lactation in these pups and delay in the physiological involution of the mammary gland in their dams, which resulted in suppression of the physiological decrease of maternal body weights in the later stage of the lactation period. Therefore, the increased maternal body weight gain on lactation Day 21 at 10000 ppm was considered to be due to growth retardation in their pups, and was judged to be not toxicologically relevant.

males and females throughout the observation period, including the gestation and lactation periods of pregnant females. Significantly higher food consumption was observed on Day 7 of dosing in the 3000 ppm group; however, it was judged to be incidental because of a transient change with the lack of dose-dependency.

F1 animals

In the 10000 ppm group, significantly lower food consumption was noted in males on postnatal Days 28 and 35 of dosing; however, there were no significant differences thereafter. In females, significantly lower food consumption was also observed sporadically, with statistical significance on postnatal Days 28, 70, and 91 during the pre-mating period as well as on Day 7 of gestation and on Days 4 to 14 of lactation. In the 1000 and 3000 ppm groups, food consumption was comparable to that in the control group in males and females throughout the observation period, including gestation and lactation periods, with no significant differences.

Oestrus cycle:

In the F0 generation as well as in the F1 generation, no treatment-related changes were observed in the oestrous cycles in females of any treated group. Almost all females in each group had an oestrous cycle of 4 to 5 days, and no significant differences were found in the mean oestrous cycle length. There were no females with irregular oestrous cycles in any group, including the control group.

Reproductive performance:

As a result of the first mating, all mating pairs in each group in both generations showed evidence of copulation by 14 days after the start of mating, except for 1 pair in the 10000 ppm group in the F0 generation. Therefore, the test compound was judged to have no effect on the mating ability, and the mating was terminated without the second mating in both generations.

F0 animals

No treatment-related changes were observed in the mating ability or fertility in males or females of any treated group. All the mating pairs of each group, except for one pair in the 10000 ppm, copulated during the first or second oestrus stage of females within 13 days after the start of mating. There were no significant differences in the copulation index, in the number of oestrus stages without copulation or in the days until copulation between the control and treated groups. There was only 1 non-pregnant female in the control group and in the 10000 ppm group, respectively, and no significant difference was found in the fertility index between the control and treated groups.

The uncopulated pair in the 10000 ppm group did not copulate during 3 oestrus cycles in the mating period. In addition, 1 female each in the 1000 and 10000 ppm groups showed continuous dioestrus after the start of mating; however, both females returned to oestrus by 12 or 13 days after the start of mating, and copulated and conceived. They were judged to be incidental because only one case was observed in each group with no dose-dependency.

F1 animals

No treatment-related changes were observed in the mating ability or fertility in males or females of any treated group.

All the mating pairs of each group copulated during the first or second oestrus stage of females within 5 days after the start of mating, and there were no significant differences in the copulation index, in the number of oestrus stages without copulation or in the days until copulation between the control and treated groups.

There were only 1 and 2 non-pregnant females in the 1000 and 3000 ppm groups, respectively, and no significant difference was found in the fertility index between the control and treated groups.

Sperm analysis:

In both the F0 and the F1 generation, no treatment-related changes suggesting effects on spermatogenesis were detected in any treated group. All parameters, including the 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 significant differences. Furthermore, no abnormalities were observed in males that had no copulation or did not impregnate their mating partners, except for 1 male in the control group of the F0 generation, which showed low sperm motility of 40%.

Observation of parturition and nursing:

F0 animals

No treatment-related changes were detected in parturition or nursing in any treated group. All dams in each group delivered normally between Days 21 and 23 of gestation and no significant differences were found in the gestation length, number of implantations or offspring born alive, birth index, or gestation index between the control and treated groups.

In the observation of nursing, loss of retrieving was observed in 1 dam in the 1000 ppm group on Day 3 of lactation; however, it was judged not to be treatment-related because only one case was observed with the lack of dose-dependency in the incidence. There were no abnormalities in nursing behaviour in the other dams of any group.

F1 animals

No treatment-related changes were detected in parturition or nursing in any treated group. All dams in each group delivered between Days 21 and 23 of gestation, except for 1 dam that delivered on Day 24 of gestation in the control group. No abnormalities were observed in parturition in any group, including the dam that delivered on Day 24 of gestation in the control group.

There were no significant differences in the number of implantations or offspring born alive, birth index, or gestation index between the control and treated groups, although the gestation length in the 10000 ppm group (21.8 days) was significantly shorter than in the control group (22.2 days). However, there was no treatment-related change in gestation length of F0 dams. In F1 dams, the difference from the control group in gestation length was extremely small and the gestation length of all dams in the 10000 ppm group was 21 or 22 days, which is within the normal range (21 to 23 days) of this strain. Moreover, the mean gestation length in the 10000 ppm group was almost equal to the lower limit of the historical control range of the test facility (mean length: 21.9-22.2 days, range of individual length: 21-23 days, 2002-2009), while that in the control group was equal to the upper limit of the historical control range. Individually, 5 out of 24 dams had a gestation length of 21 days in the 10000 ppm group, whereas in the control group none of the dams delivered before Day 22 of gestation and 1 dam had a gestation length of 24 days, which has not been observed in the historical control data of the test facility. In F0 dams of the control group, 2 out of 25 dams had a gestation length of 21 days, while further 2 of them had already started parturition at 16:00 on Day 21 of gestation. However, since parturition was not completed at 16:00, they were judged as dams giving birth on Day 22 of gestation (data not shown), indicating that the occurrence of parturition on Day 21 of gestation in F1 dams of the 10000 ppm group was almost the same as that in F0 dams in the control group. Therefore, the significant difference observed in the gestation length in the 10000 ppm group was considered to be a variation within the normal range, and was judged to be of no toxicological significance.

In the observation of nursing, there were no abnormalities in nursing behaviour in any dam of any group.

Pup development:

Viability:

In F1 and F2 pups, no treatment-related changes were detected in viability in any treated group. All parameters in each treated group, including the number of offspring born and born alive, sex ratio, live birth index, viability index on Days 4, 7, and 14, and weaning index, were comparable to those in the control group, with no significant differences.

Moreover, there was no death after weaning in any F1 group, including the control group

External examination:

There were no external anomalies in F1 or F2 pups of any group, including the pups that died.

Sexual maturation (F1 animals):

At 10000 ppm, preputial separation (cleavage of balanopreputial gland) in males and vaginal opening in females showed a significant delay of 1.6 and 1.5 days on average (males: Day 42.7, females: Day 32.5) compared to the control group (males: Day 41.1, females: Day 31.0), respectively. The body weights in the 10000 ppm group on the corresponding days were lower than those in the control group in both sexes (% of the control value: males, 96.1%, females, 94.1%), and a significant difference was observed in females.

In the 1000 and 3000 ppm groups, both, the days of preputial separation in males and vaginal opening in females, were comparable to those in the control group, with no significant differences. Moreover, no significant differences were found in body weights in males or females on the corresponding days.

Pathological examination:

Necropsy:

F0 animals

Dark brownish change of the liver was noted in 24 females in the 10000 ppm group. Enlargement of the liver was noted in 20 females in the 10000 ppm group. In the non-copulated and non-pregnant females, there were no treatment-related changes.

Greenish change in the cortex of the kidney was noted in 5 females in the 10000 ppm group. However, there were no histopathological changes attributed to the necropsy finding.

Other changes were considered to be incidental, because of the lack of dose-response and/or their pathological nature.

F1 animals

F1 pups culled on postnatal Day 4:

There were no abnormal findings in offspring of any group, including the control group.

F1 pups necropsied at weaning:

No treatment-related abnormal findings were detected in males or females in any treated group. The single pup that died after the litter size adjustment (one female in the 10000 ppm group) could not be examined due to cannibalization by the dam.

F1 parental animals:

Liver: Dark brownish change of the liver was noted in 20 females in the 10000 ppm group. Enlargement of the liver was noted in 6 females in the 10000 ppm group. In the non-pregnant females, there were no treatment-related changes.

The other changes were considered to be incidental due to the lack of dose-response and/or their pathological nature. Furthermore, enlargement of the thyroid observed in 1 and 2 males at 3000 and 10000 ppm, respectively, was judged to be incidental, because vacuolation of the follicular cells was confirmed histopathologically in all of the males in the 10000 ppm group, which is known to occur spontaneously in this strain of rats.

F2 pups

F2 pups culled on postnatal Day 4:

There were no abnormal findings in offspring of any group, including the control group.

F2 pups necropsied at weaning:

No treatment-related abnormal findings were found in males or females in any treated group.

The pups that died after the litter size adjustment (one male and one female in the 10000 ppm group) could not be examined due to cannibalization by their dams.

Organ weight:

F0 animals

In males, significant increases in the absolute and relative weights of the thyroid and liver were noted in the 3000 ppm group and above. Significantly higher relative weights of the brain and seminal vesicle were observed in the 10000 ppm group. However, the absolute weights of both organs were comparable to those in the control group, with no significant differences; therefore, these changes were considered to be due to the lower body weights at the necropsy in this group, and were judged to be of no toxicological significance.

In females, significant increases in the absolute and relative weights of the liver were noted in the 1000 ppm group and above, as were increases in the absolute and relative weights of the kidney and decreases in the absolute and relative weights of the uterus in the 10000 ppm group. Moreover, the ovary weights in the 10000 ppm group tended to be lower than those in the control group and a significant difference was observed in the relative weights. Regarding the oestrous stage at the necropsy in females, the distribution of each stage was similar among all groups. Most of the females in each group showed dioestrus and the others exhibited proestrus or oestrus, with no constant trend among the groups.

F1 animals

F1 pups necropsied at weaning:

Significant decreases in the absolute and relative weights of the spleen were noted in males in the 3000 and 10000 ppm groups. In addition, at 10000 ppm, significantly lower absolute weights were observed in the thymus in both sexes and the spleen and uterus in females, as were significantly higher relative weights of the brain in both sexes in the 10000 ppm group. However, the changes observed only in either absolute or relative weights and the corresponding absolute or relative weights of these organs were almost the same as those in the control group; therefore, they were considered to be due to the lower body weights at the necropsy in this group, and were judged to be of no toxicological significance.

F1 Parental animals:

In males, significant increases in the absolute and relative weights of the thyroid and liver were noted in the 10000 ppm group. Absolute weights of the liver in the 3000 ppm group also tended to be higher than those in the control group and a significant difference was observed in the relative weights. In addition, significantly higher relative weights of several other organs (brain, spleen, kidney, adrenal, testis, seminal vesicle, and epididymis) were observed in the 10000 ppm group. However, the absolute weights of these organs were comparable to those in the control group, with no significant differences; therefore, these changes were considered to be due to the lower body weights at the necropsy in this group, and were judged to be of no toxicological significance.

In females, significant increases in the absolute and relative weights of the liver were noted at 3000 and 10000 ppm. The absolute and relative weights of the liver in the 1000 ppm group were also higher than those in the control group, with a significant difference in the absolute weights. In addition, absolute and relative ovary weights were significantly decreased in the 10000 ppm group. Furthermore, significantly lower values were observed in the absolute weights of the brain in the 10000 ppm group and relative brain weights in the 1000 and 3000 ppm groups, in the relative weights of the pituitary in the 3000 and 10000 ppm groups, and in the relative weights of the left adrenal in the 3000 ppm group. However, they were changes observed only in either absolute or relative weights, with the lack of clear dose-dependency; therefore, they were judged to be not treatment-related or of no toxicological significance.

Regarding the oestrous stage at the necropsy in females, the distribution of each stage was similar among all groups. Most of the females in each group showed metoestrus or dioestrus and the others exhibited proestrus or oestrus, with no constant trend among the groups.

F2 pups

Significant decreases in the absolute and relative weights of the spleen were noted in males and females in the 10000 ppm group. Moreover, the spleen weights of females in the 3000 ppm group also tended to be lower than those in the control group, and a significant difference was noted in the relative weights. In addition, significantly lower absolute weights were observed in the thymus in males and females, as well as

significantly higher relative weights of the brain in both sexes in the 10000 ppm group. However, these changes were only observed only in either the absolute or relative weight and the corresponding relative or absolute weights of these organs were similar to the control group; therefore, they were considered to be due to the lower body weights at the necropsy in this group, and were judged to be of no toxicological significance.

Histopathological examination:

F0 animals

Liver: Minimal to moderate brown pigment in the bile duct/periportal area was observed in 9 males and 20 females in the 10000 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 birefringent according to an examination using a polarizing lens. It had no clear red autofluorescence by a fluorescence microscope, tentatively attributed to the microscope conditions not being optimised. At 10000 ppm, minimal brown pigment deposition in the peribubular hepatocyte was observed in 4 females, minimal focal periductular inflammatory cell infiltration was observed in 4 males and 10 females, and minimal to moderate proliferation of the bile duct was observed in 12 females. Minimal to moderate diffuse hypertrophy of hepatocytes was observed in 6 females at 1000 ppm, in 15 males and 17 females at 3000 ppm, and in 24 males and all females at 10000 ppm.

Thyroid: Minimal diffuse hypertrophy of the follicular cells in the thyroid was observed in 4 males in the 10000 ppm group.

In the non-copulated and non-pregnant females, minimal brown pigment in the bile duct/periportal area and minimal diffuse hypertrophy of hepatocytes were observed in 1 and 2 females in the 10000 ppm group, respectively. The other changes were considered to be incidental, because of the lack of dose-response and/or their pathological nature.

F1 animals

Liver: Minimal to moderate brown pigment in the bile duct/periportal area was observed in 10 males and 11 females in the 3000 ppm group, and in all animals in the 10000 ppm group. The brown pigment stained dark blue with Schmorl and negative for Hall and Berlin blue, and had birefringence according to an examination using a polarizing lens. It had no clear red autofluorescence by a fluorescence microscope, tentatively attributed to the microscope conditions not being optimised. Minimal brown pigment deposition in the peribubular hepatocytes was observed in 5 females in the 10000 ppm group. Minimal focal periductular inflammatory cell infiltration was observed in 3 females in the 3000 ppm group, and in all animals in the 10000 ppm group. Minimal to moderate proliferation of the bile duct was observed in 16 females in the 10000 ppm group. Minimal to mild diffuse hypertrophy of hepatocytes was observed in 4 females at 1000 ppm, in 6 males and 18 females at 3000 ppm, and in 23 males and all females at 10000 ppm.

Adrenals: Minimal hypertrophy of the cortical cells in the fascicular zone was observed in 5 females in the 10000 ppm group.

Thyroid: Although histopathological examination of thyroid was extended to all groups in view of the suspicion that treatment-related changes could occur as seen among F0 males, there were no treatment-related changes in the thyroid of F1 males. In one male in the 3000 ppm group, there were no histopathological changes attributed to the enlargement of the thyroid detected at necropsy.

In the non-pregnant females, there were no treatment-related changes.

The other changes were considered to be incidental, because of the lack of dose-response and/or their pathological nature.

F1 Ovarian follicle count

A significant increase in the number of medium-sized follicles was noted in the 10000 ppm group. In addition, although there was no significant difference, the number of large follicles also tended to increase in the 10000 ppm group. However, the number of small follicles, which include primordial follicles, and the total number of ovarian follicles in the 10000 ppm group were comparable to those in the control group, with no

Table B.6.6.1-5: Summary of key findings in the F0 generation in the two-generation reproduction toxicity study

[illegible]

Liver: Enlargement	0	0	0	0	0	0	0	20**
Kidney: Greenish change, cortex	0	0	0	0	0	0	0	5*
Organ weights								
Liver (g)	12.65	13.04	14.12**	15.53**	10.42	11.45*	12.24**	17.24**
Relative liver weight (% bw)	2.87	2.97	3.23**	3.73**	3.89	4.21*	4.47**	6.30**
Kidney (g)	2.536	2.545	2.555	2.499	1.987	1.992	1.961	2.095**
Relative kidney wt. (% bw)	0.577	0.580	0.585	0.599	0.742	0.731	0.717	0.766**
Thyroid (mg)	23.75	24.04	25.29**	28.97**	20.98	21.90	21.67	21.55
Relative thyroid wt. (x10 ⁻³ %)	5.45	5.48	5.78**	7.02**	7.85	8.00	7.93	7.88
Ovary (mg)					91.34	90.35	97.48	82.33
Relative ovary wt. (x10 ⁻³ %)					34.14	33.21	35.66	30.06*
Uterus (g)					0.45	0.54	0.51	0.38*
Relative uterus wt. (% bw)					0.167	0.198	0.187	0.137**
Histopathology								
Liver (no. examined)	26	26	26	26	25	26	26	24
Brown pigment, bile duct/perportal	0	0	0	9** (grade 1)	0	0	0	20** (grade 1-3)
Brown pigment deposition in perlobular hepatocytes	0	0	0	0	0	0	0	4* (grade 1)
Focal periductular inflammatory cell infiltration	0	0	0	4* (grade 1)	0	0	0	10** (grade 1)
Proliferation of bile ducts	0	0	0	0	0	0	0	12** (grade 1-3)
Diffuse hypertrophy of hepatocytes	0	0	15** (grade 1)	24** (grade 1-2)	0	6* (grade 1)	17** (grade 1)	24** (grade 1-3)
Thyroid (no. examined)	26	26	26	26	0	0	0	0
Diffuse hypertrophy of follicular cells	0	0	0	4* (grade 1)				

Grade: 1 Minimal; 2 Mild; 3 Moderate

* p < 0.05 in comparison to controls, ** p<0.01 in comparison to controls

Table B.6.6.1-6: Summary of key findings in the F1 and F2 generations in the two-generation reproduction toxicity study

	Males				Females			
Diet concentration (ppm)	0	1000	3000	10000	0	1000	3000	10000
F1 animals								
Body weight (g)								
Postnatal Day 0	5.8	5.8	5.8	5.8	5.5	5.5	5.4	5.5
Postnatal Day 7	16.2	15.9	16.3	14.7*	15.7	15.5	15.7	14.2*
Postnatal Day 14	32.5	32.0	32.3	28.0**	31.6	31.2	31.4	27.1**

Postnatal 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
F1 pup development								
Day of cleavage of balanopreputial gland	41.1	41.8	42.0	42.7**				
Day of vaginal opening					31.0	30.9	31.0	32.5*
Bodyweight (g) at cleavage of balanopreputial gland / vaginal opening	173.96	181.38	179.62	167.17	94.96	96.46	95.50	89.33*
Reproductive Performance								
Number of pairs					25	26	26	24
Copulation index (%)					100	100	100	100
Day of conceiving					2.6	2.6	2.3	2.0
Fertility index (%)					100	96.2	92.3	100
Gestation index (%)					100	100	100	100
Gestation length (days)					22.2	22.0	22.0	21.8**
Implantations (mean)					13.8	13.5	13.1	13.2
Offspring born alive					13.0	12.9	12.5	12.3
Birth index (%)					93.96	95.29	95.44	93.04
Necropsy gross findings – parental animals								
No. of animals examined	25	26	26	24	25	25	24	24
Liver: Dark brownish change	0	0	0	0	0	0	0	20**
Liver: Enlargement	0	0	0	0	0	0	0	6**
Organ weights – at weaning								
Body weight (g)	52.0	51.4	50.5	44.0**	50.6	49.8	48.8	41.6**
Spleen (g)	0.266	0.248	0.232*	0.199**	0.252	0.250	0.232	0.200**
Thymus (mg)	204.1	199.0	203.4	167.8**	208.8	206.2	208.5	172.1**
Uterus (mg)	-	-	-	-	40.38	40.83	42.70	34.60*
Organ weights – parental animals								
Body weight (g) at necropsy	466.6	466.0	455.0	427.3**	277.4	289.9*	290.1*	279.0
Liver weight (g)	13.86	14.00	14.62	15.97**	11.14	12.30**	13.33**	16.96**
Relative liver weight (% bw)	2.97	3.00	3.22**	3.74**	4.01	4.25	4.59**	6.10**
Thyroid weight (mg)	25.32	25.12	26.02	32.41*	23.61	22.06	20.62	21.03
Relative thyroid wt. (x10 ⁻³ %)	5.45	5.39	5.75	7.72**	8.49	7.65	7.12	7.55
Ovary weight (mg)	-	-	-	-	97.10	95.83	98.25	84.40**
Relative ovary wt. (x10 ⁻³ %)	-	-	-	-	35.09	33.03	33.84	30.28**
Brain (g)	2.093	2.092	2.050	2.072	1.890	1.890	1.862	1.843*
Histopathology								
Liver (no. examined)	25	26	26	24	25	25	24	24

Brown pigment, bile duct/perportal	0	0	10** (grade 1)	24** (grade 1-2)	0	0	11** (grade 1)	24** (grade 1-3)
Brown pigment deposition in perlobular hepatocytes	0	0	0	0	0	0	0	5* (grade 1)
Focal periductular inflammatory cell infiltration	0	0	0	24** (grade 1)	0	0	3	24** (grade 1)
Proliferation of bile ducts	0	0	0	0	0	0	0	16** (grade 1-3)
Diffuse hypertrophy of hepatocytes	0	0	6* (grade 1)	23** (grade 1-2)	0	4* (grade 1)	18** (grade 1)	24** (grade 1-2)
Adrenal (no. examined)	25	0	0	24	25	25	24	24
Hypertrophy of cortical cell in the fascicular zone	0			0	0	0	0	5* (grade 1)
F2 animals								
Body weight (g)								
Postnatal Day 0	5.8	5.7	5.9	5.8	5.5	5.5	5.6	5.4
Postnatal Day 7	16.0	15.8	15.9	15.0	15.5	15.3	15.5	14.3*
Postnatal Day 14	32.9	31.7	32.4	29.6**	32.1	31.0	31.8	28.5**
Postnatal Day 21	53.5	51.6	52.2	46.3**	51.4	50.3	50.4	44.5**
Organ weights								
Thymus weight (mg)	212.0	204.9	201.5	182.5**	210.7	209.0	205.3	179.0**
Spleen weight (g)	0.273	0.253	0.245	0.209**	0.274	0.262	0.234	0.203**

Grade: 1 Minimal; 2 Mild; 3 Moderate

* p < 0.05 in comparison to controls, ** p < 0.01 in comparison to controls

Conclusion:

S-2200 TG was administered orally to male and female rats via diet at doses of 1000, 3000, and 10000 ppm over two generations, and the effects on the reproductive function of parental animals and development of the next generations were assessed.

Regarding the general toxicological effects on parental animals, suppressed body weight gain and reduced food consumption were noted in males and in females in the 10000 ppm group in both F0 and F1 generations.

Pathological examination revealed treatment-related changes in the liver of both sexes. At necropsy, dark brownish change and enlargement of the liver were noted in F0 and F1 females in the 10000 ppm group. Liver weights increased in males at ≥ 3000 ppm and in females at ≥ 1000 ppm in both generations. In the histopathological examination the following findings were observed: Brown pigment in the bile duct/perportal area (in F0 animals at 10000 ppm and in F1 animals at ≥ 3000 ppm), focal periductular inflammatory cell infiltration (in F0 males and females and F1 males at 10000 ppm and in F1 females at ≥ 3000 ppm), and brown pigment deposition in the perlobular hepatocyte and proliferation of the bile duct (in F0 and F1 females in the 10000 ppm group). Brown pigment in the bile duct, the periportal area, and the perlobular hepatocyte were considered to be primary changes by the test substance. Periductular inflammatory cell infiltration and proliferation of the bile ducts were considered to be secondary changes occurred by the

pigmentation. These primary and secondary changes in F1 animals showed an increase in incidence compared to F0 animals.

Diffuse hypertrophy of the hepatocyte was also observed in males at ≥ 3000 ppm and in females at ≥ 1000 ppm in both generations. It is known that hepatocellular hypertrophy accompanied by increased liver weights is an adaptive change associated with induction of the hepatic microsomal drug metabolizing enzymes, and it is considered not to be adverse in the absence of histopathological damage indicative of hepatotoxicity and relevant clinical chemistry changes. In the 1000 ppm group, only hepatocellular hypertrophy and increased liver weights were observed in F0 and F1 females, without any other change. Therefore, the changes in the liver observed in females in the 1000 ppm group were considered to be an adaptive change, and of no toxicological significance.

Furthermore, increases in the thyroid weights were observed in F0 males at ≥ 3000 ppm and in F1 males at 10000 ppm, and hypertrophy of the follicular cell of the thyroid was observed in some F0 males in the 10000 ppm group. The increase in hepatic drug metabolizing enzyme activity is known to cause an increase of the clearance of thyroid hormones in hepatocyte, resulting in hypertrophy in the thyroid follicular cells by negative feedback. As the thyroid effects were observed only at dose levels higher than that resulting in hepatocellular hypertrophy, the hypertrophy of thyroid follicular cells in F0 males in the 10000 ppm group is considered to be secondary to the increased in hormonal turnover and the changes in the liver.

Treatment-related hypertrophy of cortical cells in the fascicular zone was observed in the adrenals in some F1 females in the 10000 ppm group. The effects of the test substance in F1 females were only slightly more severe than in F0 females and are possibly an adaptive change in response to stress.

In addition, decreases were observed in ovary weights in F0 and F1 females and uterus weights in F0 females in the 10000 ppm group, however, in absence of any histopathological changes.

Furthermore, greenish change in the cortex of the kidney was observed in some F0 females at necropsy in the 10000 ppm group, with increased organ weights. However, there were no histopathological changes related to the necropsy finding and increased organ weights in F0 females, nor were there any changes in the kidney of F1 females or F0 or F1 males. In the 13-week repeated oral dose toxicity study in rats, no abnormal changes in the kidney of females were detected in necropsy or histopathological findings or blood parameters of the renal function. Therefore, the changes in the kidney observed in F0 females were considered to be of no toxicological significance.

Based on the adverse liver effects observed at dose levels of 3000 ppm and above, the no-observed-adverse-effect level (NOAEL) in parental animals is considered to be 1000 ppm for systemic toxicity (43 mg/kg bw/d (rounded); mean substance intake of F0 males after the mating period).

Regarding the effects on the reproductive function, there were no treatment-related changes in mating ability, fertility, pregnancy, gestation length, parturition, or nursing behaviour, nor were there any changes in the oestrous cycle or sperm parameters (sperm motility, spermatid counts, sperm counts, and incidences of morphological abnormal sperm and tailless sperm) in either generation. Therefore, the no-observed-adverse-effect level (NOAEL) for the reproductive effects is set at the highest dose level tested in this study of 10000 ppm (559 mg/kg bw/day; mean substance intake of F0 males during pre-mating period).

Regarding the effects on offspring, postnatal body weight gain was suppressed in both sexes of F1 and F2 offspring in the 10000 ppm group. Since both F0 and F1 dams in the 10000 ppm group had reduced food consumption during the gestation and/or lactation period, with lower maternal body weights, the postnatal growth retardation observed in this group may be due to undernourishment of their dams.

In addition, lower spleen weights at weaning were noted in F1 males in the 3000 ppm group, in which no change was found in postnatal body weight gain, and in all F1 and F2 animals at 10000 ppm. The lower spleen weights of F1 animals at weaning completely recovered to the control level at adult in both sexes even in the 10000 ppm group, suggesting a transient retardation in growth. Moreover, both the absolute and relative spleen weights of F1 and F2 animals were within the historical control range in the test facility, indicating slight changes.

A slight delay in sexual maturation was found in both sexes (mean difference from the control group: 1.5 days for vaginal opening in F1 females and 1.6 days for preputial separation in F1 males) in the 10000 ppm group. However, there were no changes suggesting effects on the reproductive function in any observations or examinations, including oestrous cycle, sperm parameters, and histopathological findings. Therefore, the slight delay in sexual maturation in both sexes was considered to be related to the growth retardation. Under the conditions of this study, the NOAEL for effects on offspring is considered to be 1000 ppm (56 mg/kg bw/d; mean substance intake of F0 males during pre-mating period).

B.6.6.2 Developmental toxicity studies

B.6.6.2.1 Teratogenicity test by the oral route in the rat

Reference:	S-2200 TG: Oral (Gavage) Range-Finding Study of Prenatal Development in the Rat
Author(s), year:	██████████ 2009a
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. R01-0009
Guideline(s):	This study was not conducted to any specific regulatory guideline, but was conducted to support OECD TG 414, EPA OPPTS 870.3700 and Japanese MAFF 12-Nousan-8147 Teratogenicity studies (2-1-18).
GLP:	No (non-regulatory study, for which a claim of GLP compliance has not been made; however, conducted in accordance with current GLP requirements)
Deviations:	No
Validity:	Yes (supplementary study)

Material and methods:

<i>Test Material:</i>	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G
Purity:	93.4%
<i>Test animals:</i>	
Species:	Rat
Strain:	Wistar, ████████:Wl(Han)
Age at mating:	9 weeks
Weight at mating:	186.7 – 224.0 g
Source:	████████████████████
Diet:	SQC Rat and Mouse Breeder Diet No 3 <i>ad libitum</i>

The objective of the study was to assess the effects of S-2200 TG on the pregnancy of rats when administered orally by gavage, in order to select dose levels for a subsequent study of prenatal development.

Investigations were limited to maternal growth and food consumption, and pregnancy parameters to ensure that dose selection in future study would result in sufficient pups for a full evaluation of developmental toxicity.

Animal assignment and treatment:

Female rats were cohabited with male rats on a one to one basis. Once evidence of mating was seen (presence of vaginal plug *in situ*, or other evidence of mating if necessary), 7 presumed-pregnant females were allocated to each dosage group (0, 250, 500 and 1000 mg/kg bw/d).

The day on which evidence of mating was seen, was designated Day 0 of gestation.

Dose preparation, analysis and administration:

The test article was formulated as a suspension in aqueous 0.5% (w/v) methylcellulose and was stirred continuously before and throughout dosing. Solutions of the test article in the vehicle were prepared for each concentration daily.

Test and control articles were administered orally to presumed-pregnant female rats by gavage once daily from Days 6 to 19 of gestation. The females were sacrificed on Day 20 of gestation for macroscopic examination and examination of litters. Individual dose volumes were adjusted according to the most recent bodyweight, determined daily. Control animals received 0.5% w/v methylcellulose solution.

Clinical observations:

All animals were examined twice daily to detect dead or moribund animals. All animals were examined at least once daily for signs of ill health or overt toxicity. Any abnormalities of appearance or behaviour or other signs of reaction to treatment were recorded and a detailed individual record was maintained of the clinical condition of each animal on the days of body weight recording. Additionally, animals were observed immediately after dosing and at 0.5, 1, 2 and 4 hours post dose for signs of reaction to treatment.

Body weight and food consumption:

The body weight of each female was recorded on Days 4, 6, 7, 8, 9, 12, 15, 17, 19 and 20 of gestation. Food consumption was recorded for Days 4 to 5, 6, 7, 8, 9 to 11, 12 to 14, 15 to 16, 17 to 18 and 19 of gestation.

Sacrifice and pathology:

At the scheduled necropsy on Day 20 of gestation, females were sacrificed by cervical dislocation following isoflurane anaesthesia and examined macroscopically. All tissues were discarded. Gross lesions were retained in 10% neutral buffered formalin.

Uterine/implantation data:

The ovaries and uteri were removed and examined and the following data recorded: pregnancy status, gravid uterus weight, number of corpora lutea and the number and intrauterine position of implantations (subdivided into live foetuses, early intrauterine deaths, late intrauterine deaths and dead foetuses).

The uterus of any apparently non-pregnant female was immersed in a 10% ammonium sulphide solution to reveal any evidence of implantation.

Foetal examination:

Live foetuses were killed by a subcutaneous injection of sodium pentobarbitone.

Individual foetal and placental weights were recorded and foetuses were examined externally and sexed. The foetuses were stored in 10% neutral-buffered formalin. On completion of data recording, all foetuses were discarded as there was no effect of treatment.

Statistics:

All variables were analysed with a two-sided risk except where stated below.

Body weight gains, Day 20 corrected body weights, food consumption and litter weights were analysed using one-way analysis of variance (ANOVA). Levene's test was used to assess the equality of variances among the groups. Where this showed no evidence of heterogeneity ($p \geq 0.01$), pairwise comparisons with control were made using Dunnett's test. A linear contrast was performed to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was only reported where none of the pairwise comparisons was significant.

Foetal weights, placental weights, the percentage of male fetuses and the numbers of corpora lutea, implantations and fetuses per female were analysed using non-parametric methods. The non-parametric methods employed were the Kruskal-Wallis ANOVA, the Terpstra-Jonckheere test for a dose related trend and the Wilcoxon rank sum test for pairwise comparisons. Where the Kruskal-Wallis ANOVA was not significant, the pairwise comparisons were not reported in order to protect the Type I error.

Gravid uterus weights were analysed using Analysis of Covariance (ANCOVA) and Dunnett's test, using the corrected body weight on day 20 as covariate. This analysis depends on the assumption that the relationship between the organ weight and the covariate is the same for all groups, and the validity of this assumption was tested. Levene's test for equality of variances across the groups was also performed and this showed no evidence of heterogeneity ($p \geq 0.01$).

The proportions of females affected by pre- and post-implantation loss, by early and late intrauterine deaths and the number of litters affected by variations and malformations were analysed using the Cochran-Armitage test for dose-response and Fisher's exact test for pairwise comparisons. The tests were interpreted with one-sided risk for increased incidence with increasing dose. A significant trend ($p < 0.05$) was only reported where none of the pairwise comparisons was significant.

Some variables were not analysed due to all animals having the same value or there being too few distinct values for meaningful analysis.

Findings:

Mortality and clinical signs:

There were no unscheduled deaths during the study, and no treatment-related significant adverse clinical signs were observed. Piloerection was seen in all treated animals from Day 6 of gestation until the next day. On Day 7 of gestation piloerection was seen in all treated animals at half an hour post-dosing and in 2, 3 and 5 animals at doses of 250, 500 and 1000 mg/kg bw/day, respectively, for up to 1 hour post-dosing only. This typical finding in rodents was considered likely to be a reaction to the dosing procedure itself rather than evidence of toxicity.

Body weight, body weight gain and food consumption:

Mean body weight and mean body weight gain throughout the study were unaffected by treatment. There were no treatment-related effects on mean food consumption throughout the study.

Necropsy findings:

There were no significant adverse findings at necropsy.

Uterine/implantation data:

There were no adverse effects observed at examination of the uterus. Mean gravid uterus weight showed no effect of treatment. The number of pregnancies was 6, 7, 6 and 7 in the control, low dose, intermediate dose and high dose groups, respectively. The mean numbers of corpora lutea, implantations and the mean incidence of pre- and post-implantation loss showed no adverse effects of treatment.

The mean litter size was unaffected by treatment.

Foetal data:

There was no effect of treatment on sex ratio, mean litter weight, mean placental weight or mean foetal weight; differences between the groups did not show a dose-related trend and were therefore not considered to be adverse.

In the dose group of 250 mg/kg bw/day, there were two malformed fetuses in two litters; one foetus had a severely reduced orbit of the eye, the other foetus had no patent anal opening. There was one malformed foetus in the dose group of 500 mg/kg bw/day; a severely displaced umbilical opening associated with

absent muscle tissue. In animals dosed at 1000 mg/kg bw/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 foetal variations and malformations.

Table B.6.6.2.1-1: Key findings for dose range finding teratogenicity study in rats

Dose group (mg/kg bw/day)	0	250	500	1000
Number of females mated	7	7	7	7
Non-pregnant	1	0	1	0
Mortality	0	0	0	0
Number of females with live foetuses on Day 20	6	7	6	7
Maternal body weight on Day 20 (g)	306.9	317.1	312.3	310.3
Maternal weight gain from Days 6 to 20 (%)	37.7	43.5	42.9	41.4
Mean number of corpora lutea per female	13.2	12.7	12.8	14.4
Mean number of implantations per female	11.7	11.9	11.8	10.4
Mean number of foetuses	11.0	11.6	11.3	10.4
Mean placental weight (g)	0.44	0.50*	0.45	0.49*
Mean foetal weight (g)	3.64	3.73	3.84	3.67
Total number of foetuses showing malformations (% of foetuses examined)	0 (0)	2 (2.5)	1 (1.5)	0 (0)
Number of litters with external malformations	0/6	2/7	1/6	0/7

* p < 0.05 in comparison to controls

Conclusion:

In a preliminary study designed to investigate doses for further developmental toxicity testing, groups of 7 pregnant rats were administered daily doses of 0 (control), 250, 500 or 1000 mg/kg bw/day S-2200 TG from Days 6 to 19 of gestation. Administration of S-2200 TG by oral gavage to pregnant rats at dose levels up to 1000 mg/kg bw/day elicited no significant adverse effects of maternal or embryo-foetal toxicity. Under the conditions of this preliminary study, the highest dose level tested of 1000 mg/kg bw/day can be considered to be the maternal and developmental no-observed-adverse-effects-level (NOAEL).

Based on the results of this study, a top dose of 1000 mg/kg bw/day was recommended for a subsequent study of prenatal development.

Reference:

S-2200 TG: Oral (Gavage) Prenatal Development Toxicity Study in the Rat
 Author(s), year: [REDACTED] 2012a
 Report/Doc. number: Sumitomo Chemical Co., Ltd. Report No. ROT-0051
 Guideline(s): Japanese MAFF 12 Nousan 8147 Teratogenicity studies (2-1-18), EPA OPPTS 870.3700, OECD TG 414 (2001), EC 2004/73 B.31
 GLP: Yes (laboratory certified by National Authority)
 Deviations: No
 Validity: Yes

Material and methods:

Test Material: S-2200 Technical Grade (S-2200 TG)
 Lot/Batch: ST-0811G

Purity: 93.4%

Test animals:

Species: Rat
Strain: Wistar, [REDACTED]:WI (Han)
Age at mating: 9-10 weeks
Weight at mating: 175.5 - 224.0 g
Source: [REDACTED]
Diet: SQC Rat and Mouse Breeder Diet No 3, Expanded, *ad libitum*

The objective of the study was to assess the effects of S-2200 TG on the pregnant rat and on the embryonic and foetal development when administered orally, by gavage.

Animal assignment and treatment:

On Day 3 of gestation the animals were assigned to four groups (24 animals/group) using a randomisation procedure based on body weight. Each group received dose preparations containing the control article (vehicle) or 100, 300 or 1000 mg/kg bw/day of test article. Treatment group positions in the room were assigned using a set of random number permutations. The test and control articles were administered orally, by gavage, to mated female rats daily from Days 6 to 19 of gestation, inclusive at approximately the same time each day. The females were maintained to Day 20 of gestation when they were killed and examined macroscopically. The foetuses were removed, killed and examined.

Dose preparation, analysis and administration:

Formulations of the test article in the vehicle were prepared weekly and dispensed as daily aliquots. The test article was formulated as a suspension in aqueous 0.5% w/v methylcellulose. The formulations were stored at room temperature in a sealed container. They were stirred on arrival at the animal room and were stirred continuously before and throughout dosing. Stability was analysed in [REDACTED] Study Number [REDACTED] where concentrations of 2 and 250 mg/mL were found to be stable for up to 17 days when stored at room temperature. Homogeneity of samples from formulations prepared for the first and last day of dosing was analysed at [REDACTED]. The mean of the homogeneity results was used as the achieved concentration result. The test and control articles were administered orally, by gavage at a dose volume of 5 mL/kg, to mated female rats daily from Days 6 to 19 of gestation, inclusive, at approximately the same time each day.

Clinical observations:

All animals were examined twice daily to detect dead or moribund animals. All animals were examined at least once daily for signs of ill health or overt toxicity. Any abnormalities of appearance or behaviour or other signs of reaction to treatment were recorded and a detailed individual record was maintained of the clinical condition of each animal on the days of body weight recording. Additionally, animals were observed immediately after dosing and at 0.5 and 1 hours post dose for signs of reaction to treatment.

Body weight and food consumption:

The body weight of each female was recorded on Days 3, 6, 7, 8, 9, 12, 15, 17, 19 and 20 of gestation. Food consumption was recorded for Days 3-5, 6, 7, 8, 9-11, 12-14, 15-16, 17-18 and 19 of gestation.

Sacrifice and pathology:

At the scheduled necropsy on Day 20 of gestation, females were sacrificed by cervical dislocation following isoflurane anaesthesia. Post mortem examination included gross macroscopic examination of all internal organs, with gross lesions retained in relevant fixative.

Uterine/implantation data:

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. Implantations were subdivided into: live foetuses, early intrauterine deaths, late intrauterine deaths and dead foetuses. Early intrauterine deaths were classified as those which showed decidual or placental tissue only. Late intrauterine deaths showed embryonic or foetal tissue in addition to placental tissue. Dead foetuses were classified as those which appeared to have died shortly before necropsy.

The uterus of any apparently non-pregnant female was immersed in a 10% ammonium sulphide solution to reveal any evidence of implantation.

Foetal examination:

Live foetuses were killed by a subcutaneous injection of sodium pentobarbitone and subsequently immersed in fixative.

Individual foetal and placental weights were recorded and foetuses were examined externally and sexed. Approximately one half of the foetuses in each litter, selected by systematic sampling, were dissected and the viscera were examined. They were then eviscerated and the carcasses were processed to stain the ossified skeleton by the Alizarin technique and cartilage processed to stain using Alcian Blue. The skeletons were examined, preserved and stored in glycerol/propylene glycol.

The remaining foetuses were placed in Bouin's solution for at least two weeks to allow fixation and partial decalcification. At examination, the head was removed by a cut through the mouth, pharynx and the back of the head. The coronal sections of the head were examined. The remaining portion of the foetus was examined by dissection and was preserved, with the head sections, in 10% neutral buffered formalin and stored in plastic vials.

The dissection of the foetuses and the examination of the stained skeletons were performed using low power binocular magnification.

Foetal abnormalities were classified as malformations (rare and/or potentially lethal defects) and variations (commonly occurring non-lethal abnormalities).

Statistics:

The control group was taken as the baseline group with which the treated groups were compared. All variables were analysed with a two-sided risk except where stated below.

Body weight gains, Day 20 corrected body weights, food consumption and litter weights were analysed using one-way analysis of variance (ANOVA). Levene's test was used to assess the equality of variances among the groups. Where this showed no evidence of heterogeneity ($p \geq 0.01$), pairwise comparisons with control were made using Dunnett's test. A linear contrast was performed to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was only reported where none of the pairwise comparisons was significant.

Foetal weights, placental weights, the percentage of male foetuses and the numbers of corpora lutea, implantations and foetuses per female were analysed using non-parametric methods. The non-parametric methods employed were the Kruskal-Wallis ANOVA, the Terpstra-Jonckheere test for a dose related trend and the Wilcoxon rank sum test for pairwise comparisons. Where the Kruskal-Wallis ANOVA was not significant, the pairwise comparisons were not reported in order to protect the Type I error.

Gravid uterus weights were analysed using Analysis of Covariance (ANCOVA) and Dunnett's test, using the corrected body weight on Day 20 as covariate. This analysis depends on the assumption that the

relationship between the organ weight and the covariate is the same for all groups, and the validity of this assumption was tested. Levene's test for equality of variances across the groups was also performed and this showed no evidence of heterogeneity ($p \geq 0.01$).

The proportions of females affected by pre- and post-implantation loss, by early and late intrauterine deaths and the number of litters affected by variations and malformations were analysed using the Cochran-Armitage test for dose-response and Fisher's exact test for pairwise comparisons. The tests were interpreted with one-sided risk for increased incidence with increasing dose. A significant trend ($p < 0.05$) was only reported where none of the pairwise comparisons was significant.

Some variables were not analysed due to all animals having the same value or there being too few distinct values for meaningful analysis.

The percentage of foetuses in each litter affected by variations and malformations were also analysed using the non-parametric methods, as previously described.

Findings:

Mortality and clinical signs:

There were no unscheduled deaths during the study.

Clinical observations were unremarkable and showed no dose-related trend. The postdose observation mouth rubbing (and very occasionally salivation) was seen at 300 and 1000 mg/kg bw/day only immediately after dosing, but not at 0.5 and 1 hour post-dose. This postdose observation was seen with increasing frequency at 300 mg/kg bw/day from Day 9 of gestation onwards and at 1000 mg/kg/day from Day 7 of gestation onwards. This typical finding in rodents commonly observed in oral (gavage) prenatal developmental toxicity studies was considered likely to be due to taste aversion rather than evidence of toxicity. Two animals receiving 1000 mg/kg bw/day were thin on Day 20 of gestation and this finding was confirmed at necropsy.

Body weight, body weight gain and food consumption:

Group mean body weight gain and mean gravid uterus weight adjusted for body weight were both unaffected by treatment.

Over the first 3 days of dosing only mean food consumption at 1000 mg/kg/day was very slightly lower than control and this was statistically significant ($p < 0.05$, $p < 0.01$, $p < 0.05$ on Days 6, 7 and 8 of gestation, respectively). Over the entire dose period, mean food consumption was similar in all groups.

Necropsy findings:

Two animals receiving 1000 mg/kg bw/day were thin on Day 20 of gestation and this finding was confirmed at necropsy. Other findings at necropsy were typical for this strain of rat at these laboratories and showed no dose relationship and therefore are viewed as non-adverse.

Uterine/implantation data:

The number of pregnancies was 24, 23, 24 and 24 in the 0, 100, 300 and 1000 mg/kg bw/day dose groups respectively. The mean numbers of corpora lutea, implantations and the mean incidence of pre- and post-implantation loss showed no adverse effect of treatment. The number of dams with early intrauterine deaths or post-implantation loss at 1000 mg/kg bw/day showed an apparent statistical significance, however both mean number of early intrauterine deaths and mean percentage of post-implantation loss were within the background range and showed no dose relationship or statistical significance. Therefore, this was not considered to be biologically significant.

Foetal data:

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

Malformations were noted in one foetus in the group receiving 300 mg/kg bw/day and in five foetuses from five litters in the group receiving 1000 mg/kg bw/day (detailed in table B.6.6.2.1-2). The incidence and intergroup distribution of these foetal malformations do not indicate an adverse effect of treatment because there was no statistical significance or dose-related increase for the individual specific lesions. It is not considered appropriate to combine these diverse types of malformation for analysis. Overall, there was no adverse effect of treatment on the incidence of foetal variations and malformations, which were all within, or close to, expected ranges for this strain of rat.

Table B.6.6.2.1-2: Key findings of the teratogenicity study in rats

Dose (mg/kg bw/day)	0	100	300	1000
Maternal bodyweight (g) on Day 20	303.2	300.2	300.9	300.4
Maternal weight gain, Days 6-20 (%)	41.4	39.2	43.3	38.4
Food consumption, Day 6 (g/rat/day)	19.1	17.8	18.6	16.7*
Food consumption, Day 7 (g/rat/day)	20.5	19.2	19.4	17.7**
Food consumption, Day 8 (g/rat/day)	20.2	19.7	20.3	17.7*
Food consumption, Days 6-18 (g/rat/day)	21.4	20.8	21.0	20.5
Number of females mated	24	24	24	24
Non-pregnant	0	1	0	0
Pregnant (%)	24 (100)	23 (95.8)	24 (100)	24 (100)
Number of females with live foetuses	24	23	24	24
Mean number of early intrauterine deaths (number of dams affected)	0.3 (7)	0.8 (10)	0.4 (8)	0.7 (14*)
% Post-implantation loss (number of dams affected)	2.8 (7)	8.6 (10)	4.4 (10)	6.9 (14*)
Mean litter size	10.5	10.0	11.2	10.0
Mean foetal weight (g)	3.75	3.77	3.77	3.83
Mean placenta weight (g)	0.48	0.5	0.48	0.5
Sex ratio (% of male)	50.0	47.3	53.8	55.6
External or visceral malformations: number of foetuses affected (% of foetuses) / litter incidence	0	0	0	2 (0.7)/2
<ul style="list-style-type: none"> Kidney, severely increased pelvic cavitation 	0	0	0	2
External or visceral variations: number of foetuses affected (% of foetuses) / litter incidence	77 (30.9)/24	78 (35.7)/23	103 (37.4)/24	101 (42.4)/24
Skeletal malformations: number of foetuses affected (% of foetuses) / litter incidence	0	0	1 (0.8)/1	3 (2.9)/3
<ul style="list-style-type: none"> Rib cartilage shortened 	0	0	0	1
<ul style="list-style-type: none"> Sternebrae, cleft xiphoid cartilage 	0	0	0	1

• Vertebral cervical arch and centrum, additional ossification site fused	0	0	1	0
• Vertebral cervical arch, additional cartilaginous ventral plate fused	0	0	0	1
Skeletal variations: number of fetuses affected (% of fetuses) / litter incidence	122 (94.8)/24	108 (93.7)/23	128 (96.4)/24	120 (99.2)/24
Total number of fetuses with malformations, n (%)	0	0	1 (0.4)	5 (2.1)
Litter incidence	0	0	1	5*

* p<0.05, ** p<0.01, *** p<0.001

Conclusion:

Groups of 24 presumed-pregnant female rats were administered S-2200 TG at dose levels of 0, 100, 300, and 1000 mg/kg bw/day from Days 6 to 19 of gestation. All animals survived to the scheduled sacrifice on Day 20 and there were no treatment-related clinical signs or necropsy findings. Mean food consumption at 1000 mg/kg bw/day was slightly reduced over the first 3 days of dosing only. 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. Sex ratio, mean litter weight, mean placental weight and mean foetal weight were all unaffected by treatment and there was no adverse effect of treatment on the incidences of foetal variations or malformations at any dose level tested.

Based on these results, both the maternal no-observed-adverse-effect-level (NOAEL) and the embryo-foetal NOAEL were set at 1000 mg/kg bw/day.

B.6.6.2.2 Teratogenicity test by the oral route in the rabbit

Reference:	S-2200 TG: Oral (Gavage) Range-Finding Study of Prenatal Development in the Rabbits
Author(s), year:	2009b
Report/Doc. number:	Sumitomo Chemical Co. Ltd. Report No. ROT-0008
Guideline(s):	None stated (range finding study)
GLP:	No
Deviations:	Supplementary study
Validity:	Yes

Material and methods:

Test Material:	S-2200 TG
Lot/Batch:	ST-0811G (Batch Specification 01)
Purity:	93.4%
Test animals:	
Species:	Rabbit
Strain:	New Zealand White (Hsd:IfNZW)
Age at receipt:	18-22 weeks at mating
Weight:	3.10 - 3.90 kg at mating

Source:	██
Diet:	Pelleted Harlan Teklad 2930C Rabbit Diet (Harlan Teklad, Madison, USA); available <i>ad libitum</i> .
Acclimation:	Time-mated by the supplier. Day of mating designated day 0 of gestation. Animals were delivered to the test laboratory on day 3 of gestation. On arrival the animals were examined and found to be in good health. An inspection by a veterinary surgeon before the start of treatment confirmed their suitability for experimental use.

The purpose of the study was to select proper dose levels of mandestrobin for the main developmental study in rabbits.

Animal assignment and treatment:

On the day of arrival the time-mated and presumed pregnant animals were assigned to treatment groups each of 7 animals using a randomisation procedure based on body weight. The day of insemination was designated day 0 of gestation. Rabbits were dosed once each day during days 7 to 28 of gestation. The dose volume was 5 mL/kg.

Diet preparation, analysis and administration:

S-2200 TG was administered as a suspension in a 0.5% w/v aqueous methylcellulose solution, at dose levels of 0, 250, 500, and 1000 mg/kg bw/day. The test article was formulated as suspension in aqueous 0.5% (w/v) methylcellulose. Solutions of the test article in the vehicle were prepared daily.

Presumed-pregnant female rabbits were administered S-2200 TG by gavage at a dose volume of 5 mL/kg once daily during days 7 to 28 of gestation, and sacrificed on day 29 of pregnancy for macroscopic examination and examination of litters. Dose volumes were determined on the basis of the most recent bodyweight, determined daily. Control animals received 0.5% w/v methylcellulose solution.

Statistics:

Body weight gains, day 29 corrected body weights, food consumption and litter weights were analysed using one-way analysis of variance (ANOVA). Levene's test was performed to assess the equality of variances among the groups. Where this showed no evidence of heterogeneity ($p \geq 0.01$), pairwise comparisons with control were made using Dunnett's test. A linear contrast was performed to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was only reported where none of the pairwise comparisons was significant. Foetal weights, placental weights, the percentage of male foetuses and the numbers of corpora lutea, implantations and foetuses per female were analysed using nonparametric methods. The non-parametric methods employed were the Kruskal-Wallis ANOVA, the Terpstra-Jonckheere test for a dose related trend and the Wilcoxon rank sum test for pairwise comparisons. Where the Kruskal-Wallis ANOVA was not significant, the pairwise comparisons were not reported in order to protect the Type I error. Gravid uterus weights were analysed using Analysis of Covariance (ANCOVA) and Dunnett's test, using the corrected body weight on day 29 as covariate. This analysis depends on the assumption that the relationship between the organ weight and the covariate is the same for all groups, and the validity of this assumption was tested. Levene's test for equality of variances across the groups was also performed and this showed no evidence of heterogeneity ($p \geq 0.01$). The proportions of females affected by pre- and post-implantation loss and by early and late intrauterine deaths and the number of litters affected by variations and malformations were analysed using the Cochran-Armitage test for dose-response and Fisher's exact test for pairwise comparisons. The tests were interpreted with one-sided risk for increased incidence with increasing dose. A significant trend ($p < 0.05$) was only reported where none of the pairwise comparisons was significant.

Clinical observations:

All animals were checked twice daily for any mortalities. All animals were observed at least once daily for signs of reaction to treatment and/or symptoms of ill health. Additionally, animals were observed immediately after dosing and at 0.5, 1, 2 and 4 hours post dose.

Food consumption and body weight:

Individual food consumption was recorded daily from day 3 to 29 of gestation. Body weights were measured on days 3, 7, 8, 9, 12, 15, 17, 19, 22, 25, 28 and 29 of gestation.

Sacrifice and pathology:

At the scheduled necropsy on day 29 of gestation, females were sacrificed by intravenous injection of sodium pentobarbitone solution. Post mortem examination included gross macroscopic examination of all internal organs, with emphasis on the ovaries, uterus and foetus. The ovaries and uteri were removed and examined and the following data recorded: pregnancy status, gravid uterus weight, number of corpora lutea and the number and intrauterine position of implantations.

The uterus of any apparently non-pregnant female was immersed in a 10% ammonium sulphide solution to reveal any evidence of implantation.

Foetal examination:

Live foetuses were killed by intraperitoneal injection of sodium pentobarbitone solution. Individual foetal and placental weights were recorded and foetuses were examined externally and sexed by internal gonadal inspection.

The foetuses were stored in 10% neutral-buffered formalin.

Findings:

Clinical signs and mortality:

There were no post-dosing observations or treatment-related clinical observations.

One animal of the 1000 mg/kg/day dose group (animal no. 25) presented the clinical observation 'head tilt'. This animal was further examined by a veterinary surgeon and no infection or other health problems were found. This finding was not considered to be treatment-related.

There were no treatment-related deaths during the study.

One animal was found dead on day 5 of gestation, necropsy revealed findings fairly common in this strain of rabbit. The death of this animal resulted in the control group having 6 animals, rather than 7.

Food consumption, body weight and body weight gain:

Mean body weight, mean body weight gain and mean gravid uterus weight were unaffected by treatment.

A statistically significant dose-response ($p < 0.05$) in body weight loss among animals treated at 500 and 1000 mg/kg bw/day was observed on the first day of dosing. However, this was considered not to be treatment-related due to the body weight loss seen prior to treatment in these groups.

Mean food consumption at the high dose level was very slightly lower than controls throughout the study, but was only statistically significant as a dose-response on the first day of dosing ($p < 0.05$).

Sacrifice and pathology:

There were no dose-related gross necropsy findings.

Terminal necropsy on day 29 of pregnancy found a large placenta in one animal of the high dose group. In the absence of any other observations this isolated finding was not considered to be related to treatment.

The number of pregnancies was 5, 6, 7 and 6 in the control, 250, 500 and 1000 mg/kg bw/day groups, from group sizes of 6, 7, 7, and 7, respectively. The mean numbers of corpora lutea, implantations and the mean incidence of pre- and post-implantation loss showed no treatment-related adverse effects. Mean litter size was unaffected by treatment.

Foetal data:

There was no effect of treatment on sex ratio, mean litter weight or mean placental weight. In the high dose group mean foetal weight was slightly, but not significantly lower than the controls and this was considered to be a result of higher litter size at this dose level.

There was no effect of treatment on the mean incidence of external foetal variations and malformations. In the high dose group there was one malformed foetus 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 authors.

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

Table B.6.6.2.2-1: Results of the preliminary developmental study in rabbit

Dose (mg/kg bw/day)	0	250	500	1000
Number of females inseminated	6	7	7	7
Mean food consumption: Day 7 (g/animal/day)*	150	126	99	95
Mean food consumption: Day 28 (g/animal/day)	140	122	133	118
Non-pregnant	1	1	0	1
Pregnant does dead or moribund	0	0	0	0
Abortion/ premature delivery	0	0	0	0
Number of litters for evaluation	5	6	7	6
Maternal bodyweight: Day 7 (kg)	3.54	3.56	3.53	3.50
Maternal bodyweight: Day 29 (kg)	3.92	3.97	3.87	3.85
Number of implantations/doe	7.0	8.7	6.3	10.2
Mean litter size (live foetuses)	6.6	7.2	5.9	9.0
Number of foetuses examined	33	43	41	54
Number of litters examined	5	6	7	6
Number of foetuses showing malformations	0	0	0	1
Mean % of foetuses examined	0.0	0.0	0.0	1.3
Number of litters affected	0	0	0	1

Number of fetuses showing variations	0	0	1	5
Mean % of fetuses examined	0.0	0.0	1.3	8.3
Number of litters affected	0	0	1	3

*: Significant dose response test ($p < 0.05$).

Conclusion:

Small groups of presumed-pregnant female rabbits were administered S-2200 TG at doses of 0, 250, 500 or 1000 mg/kg bw/day as a suspension in 0.5% w/v aqueous methylcellulose solution from days 7 to 29 of pregnancy, sacrificed shortly before term, and uterine contents examined for indications of effects on development.

Dose-related reduced food consumption was seen at the top dose level (1000 mg/kg bw/day), which was statistically significant as a dose-response only on the first day of dosing. Otherwise, no effect on progression of pregnancy or on development of the foetus was detected.

One foetus in the high dose group showed Spina bifida, severely malformed head structures and severely flexed forelimb wrist joints. This was an isolated finding and was not considered to be treatment-related.

Based on these results, both the maternal and the developmental no-observed-adverse-effect-level (NOAEL) were set at 1000 mg/kg bw/day.

On the basis of this study, a limit dose level of 1000 mg/kg bw/day was recommended as an appropriate high dose level for a further study.

Reference:	S-2200 TG: Oral (Gavage) Prenatal Development Toxicity Study in the Rabbit. Covance Laboratories Ltd. Study No. 8202046
Author(s), year:	2012b
Report/Doc. number:	Sumitomo Chemical Co. Ltd. Report No. ROT-0052
Guideline(s):	Japanese MAFF 12 Nousan 8147, EPA OPPTS 870.3700, OECD 414
GLP:	Yes (lab certified by National Authority)
Deviations:	None
Validity:	Yes

Material and methods:

Test Material:	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G
Purity:	93.4%
Expiry date:	21 November 2011 (after completion of treatment)
Vehicle:	0.5% w/v aqueous methylcellulose

Test animals:

Species:	Rabbit (time mated)
Strain:	New Zealand White (Hsd:IfNZW)
Age at receipt:	4-5 months at time of mating, received time mated
Weight:	2.6 – 4.05 kg at time of mating
Source:	
Diet:	Harlan Teklad 2930C Rabbit Diet, Pelleted, (Harlan Teklad, Madison, USA) <i>ad libitum</i>
Acclimation period:	Time mated rabbits received day 1 or 2 of gestation (day of mating = day 0).

Housing: First dose administered on day 7 of gestation
Individually

The purpose of the study was to detect adverse effects of S-2200 TG on pregnant rabbits and on the development of embryos and the foetuses consequent to exposure of the females to the test substance (0, 100, 300, and 1000 mg/kg bw/d).

Animal assignment and treatment:

On the day of arrival the time-mated and presumed pregnant animals were assigned to treatment groups each of 24 animals using a randomisation procedure based on day of gestation and body weight.

The day of insemination was designated day 0 of gestation. Rabbits were dosed once each day during days 7 to 28 of gestation. The dose volume was 5 mL/kg.

The high dose level (1000 mg/kg/day) was selected on the basis of the results of a range-finding study (Covance study number 8202040).

Table B.6.6.2.2-2: Study design of the developmental study in rabbits

Group	Dose level (mg/kg bw/day)	No. Females
1	0	24
2 (low)	100	24
3 (mid)	300	24
4 (high)	1000	24

Diet preparation, analysis and administration:

Formulations of the test article in the vehicle were prepared weekly and dispensed as daily aliquots. Stability analyses were performed at the test laboratory where concentrations of 2 and 250 mg/mL were found to be stable for up to 17 days when stored at room temperature. Homogeneity was determined from formulations prepared for the first and last day of dosing.

Animals were administered the test material by gavage, once per day on days 7 – 28 of pregnancy. Control animals received 0.5% aqueous methyl cellulose.

Statistics:

Body weight gains, day 29 corrected body weights, food consumption and litter weights were analysed using one-way analysis of variance (ANOVA). Levene's test for equality of variances among the groups was performed. Where this showed no evidence of heterogeneity ($p \geq 0.01$), pairwise comparisons with control were made using Dunnett's test. A linear contrast was performed to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was only reported where none of the pairwise comparisons was significant.

Where Levene's test showed evidence of heterogeneity ($p < 0.01$), the data were analysed using the same methods after applying a log-transformation.

Foetal weights, placental weights, the percentage of male foetuses and the numbers of corpora lutea, implantations and foetuses per female were analysed using nonparametric methods. The non-parametric methods employed were the Kruskal-Wallis ANOVA, the Terpstra-Jonckheere test for a dose related trend and the Wilcoxon rank sum test for pairwise comparisons. Where the Kruskal-Wallis ANOVA was not significant, the pairwise comparisons were not reported in order to protect the Type I error.

Gravid uterus weights were analysed using Analysis of Covariance (ANCOVA) and Dunnett's test, using the

corrected body weight on day 29 as covariate. This analysis depends on the assumption that the relationship between the organ weight and the covariate is the same for all groups, and the validity of this assumption was tested. Levene's test for equality of variances across the groups was also performed. Where this showed evidence of heterogeneity ($p < 0.01$), the organ was analysed using one-way ANOVA on absolute organ weights and organ to necropsy body weight ratios.

The proportions of females affected by pre- and post-implantation loss, by early and late intrauterine deaths, the proportion with one or more dead fetuses and the number of litters affected by variations and malformations were analysed using the Cochran-Armitage test for dose-response and Fisher's exact test for pairwise comparisons. The tests were interpreted with one-sided risk for increased incidence with increasing dose. A significant trend ($p < 0.05$) was only reported where none of the pairwise comparisons was significant.

The percentage of fetuses in each litter affected by variations and malformations were also analysed using non-parametric methods, as previously described.

Clinical observations:

All animals were examined at least once daily for signs of ill health or overt toxicity. Any abnormalities of appearance or behaviour or other signs of reaction to treatment or ill health were recorded. An individual record was maintained of the clinical condition of each animal on each day from days 3 to 29 of gestation. On occasion, minor observations were recorded for the cage tray liner, and these were not reported.

In addition, the animals were observed immediately upon return to the home cage after dosing and at approximately 0.5 and 1 hour post dose.

Food consumption and body weight:

Individual food consumption was recorded daily from day 3 to 29 of gestation. The body weight of each female was recorded on days 3, 7, 8, 9, 12, 15, 17, 19, 22, 25, 28 and 29 of gestation.

Sacrifice and pathology:

All surviving females were sacrificed on gestation day 29, by i.v. injection of sodium pentobarbitone.

Once death had been confirmed, major blood vessels were severed to exsanguinate the animal. Animals were killed in cage order and examined macroscopically. Gross lesions were retained in 10% neutral buffered formalin.

The ovaries and uteri were removed and examined and the following data recorded:

pregnancy status, gravid uterus weight, number of corpora lutea, number and intrauterine position of implantations (subdivided into: live fetuses, early intrauterine deaths, late intrauterine deaths, dead fetuses)

Early intrauterine deaths were classified as those which showed decidual or placental tissue only. The uterus of any apparently non-pregnant female was immersed in 10% ammonium sulphide solution to reveal any evidence of implantation.

Foetal examination:

Live fetuses were killed by an intraperitoneal injection of sodium pentobarbitone solution. Individual foetal and placental weights were recorded and fetuses were examined externally.

Approximately one half of the fetuses in each litter (selected by systematic sampling) were decapitated by a cut through the neck at the base of the skull. The heads were placed in Bouin's solution, for fixation and partial decalcification. Serial sections were examined and were preserved in 10% neutral buffered formalin (NBF).

The hearts of approximately one half of the foetuses in each litter (selected by systematic sampling) were placed in Bouin's solution, for fixation. Several coronal slices of each heart were made to reveal the internal structure. These heart sections were examined and then preserved in NBF.

All foetuses were dissected, sexed and the viscera examined. They were then eviscerated and the carcasses fixed in 70% industrial methylated spirits.

Each carcass was processed to stain the ossified skeleton by the Alizarin technique and the skeletons were examined, including a basic evaluation of cartilage. They were preserved in glycerol/propylene glycol.

Foetal abnormalities were classified as malformations (rare and/or potentially lethal) and variations (commonly occurring non-lethal abnormalities).

Findings:

Clinical signs and mortality:

There were no unscheduled deaths during the study.

There were no post-dose observations and no significant findings seen at necropsy.

Clinical observations were generally unremarkable and showed no dose-related trend. Animal number 43 receiving 100 mg/kg/day was recorded as being thin from Days 7 to 15 of gestation.

Animal number 62, receiving 300 mg/kg/day aborted its pregnancy on Day 20 of gestation. At necropsy, this animal was found to have dark foci on its lung. This abortion was not considered to be an effect of treatment due to it being in one animal only and not seen at the high dose level of 1000 mg/kg bw/day.

Food consumption, body weight and body weight gain:

Group mean body weight gain during the study was highly variable with no marked adverse effect seen. During the dose period mean body weight gain was slightly reduced compared to controls at all dose levels, although not in a dose-proportional manner and this showed apparent statistical significance as a dose-response over Days 7 to 8 and Days 17 to 19 of gestation (both $P < 0.05$). Mean body weight gain after dosing ceased was slightly lower than controls at 300 and 1000 mg/kg/day, and this was statistically significant (dose response, $p < 0.05$).

Gravid uterus weight adjusted for body weight, was unaffected by treatment.

Over the dose period, mean food intake at all dose levels was slightly lower than control and this was statistically significant at 300 and 1000 mg/kg bw/day ($p < 0.01$, $p < 0.05$, respectively). However, in the pre-dose period, food intake at 1000 mg/kg/day was also slightly lower than controls, and the difference was comparable with that during the treatment phase. Mean food intake after dosing ceased was lower than controls at all dose levels, statistically significant at 300 and 1000 mg/kg bw/day ($p < 0.05$, $p < 0.01$ respectively). These slight differences from control are considered unlikely to be indicative of significant systemic toxicity to the pregnant rabbit.

Caesarean data:

Uterine/implantation data:

The number of pregnancies was 23, 23, 22 and 18, and there were 23, 23, 21 and 17 surviving females with litters on Day 29 of gestation in the control, low, intermediate and high dose groups respectively. The distribution of non-pregnant animals in the high dose group (6) was considered a chance event by the authors, and unrelated to treatment, as any increase in very early post-implantation 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 (Developmental and Reproductive Toxicology- A Practical Approach, Second Edition edited by Ronald D Hood, Taylor and Francis 2006, p163).

One animal receiving 1000 mg/kg bw/day (number 93) had total embryo-foetal loss; this isolated incidence was not considered to be an effect of treatment by the study authors. The mean numbers of corpora lutea,

implantations and the mean incidence of pre- and post-implantation loss showed no effect of treatment. The number of dams with post-implantation loss at 100 mg/kg bw/day showed an apparent statistical significance, however all data were within expected ranges and this was not considered to be biologically significant.

Mean litter size was unaffected by treatment.

Table B.6.6.2.2-3: Uterine/ implantation data

Dose (mg/kg bw/day)	0	100	300	1000*	HCD range**
Mean number of corpora lutea	10.3	10.1	10.0	9.7 (9.4)	10.0 - 11.9
Mean number of implantations	8.9	9.4	8.8	8.8 (8.4)	7.6 - 9.4
Pre-implantation loss (%)	15.0	7.7	12.9	9.1 (13.0)	9.5 – 27.6
Intrauterine deaths early	0.6	0.9	0.8	0.9 (0.9)	0.4 – 0.8
Intrauterine deaths late	0.2	0.3	0.2	0.3 (0.3)	0.1 – 0.3
Dead fetuses	0.0	0.0	0.0	0.1 (0.1)	0.0
Post implantation loss (%)	8.7	13.6	11.0	13.3 (18.1)	7.1 – 14.2
Live fetuses	8.0	8.2	7.8	7.5 (7.1)	6.9 – 8.7

*The animal with total embryo-foetal loss was excluded from the analysis; values in brackets contain this doe in the analysis
 **Historical control data was derived from 6 embryo foetal studies performed with New Zealand White rabbits at Covance from April 2004

Foetal data:

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

Malformations were noted in eight foetuses from six litters in the control group, six foetuses from four litters in the group receiving 100 mg/kg bw/day, nine foetuses from seven litters in the group receiving 300 mg/kg bw/day and three foetuses from three litters in the group receiving 1000 mg/kg bw/day.

Table B.6.6.2.2-34: Necropsy findings in the developmental study in rabbit

Dose (mg/kg bw/day)	0	100	300	1000	HCD*
Number of females inseminated	24	24	24	24	-
Non-pregnant	1	1	2	6	-
Pregnant (%)	23 (95.8)	23 (95.8)	22 (91.7)	18 (75.0)	(83.3 – 100)
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	-
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 & foetal death (%)	8.7	13.6	11.0	13.3	-
Mean litter size (live foetuses)	8.0	8.2	7.8	7.5	6.9 – 8.7
Mean foetal weight (g): Male	44.4	43.9	43.0	45.9	-
Female	45.1	43.4	41.7	45.4	-
Sex ratio (% of male)	49.1	50.3	57.6	50.0	45.1 – 50.7

Foetuses with external or visceral malformations, n (%)	7 (3.3)	4 (1.5)	2 (1.2)	2 (1.7)	(2.6)
Litter incidence	6/23	3/23	2/21	2/17	-
Foetuses with external or visceral Variations, n (%)	98 (55.3)	85 (46.0)	80 (49.4)	54 (43.0)	(48.7)
Litter incidence	22/23	23/23	21/21	15/17	-
Foetuses with skeletal malformation n (%)	3 (1.5)	4 (1.7)	7 (4.9)	2 (1.6)	(2.7)
Litter incidence	2/23	3/23	6/21	2/17	-
Foetuses with skeletal variations n (%)	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 foetuses with malformations, n (%)	8 (4.3)	6 (3.2)	9 (5.5)	3 (2.3)	-
Litter incidence	6/23	4/23	7/21	3/17	-
* Historical control data was derived from 6 embryo foetal studies performed with New Zealand White rabbits at Covance from April 2004					

Overall, there was no effect of treatment on the incidence of foetal variations and malformations, which were all within expected ranges for this strain of rabbit.

Conclusion:

In a developmental toxicity study, groups of 24 presumed-pregnant female rabbits were administered S-2200 TG at doses of 0, 100, 300 or 1000 mg/kg bw/day by gavage from days 7 to 28 of pregnancy.

There were no significant adverse post-dosing observations, and no significant treatment-related clinical signs or necropsy findings. Mean food consumption and mean body weight gain at all dose levels were variable and generally were slightly reduced compared to controls during the dosing period, and upto necropsy. However, these slight differences were very small in magnitude and are considered unlikely to represent significant systemic toxicity.

One animal receiving 300 mg/kg bw/day aborted its pregnancy on day 20 of gestation with no significant clinical signs or necropsy findings, and was not considered to be treatment-related. There were no other unscheduled deaths during the study.

Mean gravid uterus weight adjusted for body weight, was unaffected by treatment. There was no effect of treatment on mean uterine/implantation data.

Sex ratio, mean litter weight, mean placental weight and mean foetal weight were all unaffected by treatment and there was no effect of treatment on the incidences of foetal variations or malformations.

In conclusion, administration of S-2200 TG by oral gavage to pregnant rabbits elicited no systemic toxicity to maternal female rabbits. There was no evidence of embryotoxicity or developmental effects at any dose level tested. Under the conditions of this study, both the maternal and the embryo-foetal no-observed-adverse-effect-level (NOAEL) were set at 1000 mg/kg bw/day.

B.6.6.3 Summary of reproductive toxicity

Mandestrobin was examined in a two generation reproductive toxicity study in the rat and in teratogenicity studies in the rat and rabbit.

In the 2-generation study (doses of 1000, 3000, and 10000 ppm via diet), mandestrobin showed no evidence of an effect on fertility or reproductive function, resulting in a NOAEL for the reproductive effects at the highest dose level tested in this study of 10000 ppm (559 mg/kg bw/day).

Regarding the general toxicological effects on parental animals, suppressed body weight gain and reduced food consumption were noted in males and in females in the 10000 ppm group in both F0 and F1 generations.

Pathological examination revealed treatment-related changes in the liver of both sexes. At necropsy, dark brownish change and enlargement of the liver were noted in F0 and F1 females in the 10000 ppm group. Liver weights increased in males at ≥ 3000 ppm and in females at ≥ 1000 ppm in both generations. In the histopathological examination the following findings were observed: Brown pigment in the bile duct/periportal area (in F0 animals at 10000 ppm and in F1 animals at ≥ 3000 ppm), focal periductular inflammatory cell infiltration (in F0 males and females and F1 males at 10000 ppm and in F1 females at ≥ 3000 ppm), and brown pigment deposition in the peribulbar hepatocyte and proliferation of the bile duct (in F0 and F1 females in the 10000 ppm group). Diffuse hypertrophy of hepatocytes was also observed in males at ≥ 3000 ppm and in females at ≥ 1000 ppm in both generations. In the 1000 ppm group, only hepatocellular hypertrophy and increased liver weights were observed in F0 and F1 females, without any other change. Therefore, the changes in the liver observed in females in the 1000 ppm group were considered to be an adaptive change, and of no toxicological significance.

Furthermore, increases in the thyroid weights were observed in F0 males at ≥ 3000 ppm and in F1 males at 10000 ppm, and hypertrophy of the follicular cell of the thyroid was observed in some F0 males in the 10000 ppm group, which was considered to be secondary to the increased in hormonal turnover and the changes in the liver.

Treatment-related hypertrophy of cortical cells in the fascicular zone was observed in the adrenals in some F1 females in the 10000 ppm group. In addition, decreases were observed in ovary weights in F0 and F1 females and uterus weights in F0 females in the 10000 ppm group, however, in absence of any histopathological changes.

Furthermore, greenish change in the cortex of the kidney was observed in some F0 females at necropsy in the 10000 ppm group, with increased organ weights but without any histopathological changes. Therefore, the changes in the kidney observed in F0 females were considered to be of no toxicological significance.

Based on the adverse liver effects observed at dose levels of 3000 ppm and above, the parental NOAEL is considered to be 1000 ppm (43 mg/kg bw/d).

Regarding the effects on offspring, postnatal body weight gain was suppressed in both sexes of F1 and F2 offspring in the 10000 ppm group, and was probably due to undernourishment of their dams.

In addition, lower spleen weights at weaning were noted in F1 males in the 3000 ppm group, in which no change was found in postnatal body weight gain, and in all F1 and F2 animals at 10000 ppm. The lower spleen weights of F1 animals at weaning completely recovered to the control level at adulthood in both sexes, even in the 10000 ppm group, suggesting a transient retardation in growth. Moreover, both the absolute and relative spleen weights of F1 and F2 animals were within the historical control range in the test facility, indicating slight changes.

A slight delay in sexual maturation was found in both sexes (mean difference from the control group: 1.5 days for vaginal opening in F1 females and 1.6 days for preputial separation in F1 males) in the 10000 ppm group, which was considered to be related to the growth retardation.

Under the conditions of this study, the NOAEL for effects on offspring is considered to be 1000 ppm (56 mg/kg bw/d).

In a gavage developmental toxicity study, administration of 0, 100, 300, and 1000 mg/kg bw/day from Days 6 to 19 of gestation to pregnant rats elicited no treatment-related adverse effects up to the highest dose level tested. Therefore, both the maternal and the foetal NOAEL were set at 1000 mg/kg bw/day

Administration of mandestrobin by oral gavage to pregnant rabbits at doses of 0, 100, 300 or 1000 mg/kg bw/day from Days 7 to 28 of pregnancy elicited no systemic toxicity to maternal female rabbits. There was no

evidence of embryotoxicity or developmental effects at any dose level tested. Under the conditions of this study, both the maternal and the foetal NOAEL were set at 1000 mg/kg bw/day.

Based on the results of one 2-generation reproduction toxicity study in the rat and two developmental studies in rat and rabbit, no classification and labelling is triggered for mandestrobin.

Table B.6.6.3-1: Summary of reproductive toxicity studies with mandestrobin

Type of study reference	Concentrations tested	NOAEL (mg/kg bw/d)		Adverse effects / target organs at LOAEL
Dose range-finding study for Two- generation reproduction toxicity study (rat) [REDACTED] 2010	0, 5000, 10000, 20000 ppm equivalent to: 0, 244 – 782, 499 – 1429, 1033 – 2441 mg/kg bw/d (rounded)	Females: 317 (5000 ppm)	Parents	Females: <ul style="list-style-type: none"> Decreased bw, bw gain and food consumption Liver weight increase, Brown pigment in bile duct/perilobular hepatocytes, Inflammatory cell infiltration in periductular region Decreased vacuolation in the interstitial gland in the ovary Decreased uterus weight and atrophy of the uterus Males: <ul style="list-style-type: none"> Liver weight increase greater than 20%
		317 (5000 ppm)	Offspring	Suppressed body weight and body weight gain Reduced spleen weight
		1229 (20000 ppm)	Repro- duction	No effects at highest dose tested
Two-generation reproduction toxicity study (rat) [REDACTED] 2012	0, 1000, 3000, 10000 ppm equivalent to: 0, 43 – 163, 132 – 511, 452 – 1688 mg/kg bw/d (rounded)	43 (1000 ppm)	Parents	Liver: Increased liver weight and diffuse hepatocellular hypertrophy Brown pigment in bile duct/periportal area Focal periductular inflammatory cell infiltration Thyroid: Increased thyroid weight (males)
		56 (1000 ppm)	Offspring	Lower spleen weights at weaning (males)
		559 (10000 ppm)	Repro- duction	No effects at highest dose tested
Developmental toxicity range-finding study (rat) [REDACTED] 2009a	0, 250, 500, 1000 mg/kg bw/d	1000	Maternal	No treatment-related effects at highest dose tested
		1000	Foetal	No treatment-related effects at highest dose tested

Type of study reference	Concentrations tested	NOAEL (mg/kg bw/d)		Adverse effects / target organs at LOAEL
Developmental toxicity study (rat) [REDACTED] 2012a	0, 100, 300, 1000 mg/kg bw/d	1000	Maternal	No treatment-related effects at highest dose tested
		1000	Foetal	No treatment-related effects at highest dose tested
Developmental toxicity range finding study (rabbit) [REDACTED] 2009b	0, 250, 500, 1000 mg/kg bw/d	1000	Maternal	No treatment-related effects at highest dose tested
		1000	Foetal	No treatment-related effects at highest dose tested
Developmental toxicity study (rabbit) [REDACTED] 2012b	0, 100, 300, 1000 mg/kg bw/d	1000	Maternal	No treatment-related effects at highest dose tested
		1000	Foetal	No treatment-related effects at highest dose tested

B.6.7 Neurotoxicity (Annex IIA 5.7)

B.6.7.1 Acute neurotoxicity – rat

Reference:	An Oral (Gavage) Dose Range-Finding Acute Neurotoxicity Study of S-2200 TG in Wistar Rats
Author(s), year:	[REDACTED] 2011a
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0036
Guideline(s):	No appropriate guideline existing for this study
GLP:	Yes (laboratory certified by National Authority)
Deviations:	No appropriate guideline existing, preliminary study
Validity:	Yes

Material and methods:

Test Material:	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G
Purity:	93.4%
Stability of test compound:	expiration date: November 21, 2011 (after completion of treatment)
Vehicle:	0.5% (w/v) methylcellulose in deionized water

Test animals:	
Species:	Rat
Strain:	Wistar, [REDACTED]:WI(Han)
Age:	approximately 6 weeks at start of treatment
Weight at dosing:	139 – 176 g (males) and 114 – 148 g (females)
Source:	[REDACTED]
Diet:	LLC Certified Rodent LabDiet® 5002 <i>ad libitum</i>

The objective of the study was to select dose levels for use in a definitive acute neurotoxicity study with S-2200 TG and to determine the appropriate time following dose administration at which neurobehavioural endpoints, such as a functional observational battery, should be assessed in that study.

Animal assignment and treatment:

Approximately 6 week-old animals were assigned to groups using a computer generated randomization program. Each group (Groups 1-4) consisted of 3 males and 3 females. These animals were then randomized into 3 study replicates to allow for the reasonable conduct of the detailed clinical observations. On study day 0 prior to dose administration, the animals were weighed to determine the dose. Treatment groups received one single oral (gavage) dose of 300, 1000, or 2000 mg/kg bw, and controls received vehicle only (0.5% (w/v) methylcellulose in deionized water). Animals were not fasted before dosing to avoid any potential confounding effects of inanition on the behaviour of the animals. On the day following dosing, animals were euthanized by carbon dioxide inhalation and discarded without macroscopic examination.

Detailed clinical observations:

All animals were examined twice daily to detect dead or moribund animals. Clinical examinations were performed on all animals prior to dosing on study day 0 including (but not limited to) evaluations for changes in appearance of skin, fur, eyes, mucous membranes, respiratory and circulatory systems, autonomic and central nervous systems, somatomotor activity, and behaviour. Body weights were determined on the day of dosing (day 0) for the purpose of dose calculations only.

Detailed clinical observations were recorded for all animals at approximately 1, 2, 3, 4, 5, 6, 7, and 8 hours after dosing. All animals were observed for the following parameters:

Ease of removal from cage	Ease of handling animal in hand
Lacrimation/chromodacryorrhoea	Salivation
Piloerection	Fur appearance
Palpebral closure	Respiratory rate/character
Red/crusty deposits	Mucous membranes/eye/skin colour
Eye prominence	Muscle tone
Mobility	Gait
Convulsions/tremors	Arousal
Grooming	Urination/defecation
Bizarre/stereotypic behaviour	Backing

Statistics:

All statistical tests were performed using [REDACTED] Data Management System™ unless otherwise noted. Analyses were conducted using two-tailed tests (except as noted otherwise) for minimum significance levels of 1% and 5%, comparing each test compound-treated group to the control group by sex. Body weight and continuous detailed clinical observation data were subjected to a parametric one-way ANOVA to determine intergroup differences. If the ANOVA revealed significant ($p < 0.05$) intergroup variance, Dunnett's test was used to compare the test compound-treated groups to the control group. Detailed clinical observation parameters that yielded scalar or descriptive data were analysed using Fisher's Exact Test.

Findings:

Clinical observations and survival:

All animals survived to the scheduled euthanasia on study day 1. No clinical findings were noted for animals in the 300, 1000 and 2000 mg/kg bw dose groups prior to dose administration.

Body weight:

Body weights were similar across all groups prior to dose administration on study day 0; no statistically significant differences were noted.

Detailed clinical observations:

No remarkable findings were observed at any dose level during the detailed clinical observations conducted at approximately 1, 2, 3, 4, 5, 6, 7, and 8 hours following dose administration on study day 0. Statistically significant findings were restricted to higher mean defecation count (1.3 counts) and higher number of urine pools (1.0 pool), noted for males treated at 2000 mg/kg when compared with the control group (0.0 defecation counts and 0.0 urine pools) at approximately 1 hour following dose administration on study day 0. However, these findings were transient (observed only 1 hour post-dosing), the magnitude of change from the control group was small, and there were no correlating findings for the females dosed at 2000 mg/kg. Therefore, these changes were not considered test compound-related.

Conclusion:

Based on the results of the detailed clinical observations, no neurotoxic effects were observed following a single dose of S-2200 TG at 300, 1000 or 2000 mg/kg bw. As a result, dose levels of 500, 1000 and 2000 mg/kg bw were selected for evaluation of the acute neurotoxic potential of S-2200 TG. In the definitive study, the time at which functional observational battery and locomotor activity will be observed on study day 0 (time of peak effect) will be 8 hours post-dosing.

Reference:	An Oral (Gavage) Acute Neurotoxicity Study of S-2200 TG in Wistar Rats
Author(s), year:	██████████ 2011b
Report/Doc. number:	Sumitomo Chemical Co. Ltd. Report No. ROT-0037
Guideline(s):	OECD Guideline 424 (1997), US EPA OPPTS 870.6200 Neurotoxicity Screening Battery (1998), Japanese MAFF 12 Nohsan No. 8147 (2000)
GLP:	Yes (laboratory certified by National Authority)
Deviations:	No
Validity:	Yes

Material and methods:

Test Material:	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G
Purity:	93.4%
Stability of test compound:	November 21, 2011 expiration date (after completion of treatment); storage stability of the test compound formulations for at least 19 days of room temperature and refrigerated storage was established in a previous study (Bodle, 2011, WIL-118054)
Vehicle:	0.5% (w/v) methylcellulose in deionized water
Test animals:	
Species:	Rat
Strain:	██████:Wl(Han)
Age:	approximately 6 weeks at start of treatment
Weight at dosing:	males: 151 – 218 g; females: 119 – 166 g
Source:	██
Diet:	LLC Certified Rodent LabDiet® 5002 <i>ad libitum</i>

Animal assignment and treatment:

Approximately 6 week-old animals were assigned to groups using a computer generated randomization procedure. Each group (Groups 1-4) consisted of 12 males and 12 females. On study day 0, the animals were weighed to determine the individual doses. Treatment groups received one dose of 500, 1000 or 2000 mg/kg bw (without overnight fasting) by oral gavage, and controls received vehicle only (0.5% w/v methylcellulose in deionized water, 5 mL/kg bw).

Clinical observations and survival:

All animals were observed twice daily, once in the morning and once in the afternoon, for mortality and moribundity.

Clinical examinations were performed once daily on all animals. The observations included, but were not limited to, evaluations for changes in the appearance of skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous system function, somatomotor activity, and behaviour patterns. Detailed physical examinations were conducted once at approximately 24 hours following dose administration to assess the potential of the test compound to cause delayed effects. The animals were removed from their home cages and placed in a standard arena for observation of changes in gait, posture, or clonic or tonic movements. Stereotypies (e.g., excessive grooming, repetitive circling), bizarre behaviour (e.g., self-mutilation, walking backwards) and permanent or semi-permanent signs, such as skin lesions or hair loss, were also recorded.

Body weight:

Individual body weights were recorded at least weekly, beginning approximately 1 week prior to test substance administration. Mean body weights and mean body weight changes were calculated for the corresponding intervals.

Functional observational battery (FOB):

FOB findings were recorded for all animals prior to the initiation of dose administration (study day -6), at the time of peak effect (set arbitrarily at 8 hours post-dosing, the maximum lapse allowed by guideline, based on the absence of effects in a range-finding study) on study day 0, and again on study days 7 and 14. Testing was performed by the same biologists, to the extent possible, without knowledge of the animal's group assignment. The FOB was performed in a sound-attenuated room equipped with a white-noise generator set to operate at 70 ± 10 dB. All animals were observed for the following parameters:

- **Home Cage Observations:** Posture, convulsions/tremors, faeces consistency, biting, palpebral (eyelid) closure.
- **Handling Observations:** Ease of removal from cage, lacrimation/chromodacryorrhoea, piloerection, palpebral closure, eye prominence, red/crusty deposits, ease of handling animal in hand, salivation, fur appearance, respiratory rate/character, mucous membranes/eye/skin colour, muscle tone.
- **Open Field Observations** (evaluated over a 2-minute observation period): Mobility, rearing, convulsions/tremors, grooming, bizarre/stereotypic behaviour, time to first step (seconds), gait, arousal, urination/defecation, gait score, backing.
- **Sensory observations:** Approach response, startle response, pupil response, forelimb extension, air righting reflex, touch response, tail pinch response, eyeblink response, hindlimb extension, olfactory orientation.
- **Neuromuscular Observations:** Hindlimb extensor strength, hindlimb foot splay, grip strength hind- and forelimb, rotarod performance.
- **Physiological Observations:** Catalepsy, body temperature, body weight.

Locomotor activity:

Locomotor activity was measured after each completion of the FOB, on study days -6, 0 (8 hours post dosing), 7 and 14, using a personal computer-controlled system that utilizes a series of infrared photobeams surrounding an amber plastic, rectangular cage to quantify the motor activity of each animal. Four-sided black plastic enclosures were used to surround the transparent plastic boxes and decrease the potential for distraction from extraneous environmental stimuli or activity by technicians or adjacent animals. The black enclosures rested on top of the photobeam frame and did not interfere with the path of the beams. The locomotor activity assessment was performed in a sound-attenuated room equipped with a white-noise generator set to operate at 70 ± 10 dB. The testing of treatment groups was conducted according to replicate sequence. Each animal was tested separately. Data were collected in 5-minute periods, and the test session duration was 60 minutes. These data were compiled as six 10-minute subintervals for tabulation. Data for ambulatory and total motor activity were tabulated. Total motor activity was defined as a combination of fine motor skills (i.e., grooming, interruption of 1 photobeam) and ambulatory motor activity (interruption of 2 or more consecutive photobeams).

Pathology:

On study day 15, all animals were anesthetized by an intraperitoneal injection of sodium pentobarbital and then perfused *in situ* with 4.0% paraformaldehyde in a 0.1 M phosphate buffer solution. The central and peripheral nervous system tissues were dissected and preserved. Fixed brain weight and brain dimensions (length [excluding olfactory bulbs] and width) were recorded. Any observable gross changes and abnormal coloration or lesions of the brain and spinal cord were recorded.

The following nerve tissues were prepared for a microscopic neuropathologic examination from 6 randomly selected animals/sex in the control and 2000 mg/kg bw dose groups:

- Brain (olfactory bulbs, cerebral cortex (2 levels), hippocampus/dentate gyrus, basal ganglia, thalamus, hypothalamus, midbrain, cerebellum, pons, and medulla oblongata)
- Spinal cord (at cervical swellings C₃-C₇ and at lumbar swellings T₁₃-L₄)
- Trigeminal ganglia/nerves^a
- Lumbar dorsal root ganglia at T₁₃-L₄^b, lumbar dorsal root fibres at T₁₃-L₄^b, lumbar ventral root fibres at T₁₃-L₄^b
- Cervical dorsal root ganglia at C₃-C₇^b, cervical dorsal root fibres at C₃-C₇^b, cervical ventral root fibres at C₃-C₇^b
- Cervical spinal nerve, lumbar spinal nerve
- Sciatic nerves (mid-thigh region)(2)^c, sciatic nerves (at sciatic notch)(2)^c, sural nerves (2)^c, tibial nerves (2)^c, peroneal nerves (2)^c
- optic nerves^a, eyes^a
- skeletal muscle (gastrocnemius)

^a Both processed and evaluated microscopically;

^b Four to six tissues were collected at necropsy; two were evaluated microscopically;

^c One processed for microscopic examination;

(2) Two sections (1 transverse and 1 longitudinal) of the tissue were evaluated from the right hind leg. The tissues from the left hind leg were collected and preserved for possible future evaluation.

The tissues listed above were prepared for a qualitative histopathological examination by embedding in paraffin (central nervous system tissues) or plastic (peripheral nervous system tissues), sectioning, and staining with haematoxylin and eosin.

Statistics:

All statistical tests performed by [REDACTED] were performed using [REDACTED] Toxicology Data Management System™ unless otherwise noted. Analyses were conducted using two-tailed tests (except as noted otherwise) for minimum significance levels of 1% and 5%, comparing each test compound-treated group to the control group by sex.

Body weight, body weight change, pre-test and post-dosing continuous FOB and brain measurement data were subjected to a parametric one-way ANOVA to determine intergroup differences. If the ANOVA revealed significant ($p < 0.05$) intergroup variance, Dunnett's test was used to compare the test compound-treated groups to the control group. FOB parameters that yielded scalar or descriptive data, necropsy findings, and non-graded histopathologic findings were analysed using Fisher's Exact Test.

Statistical analysis of graded histopathologic findings was not conducted on this study due to the absence of multiple severities (i.e., those findings with more than 2 distinct severities including none/not remarkable).

All statistical analyses performed by BioSTAT Consultants, Inc. were conducted using SAS version 9.1, or higher. All repeated measures analysis of variance (RANOVA) statistical analyses for total and ambulatory locomotor activity counts recorded during pre-test and after dosing were conducted as follows. Each analysis endpoint was analysed, by sex and session, with a RANOVA. Factors in the model included treatment group (TRT), time interval (TIME), and the interaction of time interval and treatment group (TRT*TIME). The SAS procedure PROC MIXED was used for analysis with the random effect of animal included as the repeated measurement. The covariance structure across time was selected by comparing Akaike's Information Criterion (AIC).

The monotonic dose-response relationship was evaluated using sequential linear trend tests based on ordinal spacing of dose levels. The linear dose by time interaction (LinTrt*Time) was evaluated and, if significant at the 0.05 level, trend tests on treatment means were performed at the 0.05 level for each time interval. If the linear dose by time interaction was not significant, the trend test was conducted across the pooled time intervals for the entire session only.

Non-monotonic dose responses were evaluated whenever no significant linear trends were detected but TRT and/or TRT*TIME interaction was significant at the 0.01 level. Within the framework of the RANOVA, pair-wise comparisons were made for each individual test compound-treated group with the control group through linear contrasts. If TRT*TIME was significant, the comparisons were conducted for each time interval. If only the TRT effect was significant, the comparisons were conducted across the pooled time intervals for the entire session. These non-monotonic dose-response comparisons were conducted at the 0.01 significance level.

Findings:

Clinical observations and survival:

No mortality was observed in the study.

There were no treatment-related clinical findings.

Body weight:

Body weights and body weight gain were unaffected by test substance administration. There were no statistically significant differences between treated groups and control animals.

FOB assessments:

Home cage, handling, open field, sensory, neuromuscular and physiological parameters were unaffected by test substance administration.

On study day 7, home cage observations recorded that statistically significantly fewer females in the 1000 mg/kg bw group were sitting or standing normally in the home cage when compared to the control group. However, no decrease in this normal home cage behaviour was observed at 2000 mg/kg bw. Therefore, a decrease in the number of females exhibiting this behaviour was not attributed to test compound

administration. There were no other statistically significant differences between the control and test compound-treated males and females regarding home cage parameters.

During open field observations on study day 7, a statistically significant decrease in the number of rearing counts was observed for the 2000 mg/kg bw group females compared to the control group. This decrease was attributed to a single female in this group that did not rear during the evaluation while 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 in the mean number of rearing counts observed in the 2000 mg/kg bw group females was not attributed to test compound administration. There were no other statistically significant differences between the control and test compound-treated males and females regarding open field parameters.

Locomotor activity:

Some differences in group locomotor activity were seen. Statistically significant lower mean total and ambulatory counts were noted for the 1000 and 2000 mg/kg bw group males during the second subinterval (11-20 minutes) at the time of peak effect on study day 0. As a result, mean overall motor activity counts were 24.3% lower (total counts) and 34.3% lower (ambulatory counts) for the 2000 mg/kg bw group males and were statistically significant ($p = 0.003$) compared to the control group. By study day 7 and continuing to study day 14, mean total and ambulatory counts for the 2000 mg/kg bw group males were unaffected by test substance administration. There were no other findings to corroborate this effect, which was therefore attributed to transient systemic toxicity at this top dose level rather than typical neurotoxicity. No other significant changes were noted in the 1000 mg/kg bw group males during the other subintervals or study days, which was attributed to better habituation observed in this group. Therefore, the decreases in locomotor activity counts observed at 1000 mg/kg bw/day were not attributed to test substance administration.

Statistically significant lower mean overall ambulatory counts were noted for the 2000 mg/kg bw group females at the time of peak effect on study day 0, primarily due to reductions (up to 21.8%, although not statistically significant) in ambulatory counts during the first 2 subintervals (0-10, and 11-20 minutes). Although there were no differences in total motor activity counts observed for these animals on study day 0, similar reductions in ambulatory counts were noted for the 2000 mg/kg bw group males at this evaluation. Therefore, these reductions were attributed to test compound administration. There were no other test substance-related effects on locomotor activity observed for the 2000 mg/kg bw group females on study days 7 or 14.

Locomotor activity patterns (mean ambulatory and total motor activity counts) were unaffected by test substance administration for males at 500 mg/kg and females at up to 1000 mg/kg. Aside from the aforementioned better habituation noted for the 1000 mg/kg group males, no remarkable shifts in the pattern of habituation occurred in any of the test compound treated groups when the animals were evaluated on study days 0, 7, and 14.

Table B.6.7.1-1: FOB and locomotor activity

Endpoint	Day	Min	Males				Females			
			Dose (mg/kg bw)				Dose (mg/kg bw)			
			0	500	1000	2000	0	500	1000	2000
Home cage parameter: Mean number 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	0	-	6.5	7.3	6.8	5.5	12.3	7.9	9.2	8.8

parameter: Mean number of animals rearing	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	1146	1094	1052	979	1275	1283	1186	1115
		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
		51 – 60	94	93	124	63	149	159	177	125
	Cumulative	0 – 60	2268	2267	1918	1718⁺	2339	2536	2316	2042
Locomotor activity counts: Ambulatory counts	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
	Cumulative	0 – 60	522	498	424	343⁺	623	708	587	480⁺

p < 0.05 (Fisher's exact test), * p < 0.05 (Dunnett's test), ⁺ p < 0.05 (sequential linear trend tests)

Pathology:

There were no macroscopic findings observed for animals at the scheduled necropsy on study day 15. Brain weights and measurements were unaffected by administration of S-2200 TG at 500, 1000 and 2000 mg/kg bw. There were no statistically significant differences between test substance-treated groups and the control.

No test substance-related microscopic findings were observed in any of the central or peripheral nervous system tissues examined from 6 animals/sex in the 2000 mg/kg bw group.

Conclusion:

The acute oral neurotoxicity of S-2200 TG was evaluated in male and female Han Wistar rats at doses of 0 (control), 500, 1000 and 2000 mg/kg bw. There were no clinical signs, body weight losses, or mortality over the 14 day observation period post-dosing. No treatment-related changes were seen during functional observational battery and at the neuropathological examination. Decreased overall locomotor activity (total and/or ambulatory counts) was noted for males and females in the 2000 mg/kg bw group at the time of peak effect on study day 0. The acute NOAEL in this study was determined to be 1000 mg/kg bw for male and female Wistar rats.

B.6.7.2 Delayed neurotoxicity following acute exposure

No study was submitted; requirement not triggered.

B.6.7.3 Subchronic neurotoxicity – rat 90-day

Reference:	A 90-Day Oral Dietary Neurotoxicity Study of S-2200 TG in Wistar Rats
Author(s), year:	██████████ 2012
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0050
Guideline(s):	OECD 424 (1997), US EPA OPPTS 870.6200, Japanese MAFF 12 Nohsan, No. 8147
GLP:	Yes (laboratory certified by National Authority)
Deviations:	On study day 56, one male in the 5000 ppm group and one female in the control group were found in the incorrect cages and, therefore, had access to the wrong diets. Both were euthanized and sent to necropsy on study day 58. This deviation did not negatively impact the quality or integrity of the data nor the outcome of the study.
Validity:	Yes

Material and methods:

<i>Test Material:</i>	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G
Purity:	93.4%
Stability of test compound:	November 21, 2011 expiration date (after completion of treatment); storage stability of the test diet formulations for 10 days of room temperature storage was established in a previous study (Bodle, 2011, WIL-118054)
Control:	PMI Nutrition International, LLC Certified Rodent LabDiet® 5002
<i>Test animals:</i>	
Species:	Rat
Strain:	Wistar ████████:WI(Han)
Age:	approximately 6 weeks at start of treatment
Weight at dosing:	Males: 165 – 217 g; Females: 111 – 160 g (at start of treatment)
Source:	██
Diet:	PMI Nutrition International, LLC Certified Rodent LabDiet® 5002

The objective of the study was to evaluate the potential neurotoxic effects of the test substance when administered continuously in the diet to rats for 13 weeks. The neurotoxic potential of the test substance was evaluated using a neurotoxicity screening battery consisting of functional observational battery, locomotor activity, and neuropathological assessments.

Animal assignment and treatment:

Approximately 4 week-old animals were acclimatised for 14 days. One week prior to the initiation of test diet administration, animals were assigned to groups of 12 animals per sex per group using a computer generated randomization program. Male and females rats were administered the test substance in rodent diet at 0, 1500, 5000 or 15000 ppm continuously for at least 90 consecutive days (13 weeks). Study day 0 was the first day of administration and study day 91 was the day of necropsy.

WARNING: This document is part of an EC evaluation package and should not be used in isolation. Registration must be completed on the basis of this document.

Table B.6.7.3-1: Test substance intake

Group	Dietary Concentration (ppm)	Group Size		Effective test substance intake (mg/kg bw/d)	
		Males	Females	Males	Females
1	0	12	12	0	0
2	1500	12	12	99	122
3	5000	12	12	338	416
4	15000	12	12	1024	1223

Diet Preparation and Analysis:

The dietary formulations were prepared as test compound/diet mixtures. The test compound was ground to a fine powder using a mortar and pestle prior to use. The test compound was added to the diet and blended in a Hobart blender. The resulting premix was then mixed throughout the diet in a V-blender for approximately 10 minutes using an intensifier bar for the first and last 3 minutes to obtain the appropriate dietary concentrations. The test diet formulations were prepared approximately weekly, placed in labeled plastic storage bags, and stored at room temperature. The test compound was administered as a constant concentration in the diet and was not adjusted during the study. For the control group, an appropriate amount of PMI Nutrition International, LLC Certified Rodent LabDiet® 5002 was weighed approximately weekly, placed in a labeled storage bag, and stored at room temperature until use.

Stability of the test diet formulations for 10 days of room temperature storage was established in a previous study at the same laboratory. Therefore stability analyses were not conducted in this study. Homogeneity and concentration analyses were conducted in the study.

Clinical observations:

All animals were observed twice daily, once in the morning and once in the afternoon, for mortality and moribundity.

Detailed physical examinations were recorded approximately weekly, beginning 1 week prior to test diet administration and continuing until the scheduled necropsy. The observations included, but were not limited to, evaluation for changes in appearance of skin and fur, eyes, mucous membranes, respiratory and circulatory systems, autonomic and central nervous systems, somatomotor activity, and behaviour. On the days, on which the functional observational battery was conducted, no additional clinical findings were recorded.

Body weight and food consumption:

Individual body weights were recorded weekly, beginning approximately 1 week prior to test diet administration. Mean body weights and mean body weight changes were calculated for the corresponding intervals. When body weights could not be determined for an animal during a given interval (due to an unscheduled death), group mean values were calculated for that interval using the available data.

Individual food consumption was recorded weekly, beginning 1 week prior to test diet administration (study day -8 to 0). Food consumption was calculated as g/animal/day and g/kg/day for the corresponding body weight change intervals. Mean test compound consumption (mg/kg bw/day) for each group was determined by multiplying the concentration of the test compound in the diet (mg/kg of diet) by the g/kg bw/day food consumption value for each interval. When food consumption could not be determined for an animal during a given interval (due to an unscheduled death, weighing error, food spillage, etc.), group mean values were calculated for that interval using the available data.

Ophthalmic examination:

Ocular examinations were conducted on all animals prior to the initiation of the test diet administration (study week -2) and near to the end of the treatment period (week 12). All ocular examinations were conducted using an indirect ophthalmoscope and slit lamp biomicroscope preceded by pupillary dilation with an appropriate mydriatic agent.

Functional observational battery (FOB):

FOB findings were recorded for all animals during pretest (study week -1) and during study weeks 1, 3, 7, and 12. Testing was performed by the same biologists, to the extent possible, without knowledge of the animal's group assignment. The FOB was performed in a sound-attenuated room equipped with a white-noise generator set to operate at 70 ± 10 dB. All animals were observed for the following parameters:

- **Home Cage Observations:** Posture, convulsions/tremors, faeces consistency, biting, palpebral (eyelid) closure.
- **Handling Observations:** Ease of removal from cage, lacrimation/chromodacryorrhoea, piloerection, palpebral closure, eye prominence, red/crusty deposits, ease of handling animal in hand, salivation, fur appearance, respiratory rate/character, mucous membranes/eye/skin colour, muscle tone.
- **Open Field Observations** (evaluated over a 2-minute observation period): Mobility, rearing, convulsions/tremors, grooming, bizarre/stereotypic behaviour, time to first step (seconds), gait, arousal, urination/defecation, gait score, backing.
- **Sensory observations:** Approach response, startle response, pupil response, forelimb extension, air righting reflex, touch response, tail pinch response, eyeblink response, hindlimb extension, olfactory orientation.
- **Neuromuscular Observations:** Hindlimb extensor strength, hindlimb foot splay, grip strength hind- and forelimb, rotarod performance.
- **Physiological Observations:** Catalepsy, body temperature, body weight.

Locomotor activity:

Locomotor activity was recorded after the completion of the FOB during pretest (study week -1) and during study weeks 1, 3, 7, and 12. Locomotor activity was measured automatically using a personal computer-controlled system that utilizes a series of infrared photobeams surrounding an amber plastic, rectangular cage to quantify the motor activity of each animal. Four-sided black plastic enclosures were used to surround the transparent plastic boxes and decrease the potential for distraction from extraneous environmental stimuli or activity by technicians or adjacent animals. The black enclosures rested on top of the photobeam frame and did not interfere with the path of the beams. The locomotor activity assessment was performed in a sound-attenuated room equipped with a white-noise generator set to operate at 70 ± 10 dB. The testing of treatment groups was conducted according to replicate sequence. Each animal was tested separately. Data were collected in 5-minute epochs, and the test session duration was 60 minutes. These data were compiled as six 10-minute subintervals for tabulation.

Data for ambulatory and total motor activity were tabulated. Total motor activity was defined as a combination of fine motor skills (i.e., grooming, interruption of 1 photobeam) and ambulatory motor activity (interruption of 2 or more consecutive photobeams).

Pathology:

In case of unscheduled deaths, a complete necropsy was conducted on all animals euthanised (by carbon dioxide inhalation) and removed from study. The necropsy included examination of the external surface, all orifices, and the cranial, thoracic, abdominal, and pelvic cavities, including viscera.

At the termination of the study (study week 13), all surviving animals were anesthetized by an intraperitoneal injection of sodium pentobarbital and then perfused *in situ* with 4.0% paraformaldehyde in a 0.1 M phosphate

buffer solution. The central and peripheral nervous system tissues were dissected and preserved. Fixed brain weight and brain dimensions (length [excluding olfactory bulbs] and width) were recorded. Any observable gross changes and abnormal coloration or lesions of the brain and spinal cord were recorded. The following nerve tissues were prepared for a microscopic neuropathologic examination from 6 randomly selected animals/sex in the control and 15000 ppm groups:

- Brain (olfactory bulbs, cerebral cortex, hippocampus, basal ganglia, thalamus, hypothalamus, midbrain, cerebellum, pons, and medulla oblongata)
- Spinal cord (at cervical swellings C₃-C₇ and at lumbar swellings T₁₃-L₄)
- Trigeminal ganglia/nerves^a
- Lumbar dorsal root ganglia at T₁₃-L₄^b, lumbar dorsal root fibres at T₁₃-L₄^b, lumbar ventral root fibres at T₁₃-L₄^b
- Cervical dorsal root ganglia at C₃-C₇^b, cervical dorsal root fibres at C₃-C₇^b, cervical ventral root fibres at C₃-C₇^b
- Cervical spinal nerve, lumbar spinal nerve
- Sciatic nerves (mid-thigh region)(2)^c, sciatic nerves (at sciatic notch)(2)^c, sural nerves (2)^c, tibial nerves (2)^c, peroneal nerves (2)^c
- optic nerves^a, eyes^a
- skeletal muscle (gastrocnemius)
- other sites (if deemed necessary)

^a Both processed and evaluated microscopically;

^b Four to six tissues were collected at necropsy; two were evaluated microscopically;

^c One processed for microscopic examination;

(2) Two sections (1 transverse and 1 longitudinal) of the tissue were evaluated from the right hind leg. The tissues from the left hind leg were collected and preserved for possible future evaluation.

The tissues listed above were prepared for a qualitative histopathological examination by embedding in paraffin (central nervous system tissues) or plastic (peripheral nervous system tissues), sectioning, and staining with haematoxylin and eosin.

Statistics:

All statistical tests conducted by [REDACTED] LLC, were performed using [REDACTED] Data Management System™. Analyses were conducted using two-tailed tests (except as noted otherwise) for minimum significance levels of 1% and 5%, comparing each test compound-treated group to the control group by sex.

Body weight, body weight change, food consumption, pre-test and post-dosing continuous FOB, and brain measurement data were subjected to a parametric one-way ANOVA to determine intergroup differences. If the ANOVA revealed significant ($p < 0.05$) intergroup variance, Dunnett's test was used to compare the test compound-treated groups to the control group. FOB parameters that yielded scalar or descriptive data, necropsy findings, and non-graded histopathologic findings were analysed using Fisher's Exact Test.

All statistical analyses performed by BioSTAT Consultants, Inc. were conducted using SAS version 9.2, or higher. All repeated measures analysis of variance (RANOVA) statistical analyses for total and ambulatory locomotor activity counts recorded during pre-test and after dosing were conducted as follows. Each analysis endpoint was analysed, by sex and session, with a RANOVA. Factors in the model included treatment group (TRT), time interval (TIME), and the interaction of time interval and treatment group (TRT*TIME). The SAS procedure PROC MIXED was used for analysis with the random effect of animal included as the repeated measurement. The covariance structure across time was selected by comparing Akaike's Information Criterion (AIC).

The monotonic dose-response relationship was evaluated using sequential linear trend tests based on ordinal spacing of dose levels. The linear dose by time interaction (LinTrt*Time) was evaluated and, if significant at the 0.05 level, trend tests on treatment means were performed at the 0.05 level for each time interval. If the linear dose by time interaction was not significant, the trend test was conducted across the pooled time intervals for the entire session only.

Non-monotonic dose responses were evaluated whenever no significant linear trends were detected but TRT and/or TRT*TIME interaction was significant at the 0.01 level. Within the framework of the RANOVA, pair-wise comparisons were made for each individual test compound-treated group with the control group through linear contrasts. If TRT*TIME was significant, the comparisons were conducted for each time interval. If only the TRT effect was significant, the comparisons were conducted across the pooled time intervals for the entire session. These non-monotonic dose-response comparisons were conducted at the 0.01 significance level.

Findings:

Clinical observations and survival:

On study day 56, one male in the 5000 ppm group and one female in the control group were found in the incorrect cages and, therefore, had access to the wrong diets. Both were euthanized and sent to necropsy on study day 58.

All other males and females survived to the scheduled euthanasia. No test substance-related clinical findings were noted at weekly examinations. Spontaneous findings included hair loss and scabbing on various body surfaces. These findings occurred infrequently, in similar frequencies in the control group, and/or in a manner that was not dose-related.

Body weight and food consumption:

Test compound-related, significantly reduced mean body weight gains were noted for males offered diet containing 15000 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 lower (not statistically significant) 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% to 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 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 15000 ppm of the test-compound was significantly higher than 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.

Significantly reduced mean food consumption, evaluated as g/animal/day and g/kg bw/day, was noted for males offered diet containing 15000 ppm of the test-compound during study days 0-7 (g/animal/day and g/kg bw/day), days 21-28 (g/animal/day), and days 28-35 (g/animal/day). In addition, slightly lower (not statistically significant) mean food consumption (g/animal/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 test substance-related. Mean food consumption in this group was unaffected by test compound exposure thereafter. Although mean g/kg/day food consumption for males in the 15000 ppm group was significantly higher than in the control group during study days 56-63 and 63-70, these results were attributed to lower mean body weights noted in this group.

Mean food consumption of males offered diet containing 1500 and 5000 ppm of the test-compound and females at all exposure concentrations was unaffected by test compound administration. Significantly higher mean food consumption was noted for females offered diet containing 15000 ppm of the test-compound during study days 7-14 (g/animal/day and g/kg/day); however, increases in food consumption were not considered toxicologically relevant. Other differences from the control group were slight and not statistically significant.

Table B.6.7.3-2: Body weight, body weight gain and food consumption (mean values)

Endpoint	Study day	Males				Females			
		Diet concentration (ppm)				Diet concentration (ppm)			
		0	1500	5000	15000	0	1500	5000	15000
Mean body weights (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 gains (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
	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
Mean food consumption (g/animal/day)	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

* p < 0.05 (Dunnett's test), ** p < 0.01 (Dunnett's test)

FOB assessments:

Home cage parameters, handling parameters, open field parameters, sensory parameters, neuromuscular parameters and physiological parameters were unaffected by test diet consumption.

The only statistically significant differences during home cage observations were noted for females of the 1500 ppm group during study week 12. These differences consisted of a lower number of females that were alert and oriented toward the observer and a higher number of females that were sitting or standing normally. These transient findings are not considered abnormal in the home cage and showed no dose-relationship. Therefore, observations were not considered treatment-related. There were no other statistically significant differences between the control and test compound-treated males and females when home cage parameters were evaluated.

Statistically significant differences noted during open field parameter observations were limited to a lower mean time to first step for females at all test compound concentrations during study week 1 and a higher mean rearing count for males of the mid dose group (5000 ppm) during study week 3. However, regarding the lower mean time to first step, there was a small magnitude of change, findings were only noted during the week 1 assessment, there was no dose-response and no effect was observed in males. For the higher

mean rearing count, there was no dose-response. Therefore, observations were not considered test substance-related. There were no other statistically significant differences between the control and test compound-treated males and females when open field parameters were evaluated.

During neuromuscular observations, mean forelimb grip strength for females in the high dose group (15000 ppm) was found to be significantly reduced during study week 7 compared to the control group; however, these results were not noted during the study week 3 and 12 and, in the absence of additional effects on related parameters, were not considered to be test compound-related. In addition, a significantly lower mean rotarod performance during week 1 and lower mean forelimb grip strength during week 3 were noted for females of the low dose group (1500 ppm); however, no dose-response was evident. Therefore, observations were not considered to be test substance-related. There were no other statistically significant differences between the control and test compound-treated males and females when neuromuscular parameters were evaluated.

Locomotor activity:

Locomotor activity patterns (mean ambulatory and total motor activity counts) were unaffected by test diet consumption. Significant differences between the control and test compound-exposed groups were limited to lower mean total counts for females in the 15000 ppm group during 51-60 minutes at study week 1, higher mean cumulative total counts for males in the 15000 ppm group at study week 7, and higher mean ambulatory counts for females offered the 15000 ppm diet during 21-30 minutes at study week 12. However, findings were transient, did not affect mean overall counts, and/or no remarkable shifts in the pattern of habituation occurred in any of the test compound-exposed groups when the animals were evaluated on study weeks 1, 3, 7, and 12. Therefore, no relationship to the test compound intake was apparent.

Ophthalmoscopy:

No ophthalmic lesions indicative of toxicity were observed in any of the test substance-exposed groups. All findings observed were typical in prevalence and appearance for laboratory rats of this age and strain.

Pathology:

There were no macroscopic findings observed for any animal at any test substance concentration in this study. Brain weights and measurements were unaffected by administration of S-2200 TG at 1500, 5000, and 15000 ppm. There were no statistically significant differences between the control and test substance-treated groups. No test substance-related microscopic lesions indicative of neurotoxicity were observed in any of the central or peripheral nervous system tissues examined from 6 animals/sex in the 15000 ppm group.

Conclusion:

There were no neurotoxic effects noted at any dietary exposure level during functional observational battery, locomotor activity, and neuropathological assessments. Based on these results, the NOAEL for subchronic neurotoxicity in this study was considered to be 15000 ppm for male and female Wistar rats (1024 and 1223 mg/kg bw/day, respectively) when S-2200 TG was offered in the diet continuously for at approximately 90 days.

The only treatment-related finding in this study was a transient decrease in body weight, bodyweight gain and food consumption in males offered diet containing 15000 ppm of the test-compound. Based on lower mean body weight, bodyweight gain and food consumption in high dose males and the lack of effects noted for any females at any exposure concentration, the NOAEL for systemic toxicity was considered to be 5000 ppm for male and 15000 ppm for female Wistar rats (338 and 1223 mg/kg bw/day, respectively).

B.6.7.4 Postnatal developmental neurotoxicity

No study was submitted, not mandatory.

B.6.7.5 Summary on neurotoxicity

The potential for mandestrobin to cause neurotoxicity was thoroughly assessed in acute and subchronic neurotoxicity studies. It is noted that these studies were conducted for other regulatory authorities, since there is no toxicity that triggers these studies as a requirement under EU regulations.

In a range-finding assay designed to identify the time of peak effect after an acute oral limit dose (2000 mg/kg bw), no reaction was seen, so no peak effect was evident. In the subsequent definitive acute neurotoxicity study, investigations intended for the “time of peak effect” were therefore conducted at the latest timepoint compatible with guideline, i.e. 8 hours post-dose.

The only treatment-related finding in the definitive acute neurotoxicity study was a decrease in mean locomotor activity, seen at the top dose (2000 mg/kg bw) at 8 hours post-dose. In the absence of any other specific neurological findings, this is probably attributable to transient systemic toxicity rather than typical neurotoxicity. The NOAEL, set on a highly precautionary basis assuming a decrease in locomotor activity in absence of any other neurological change might be meaningful, was 1000 mg/kg bw.

In a 90-day subchronic neurotoxicity study, at doses of up to 15000 ppm, no evidence of specific neurotoxicity was found. The NOAEL for specific neurotoxicity was therefore approximately 1024 mg/kg bw/day. On the basis of body weight gain retardation at the top dose in males only, the NOAEL for systemic toxicity was 338 mg/kg bw/day.

Table B.6.7.5-1: Summary of neurotoxicity studies for mandestrobin

Type of study (reference)	Dose tested	NOAEL (mg/kg bw/day)	Adverse effects / target organs at LOAEL
An Oral (Gavage) Dose Range-Finding Acute Neurotoxicity Study of S-2200 TG in Wistar Rats (2011a)	0, 300, 1000, 2000 mg/kg bw	2000	No effect at highest dose tested
An Oral (Gavage) Acute Neurotoxicity Study of S-2200 TG in Wistar Rats (2011b)	0, 500, 1000, 2000 mg/kg bw	1000	Decreased overall locomotor activity (total and/or ambulatory counts) in males and females at 2000 mg/kg bw
A 90-Day Oral Dietary Neurotoxicity Study of S-2200 TG in Wistar Rats (2012)	0, 1500, 5000, 15000 ppm equivalent to: 0, 99, 338, 1024 mg/kg bw/d in males and 0, 122, 415, 1223 mg/kg bw/d in females	Neurotoxicity: 1024 Systemic tox.: 338 ♂ 1223 ♀	No neurotoxicity at highest dose tested ↓ body weight, body weight gain and food consumption in males at 1024 mg/kg bw/d

B.6.8 Further toxicological studies (Annex IIA 5.10)

B.6.8.1 Mechanistic studies

B.6.8.1.1 Liver and thyroid changes

In repeat dose toxicity studies the primary target organ of mandestrobin is the liver in all species examined. The thyroid was also a target organ in the rat, but not in the mouse and 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 tumourigenicity was observed in rat or mouse carcinogenicity studies.

In conjunction with evidence from the literature, mode of action studies were conducted to provide evidence that mandestrobin is a hepatic enzyme inducer via at least constitutive androstane receptor (CAR) activation in rat, in a manner similar to phenobarbital (PB).

Reference:	Short-Term Study for Mode of Action Analysis for Rat Liver and Thyroid Findings by S-2200TG – Dose Response, Time-Course and Reversibility
Author(s), year:	██████████ 2012e
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0067
Guideline(s):	No (mechanistic study)
GLP:	No. Conducted in a GLP-compliant facility
Deviations:	-
Validity:	Yes

Material and methods:

<i>Test Material:</i>	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G
Purity:	93.4%
Stability of test compound:	Expiry date: June 14 th , 2014 (after completion of treatment)
Vehicle:	None. Test material was mixed directly into diet.
<i>Test animals:</i>	
Species:	Rat
Strain:	Wistar (██████:WI)
Age at start of treatment:	10 weeks at start of treatment
Weight at start of treatment:	males: 317-404g, females: 211-282g
Source:	██
<i>Diet:</i>	CRF-1 powdered feed (Oriental Yeast Co. Ltd, Tokyo) provided <i>ad libitum</i>
<i>Housing:</i>	Suspended aluminium cages with wire-mesh front and floor
<i>In life dates:</i>	22 July 2011 – 5 August 2011 for males, 27 January – 10 February 2012 for females

Animal assignment and treatment:

10 Wistar rats/sex/group were fed diets containing 0 (control), 400, 2000, 7000 and 15000 ppm S-2200 TG for 7 days. Doses were selected to replicate the doses in the carcinogenicity study in rats.

10 rats/sex/group were fed diets containing 0 and 15000 ppm S-2200 TG for 14 days to evaluate the time course of alterations.

10 rats/sex were fed diets containing 0 and 15000 ppm S-2200 TG for 7 days followed by a 7-day recovery period, to evaluate reversibility of any findings.

10 rats/sex were treated with 1000 ppm PB as CAR activation positive controls.

Table B.6.8.1-1: Experimental design and test substance intake

	7 day treatment groups		14 day treatment groups		Recovery groups (7 day treatment followed by 7 day recovery period)	
Dose level, ppm S-2200 TG	Males	Females	Males	Females	Males	Females
control	0	0	0	0	0	0
400	23.3	25.7	-	-	-	-
2000	115.7	131.2	-	-	-	-
7000	378.9	420.2	-	-	-	-
15000	744.4	811.8	796.3	952.4	804.9	896.1
Positive control (1000 ppm PB)	57.0	66.2	55.9	63.3	52.9	64.9

Food consumption and body weight:

In the treatment period, body weights were measured on Days 1 (prior to dosing), 4, 8 and 15, and at the time of sacrifice. In the recovery period, body weights were measured on Days 1 and 8 at the time of sacrifice. Food consumption for each cage was measured on Day 1 through Day 4, Day 4 through Day 8, and Day 8 through Day 15 over a period of 72, 96 or 168 consecutive hours throughout the study.

Clinical observations:

Animals were observed for mortality once daily.

Sacrifice and pathology:

After the treatment period, rats were euthanized (decapitation without anaesthesia) without prior fasting. Blood was collected from carotid artery and cervical vein. All organs and tissues from all animals were subjected to necropsy.

Organ weights:

Liver (before fixation) and thyroid (after fixation) were weighed while wet from all animals. Relative organ weight (organ weight to body weight ratio) was calculated on the basis of the body weight on the day of sacrifice.

Histopathology:

Histopathological analysis was carried out for liver and thyroid.

Liver, thyroid and duodenum fixed in buffered formalin from all animals were dehydrated, embedded in paraffin, sectioned, stained with haematoxylin and eosin, and examined with the light microscope.

Electron microscopy: Small pieces of the left lateral lobe in livers from 2 animals/sex in the control, S-2200 TG 15000 ppm, and PB group were cut and fixed in 2.5% glutaraldehyde solution with post-fixation in 2% osmium tetroxide, then dehydrated and embedded in epoxy resin, and were examined under the electron microscope. Ultra-thin sections were prepared and stained with uranyl acetate and lead citrate, and examined with the transmission electron microscope.

Replicative DNA Synthesis:

Preparation and implantation of BrdU-containing osmotic pumps:

5-Bromo-2'-deoxyuridine (BrdU) was dissolved in 10% DMSO (20 mg BrdU/mL). Osmotic pumps were filled with 2 mL of this solution, and were implanted subcutaneously into rats of the main and satellite groups 3 days prior to sacrifice. The pump was rated to supply 10 µL/h of BrdU, for 3 days. This corresponds to a total release of:

$$200 \mu\text{g/h} \times 24 \text{ h/day} \times 3 \text{ days} = 14400 \mu\text{g BrdU.}$$

The pumps were implanted in the dorsal region under ether anaesthesia.

BrdU labelling indices:

Thin sections of liver prepared from the same paraffin embedded blocks used for histopathology in main and satellite groups were stained immunohistochemically using BrdU monoclonal antibody to determine BrdU labelling indices. BrdU labelling was analysed blind to treatment at DIMS Institute of Medical Science, Inc. (Aichi, Japan). BrdU labelling indices were calculated by dividing the number of labeled hepatocellular nuclei by the total number of hepatocellular nuclei (%). The total number of cells evaluated per liver was greater than 2000. Sections of duodenum were also prepared and stained immunohistochemically with the BrdU monoclonal antibody on the same glass slide as liver to serve as a positive control to confirm appropriate administration of BrdU, but counts were not formally recorded.

Hepatic enzyme analysis:

Preparation of liver S9 fractions

Liver S9 fractions were prepared. A portion of cryopreserved liver (approximately 0.5 g) from six animals whose numbers were low in main and recovery groups was homogenized in 4 volumes of Tris/HCl buffer. Liver homogenate was centrifuged to separate the S9 fraction. The protein level in the S9 fraction was determined using an assay kit with bovine serum albumin as the protein standard. Liver microsomal activities of 7-pentoxoresorufin O-depentyldase activity (PROD) and lauric acid hydroxylation were determined as markers of CYP2B and 4A, respectively.

7-Pentoxoresorufin O-depentyldase (PROD) activity (CYP2B)

The reaction mixture (200 µL) consisted of 3 µM 7-pentoxoresorufin, 10 µM dicoumarol, 1 mM NADPH, and 1 µL S9 fraction in Tris/HCl buffer (pH7.4), and was added to a 96-well microplate. After incubation, the reaction was stopped by addition of 100 µL acetonitrile. PROD activity was determined by fluorometric analysis. The fluorescence of the sample was measured with a microplate reader. The activity was expressed as the rate of resorufin formation, and was calculated based on the fluorescence of a standard curve of resorufin.

Lauric acid hydroxylation (CYP4A-activity)

The reaction mixture (200 µL) consisted of 100 µM [1-¹⁴C] lauric acid (3.7 kBq), 1 mM NADPH, 20 µL S9 fractions in Tris/HCl buffer (pH7.4) and was added to 96-well microplate. After incubation for 30 minutes at 37°C, reaction was stopped by addition of 100 µL methanol and an aliquot of sample was applied to thin-layer chromatography (TLC). TLC plates were developed with solvent system, and processed with a photographic film. The value was expressed as the rate of hydroxy lauric acid formation, determined by TLC.

UDP-glucuronosyltransferase (UGT) activity

UGT activity toward T4 (T4-UGT) was analysed. The reaction mixture consisted of 5 µL of liver S9, 10 mM MgCl₂, 0.05% "Brij58", 1.4 mM D-saccharic acid-1,4-lactone, 4mM UDP-glucuronic acid and 1 µM ¹²⁵I-thyroxine (5.6 kBq) in 200 µL of 66 mM Tris-HCl Buffer (pH 7.4) and was added to 96-well microplate. After incubation the reaction was stopped by addition of 100 µL methanol and an aliquot of sample was analysed

using thin-layer chromatography (TLC). TLC plates were developed with solvent system and processed with photographic film. The activity was expressed as the rate of glucuronide formation.

Serum hormone assays:

Serum hormone concentrations were determined using an immunoassay analyser for total triiodothyronine (T3) and thyroxine (T4), and a commercially available kit (Rat thyroid stimulating hormone (rTSH) [¹²⁵I] assay system for TSH).

Statistics:

Body weights were evaluated including osmotic pump after implantation. The following comparison procedures were used for analysis of data for body weight, body weight gains, food consumption, absolute and relative organ weights, microsomal protein level, hepatic enzyme activities, BrdU labelling indices and serum hormone levels.

For comparison among multiple groups, if the variables were assumed to follow a normal distribution by the Bartlett-test, the Dunnett-test was applied for a comparison of the treated groups with control groups for each sex. The Steel-test was applied instead of the Dunnett-test when the data could not be assumed to follow a normal distribution. For comparison between two groups, the F-test was applied to compare treated groups with the control group. If the variance was homogeneous, Student's t-test was used. If the variance was heterogeneous, the Aspin-Welch-test was used.

The Fisher's Exact probability test was similarly employed for analysis of gross pathological findings and non-gradable histopathological findings. The Wilcoxon rank sum test was used for analysis of the gradable histopathological findings, comparing treated and control groups. Each evaluation, except for BrdU labeling indices, was by 2-tailed tests with 0.05 and 0.01 as the levels of significance. For the BrdU labeling indices, the significance of differences was estimated by 1-tailed tests at probability levels of 1% and 5% to assess them for possible increases.

Findings:

Mortality and clinical signs:

No treatment-related abnormalities were detected. No animals died before terminal sacrifice.

Food consumption, body weight, and body weight gain:

Body weight gain during the first 4 days of treatment period was statistically significantly lower in both sexes administered 15000 ppm in both 7- and 14-day treatment groups, and these findings resulted in lower body weight. After the first 4 days, however, body weight gain was generally equivalent to or more than the control level, but total body weight gain remained lower at 15000 ppm in females of both 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 7-day treatment with S-2200 TG. Males at 7000 ppm also had decreased body weight and body weight gain after 7-day treatment.

Although suppression of body weight gains was observed in females administered PB on Day 8 in both 7- and 14-day treatment groups, the recovery group demonstrated no difference from control at Day 8.

Significant or a tendency toward suppression of food consumption was observed in both sexes administered 15000 ppm, especially in the early phase of the treatment period, however, these alterations were not observed after 14-day treatment. Less severe changes were observed in both sexes at 7000 ppm. In the recovery group, food consumption was increased after cessation of treatment with S-2200 TG.

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

Liver and thyroid weights, gross pathology and histopathology:

After 7-day treatment, liver weights were increased with statistical significance: absolute weights for both sexes at 15000 ppm, and relative weights of males at 7000 ppm and above and of females at 2000 ppm and above. For thyroid, a statistically significant increase was observed in the absolute and relative weights in females at 7000 ppm and above. 1000 ppm PB revealed statistically significant increases in the absolute and relative liver weights in both sexes.

After 14-day treatment, statistically significant increases were observed in the absolute and relative liver and thyroid weights in both sexes at 15000 ppm and PB group. In the recovery group, a statistically significant increase was observed: in the absolute and relative liver weights in males administered 15000 ppm, in the relative liver weights, and absolute and relative thyroid weights in males administered PB. However, all findings were less severe than those after 7-day treatment.

After 7-day treatment, an increased incidence of enlarged liver was visible in males receiving S-2200 TG 15000 ppm; and for females receiving PB 1000 ppm.

After 14-day treatment, the incidences of enlarged liver were increased in males and females receiving S-2200 TG 15000 ppm in males and females at PB 1000 ppm.

In the recovery group, there were no treatment-related findings.

After 7-day treatment, the incidences of diffuse hypertrophy of hepatocytes were increased in females at 2000 ppm and both sexes at 7000 and 15000 ppm. Centrilobular hypertrophy of hepatocytes was observed in all animals of PB groups. For thyroid, the incidences of diffuse follicular cell hypertrophy were statistically significantly increased in females at 7000 and 15000 ppm S-2200 TG. This finding was also observed in PB groups.

After 14-day treatment, the incidences of diffuse hypertrophy of hepatocytes were increased in both sexes at 15000 ppm. Centrilobular hypertrophy of hepatocytes was observed in all animals of the PB groups. For thyroid, the incidences of diffuse follicular cell hypertrophy were increased for males and females at 15000 ppm. This finding was also observed in the PB groups.

In the recovery group, the incidences of diffuse hypertrophy of hepatocytes were not disturbed among males at S-2200 TG 15000 ppm and PB 1000 ppm. Centrilobular hypertrophy of hepatocytes was observed in one female administered PB 1000 ppm. For thyroid, the incidences of diffuse follicular cell hypertrophy remained minimally elevated for males at PB 1000 ppm.

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 15000 ppm after 7-day treatment and 2/10 for male at 15000 ppm after 14-day treatment. No pigment was observed in the recovery animals.

The morphology of hepatocytes was assessed in controls and the highest dose level using electron microscopy. After the 7-day treatment, proliferation of smooth endoplasmic reticulum (SER) was observed in the hepatocytes from both sexes in the S-2200 TG 15000 ppm treated groups. Peroxisome size and number were not changed in the treatment group. Enlarged lipid droplets in the hepatocytes were observed in one female administered S-2200 TG at 15000 ppm.

Table B.6.8.1-2: Liver weights and (histo-) pathological findings

	Males						Females					
Dose level, ppm	0	400	2000	7000	15000	PB	0	400	2000	7000	15000	PB
	Relative weight (% control)											
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 recovery	100	ND	ND	ND	111	109*	100	ND	ND	ND	108	105
	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 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 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

* statistically significant from the control group (p < 0.05); ** statistically significant from the control group (p < 0.01)

Table B.6.8.1-3: Thyroid weights and (histo)pathological findings

	Males						Females					
Dose level, ppm	0	400	2000	7000	15000	PB	0	400	2000	7000	15000	PB
	Thyroid relative weight (% control)											
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**	124**
7 days + 7 days recovery	100	ND	ND	ND	102	124**	100	ND	ND	ND	112	105
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 recovery	1/10	ND	ND	ND	2/10	4/10	0/10	ND	ND	ND	0/10	0/10

* statistically significant from the control group (p < 0.05); ** statistically significant from the control group (p < 0.01)

Replicative DNA synthesis in the liver:

After 7 days of treatment, statistically significant increases in the BrdU labeling indices were noted in animals at 7000 ppm and above and in the PB group. After 14 days of treatment, a statistically significant increase was still observed in males administered 15000 ppm and females administered PB, but were attenuated compared to the 7-day treatment.

Table B.6.8.1-4: Replicative DNA Synthesis in the liver (BrdU labelling indices, % control)

Dose level, ppm	Males						Females					
	0	400	2000	7000	15000	PB	0	400	2000	7000	15000	PB
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*

* statistically significant from the control group (p < 0.05); ** statistically significant from the control group (p < 0.01)

Hepatic enzyme activity:

7-Pentoxoresorufin O-depentyrase (PROD) activity (CYP2B)

After 7 days of treatment, a statistically significant increase in CYP2B activity was observed in each sex 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 some recovery had taken place with a decrease in activity compared to the peak at 7-days of treatment.

Lauric acid hydroxylation (CYP4A)

After 7 days of treatment, a statistically significant increase in CYP4A activity was observed in both sexes of the PB group. However, no significant alterations were observed in the S-2200 TG groups. In the recovery group, a statistically significant increase in CYP4A activity was observed in males that had been administered 15000 ppm and statistically significant decrease in CYP4A activity was observed in females that had been administered 15000 ppm or PB. However, these alterations are marginal and are likely to be due to individual variation. Therefore, they are considered not to be of toxicological relevance.

UDP-glucuronosyltransferase (UGT) activity

After 7-days of treatment, statistically significant increases in T4-UGT activity were observed in males administered S-2200 TG at 400 ppm and higher (except for 2000 ppm) and females administered 15000 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 of T4-UGT activity was observed in each sex of the PB group. In the recovery group, a clear decrease of T4-UGT activity was detected.

Table B.6.8.1-5: Hepatic enzyme activity

	Males						Females					
Dose level, ppm	0	400	2000	7000	15000	PB	0	400	2000	7000	15000	PB
	CYP2B activity (% control)											
7 days	100	148	289*	872*	1358*	2510*	100	112	305*	2581*	7987*	26948*
7 days + 7 days recovery	100	ND	ND	ND	135	235**	100	ND	ND	ND	108	334**
	CYP4A activity (% control)											
7 days	100	98	99	120	124	167**	100	94	86	89	85	129*
7 days + 7 days recovery	100	ND	ND	ND	141**	121	100	ND	ND	ND	76*	77*
	UGT activity (% control)											
7 days	100	123*	130	150*	148*	191*	100	95	100	117	136**	123*
7 days + 7 days recovery	100	ND	ND	ND	113	136**	100	ND	ND	ND	109	110

* statistically significant from the control group ($p < 0.05$); ** statistically significant from the control group ($p < 0.01$)

Serum hormone levels:

After 7 days of treatment with S-2200 TG, a decrease in the serum concentration of T4 was observed in both genders administered 15000 ppm (statistically significant only in males) and statistically significant increase of TSH was observed in females administered 15000 ppm. Statistically significant decreases in the serum concentration of T4 and an increase in TSH were observed in females administered PB.

After 14 days of treatment, statistically significant decreases in the serum concentration of T4 and an increase in the serum concentrations of TSH were observed in both sexes of the 15000 ppm S-2200 TG and PB groups. Statistically significant decreases in the serum concentrations of T3 were observed in females of the same groups.

In the recovery group, statistically significant decreases in the serum concentration of T4 were observed in males administered 15000 ppm of S-2200 TG and statistically significant increases in T3 were observed in males administered PB.

Table B.6.8.1-5: Serum hormone levels

	Males						Females					
Dose level, ppm	0	400	2000	7000	15000	PB	0	400	2000	7000	15000	PB
	Serum [T4] (% control)											
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 recovery	100	ND	ND	ND	90*	102	100	ND	ND	ND	109	108
	Serum [TSH] (% control)											
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 recovery	100	ND	ND	ND	92	111	100	ND	ND	ND	122	98
	Serum [T3] (% control)											
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 recovery	100	ND	ND	ND	100	117*	100	ND	ND	ND	114	114

* statistically significant from the control group (p < 0.05); ** statistically significant from the control group (p < 0.01)

Conclusion:

The hypothesis that S-2200 TG induces hepatic metabolic enzymes via constitutive androstane receptor (CAR) activation similar to phenobarbital (PB), resulting in the liver and thyroid alterations seen in the rat studies, was tested. To evaluate the time course of alterations at 15000 ppm, 10 rats/sex/group were also fed diets containing 0 and 15000 ppm for 14 days. Data from both 7 and 14-day treatment groups were compared to determine whether enhancement or attenuation of alterations was observed. To evaluate reversibility of any findings, 10 rats/sex/group were also fed diets containing 0 and 15000 ppm S-2200 TG 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 PB.

Generally the mode of action (MOA) for phenobarbital-like liver and thyroid effects can be described as follows: Phenobarbital activates nuclear receptors, particularly the constitutive androstane receptor (CAR). This receptor translocates into the nucleus and dimerizes with the retinoid-X-receptor (RXR). The dimer then binds to specific response elements, resulting in transcriptional activation of genes regulating P450 expression, particularly expression of CYP2B. In turn, CYP4A expression is associated with activation of the peroxisome proliferator-activated receptor (PPAR) (Holsapple *et al*, Toxicological Sciences 2006: 89(1), 51-56). Phenobarbital-induced liver enlargement is associated with initial transient hyperplasia, and a substantial proliferation of smooth endoplasmic reticulum (SER), causing hepatocellular hypertrophy (Maronpot *et al*, Toxicologic Pathology 2010: 38, 776-795).

Another liver enzyme induced via the activated CAR receptor is UDP-glucuronosyltransferase (UGT). Induction of UGT leads to increased hepatic clearance of thyroxine (T4) by increased UDP glucuronosyltransferase activity towards T4 (T4-UGT). Circulating levels of T4 are monitored by the thyrotropic cells of the pituitary gland that are responsible for the synthesis of thyroid stimulating hormone (TSH). In the pituitary gland, T4 is metabolised to T3. Decreasing levels of T3 leads to stimulation of TSH synthesis and secretion. Sustained perturbation of the hypothalamic-pituitary-thyroid axis and the prolonged stimulation of the thyroid gland by TSH can lead to the progression of thyroid follicular cells to hypertrophy, hyperplasia, and eventually neoplasia (Dellarco *et al*, Critical Reviews in Toxicology 2006: 36, 793-801). It is generally accepted that this MOA is unlikely to be of relevance for humans, due to species differences in kinetic and dynamic factors (Holsapple *et al*, Toxicological Sciences 2006: 89(1), 51-56).

Administration of S-2200 TG resulted in increased liver weight in both genders after 7 and 14 days of administration. The changes were reversible after the 7 day recovery period. Liver weight changes caused by treatment with phenobarbital were comparable.

Whereas PB caused hepatocyte centrilobular hypertrophy in all animals in both genders, S-2200 TG caused clear increases in diffuse hepatocyte hypertrophy in both genders.

Similar stimulation of replicative DNA synthesis was caused by both substances after 7 days of substance administration, and in both cases, this response was weaker after 14 days of substance administration. Proliferation of the smooth endoplasmic reticulum was observed at 15000 ppm of S-2200 TG.

A clear induction of CYP2B activity was induced by administration of S-2200 TG and PB, which after the 7 day recovery period, was clearly decreased.

There was a weak, but statistically significant induction of CYP4A activity by PB, but not by S-2200 TG. After the 7 day recovery period, CYP4A activity was slightly increased in males and decreased in females; the biological significance of this finding is doubtful.

After 7 days, UGT activity was clearly increased in both genders after PB and S-2200 TG administration, with a clear tendency to reverse to normal after 7 days recovery.

Thyroid weight was statistically significantly increased in females after 7 days and in both genders after 14 days. This finding was associated with diffuse follicular hypertrophy. There was a tendency towards reversibility after the 7 day recovery period.

Serum T4 levels were decreased after 7 and 14 days of administration of PB and S-2200 TG, with a tendency to reverse to control levels after 7 days recovery.

Serum T3 levels were not yet decreased after 7 days of S-2200 TG treatment in both genders, 14 day treatment with S-2200 TG resulted in decreased serum T3 levels in females.

Serum TSH levels were increased in females after 7 days of treatment, and in both genders after 14 treatment days. The 7 days recovery period did lead to TSH levels had lowered.

In conclusion, the effects observed in the study are in agreement with the published MOA for phenobarbital: liver weight increase, proliferation of SER, initial stimulation of replicative DNA synthesis, specific induction of CYP2B and UGT activity, increased thyroid weight and hypertrophy and changes in serum T4, T3, and TSH levels were observed and showed clear indications of reversibility after a 7 day recovery period.

Material and methods:

Test animals:

Diet:	CRF-1 powdered feed (Oriental Yeast Co. Ltd, Tokyo) provided <i>ad libitum</i>
Housing:	Suspended aluminium cages with wire-mesh front and floor
In life dates:	25 January 2012 – 1 February 2012

Animal assignment and treatment:

10 male mice/group were fed diets containing 0 (control) and 7000 ppm S-2200 TG for 7 days.

Food consumption and body weight:

In the treatment period, body weights were measured on Days 1 (prior to dosing), 3, 6 and 8 (at the time of sacrifice). Food consumption for each cage was measured on Day 1 through Day 3, Day 3 through Day 6, over a period of 24 or 72 consecutive hours throughout the study.

Clinical observations:

Animals were observed for mortality once daily.

Sacrifice and pathology:

After the treatment period, mice were euthanized without prior fasting. Mice were decapitated under ether anaesthesia, and blood collected from the carotid artery and cervical vein. All organs and tissues from all animals were subjected to necropsy.

Organ weights:

Liver was weighed while wet from all animals. Relative organ weight (organ weight to body weight ratio) was calculated on the basis of the body weight on the day of sacrifice.

Histopathology:

Liver and duodenum fixed in buffered formalin from all animals were dehydrated, embedded in paraffin, sectioned, stained with haematoxylin and eosin, and examined with the light microscope. Histopathological analysis was carried out for liver.

Replicative DNA Synthesis:

Preparation and implantation of BrdU-containing osmotic pumps:

5-Bromo-2'-deoxyuridine (BrdU) was dissolved in 10% DMSO (40 mg BrdU/mL). Osmotic pumps were filled with 200 µL of this solution, and were implanted subcutaneously into the mice under ether anaesthesia 7 days prior to sacrifice. The pump was rated to supply 10µL/h of BrdU, for 7 days. This corresponds to a total release of:

$$40 \mu\text{g/h} \times 24 \text{ h/day} \times 7 \text{ days} = 6720 \mu\text{g BrdU}$$

BrdU labelling indices:

Thin sections of liver prepared from the same paraffin embedded blocks used for histopathology were stained immunohistochemically using BrdU monoclonal antibody to determine BrdU labelling indices. BrdU labelling was analysed blind to treatment at DIMS Institute of Medical Science, Inc. (Aichi, Japan). BrdU labelling indices were calculated by dividing the number of labeled hepatocellular nuclei by the total number of hepatocellular nuclei (%). The total number of cells evaluated per liver was greater than 2000. Sections of duodenum were also prepared and stained immunohistochemically with the BrdU monoclonal antibody on the same glass slide as liver to serve as a positive control to confirm appropriate administration of BrdU, but counts were not formally recorded. Immunohistochemical staining for BrdU of the duodenum in one control animal indicated that some problems occurred in the osmotic pump; the data for the BrdU labeling index for this animal was omitted from the analysis.

Hepatic enzyme analysis:

Preparation of liver S9 fractions

Liver S9 fractions were prepared. A portion of cryopreserved liver (approximately 0.5 g) from six animals, whose numbers were low in main and recovery groups, was homogenized in 4 volumes of Tris/HCl buffer. Liver homogenate was centrifuged to separate the S9 fraction. The protein level in the S9 fraction was determined using an assay kit with bovine serum albumin as the protein standard. Liver microsomal activity of 7-pentoxoresorufin O-depentyldase activity (PROD) was determined as marker of CYP2B.

The reaction mixture (200 µL) consisted of 3 µM 7-pentoxoresorufin, 10 µM dicoumarol, 1 mM NADPH, and 1 µL S9 fraction in Tris/HCl buffer (pH 7.4), and was added to a 96-well microplate. After incubation, the reaction was stopped by addition of 100 µL acetonitrile. PROD activity was determined by fluorometric analysis. The fluorescence of the sample was measured with a microplate reader. The activity was expressed as the rate of resorufin formation, and was calculated based on the fluorescence of a standard curve of resorufin.

Statistics:

Body weights were evaluated including the osmotic pump after implantation. The following comparison procedures were used for analysis of data for body weight, body weight gains, food consumption, absolute and relative organ weights, microsomal protein level, hepatic enzyme activities, and BrdU labeling indices. For comparison between two groups, the F-test was applied to compare treated groups with the control group. If the variance was homogeneous, the Student's t-test was used. If the variance was heterogeneous, the Aspin-Welch-test was used.

The Fisher's Exact probability test was similarly employed for analysis of gross pathological findings and non-gradable histopathological findings. The Wilcoxon rank sum test was used for analysis of the gradable histopathological findings, comparing treated and control groups.

Each evaluation, except for BrdU labeling indices, was by 2-tailed tests with 0.05 and 0.01 as the levels of significance. For the BrdU labeling indices, the significance of differences was estimated by 1-tailed tests at probability levels of 1% and 5% to assess them for possible increases.

Findings:

Mortality and clinical signs:

No treatment-related abnormalities were detected. No animals died before terminal sacrifice.

Food consumption, body weight, and body weight gain:

No remarkable changes in body weight, body weight gain, and food consumption were observed. The achieved compound intake was 814 mg/kg bw/day.

Organ weights:

After 7-day treatment, absolute liver weights in mice treated with S-2200 TG tended to increase compared to control, and relative liver weights were slightly but statistically significantly increased.

Gross pathology:

No remarkable differences between control and S-2200 TG 7000 ppm groups were observed.

Histopathology – light microscopy:

Slight eosinophilic change/hypertrophy of hepatocytes was observed in three of ten animals treated with S-2200 TG 7000 ppm in histopathology, none in the controls.

Replicative DNA synthesis:

No remarkable differences between control and S-2200TG 7000 ppm groups were observed in BrdU labeling indices.

Hepatic enzyme assay:

After 7-day treatment, statistically significant increase of PROD activity (CYP2B) (1.71-fold of control value) was observed in S-2200 TG 7000 ppm group.

Table B.6.8.1-6: Summary of study findings

Endpoints	Control	S-2200TG
Dose level (ppm S-2200 TG)	0	7000
Test item intake (mg/kg bw/day)	0	814
Liver absolute weight (% control)	100	106
Liver relative weight (% control)	100	107*
S9 protein content of liver (% control)	100	115*
Hepatic CYP2B activity (% of control)	100	171**
BrdU labeling index of hepatocytes (% of control)	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

* statistically significant from the control group ($p < 0.05$); ** statistically significant from the control group ($p < 0.01$)

Conclusion:

In the mouse, treatment with S-2200 TG resulted in increased liver weight with slight eosinophilic change/hypertrophy of hepatocytes and increased CYP2B activity. However, S-2200 TG did not enhance replicative DNA synthesis in hepatocytes during the short period of treatment.

Reference:	The toxicological relevance of the liver and thyroid alterations observed in rats treated with S-2200TG based on mode of action
Author(s), year:	██████████ 2012a
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. R01-0070
Guideline(s):	Not applicable, position paper
GLP:	-
Deviations:	-
Validity:	Yes

The unchanged position paper is included into Appendix 2 of chapter B.6 Toxicology and metabolism of this DAR.

Executive Summary:

In repeat dose toxicity studies the primary target organ of S-2200 TG is the liver in all species examined. The thyroid was also a target organ in the rat, but not in the mouse and 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 these two target organs in rat or mouse carcinogenicity studies.

In conjunction with evidence from the literature, mode of action studies were conducted to provide evidence that S-2200 TG is a hepatic enzyme inducer via at least constitutive androstane receptor (CAR) activation in the rat, in a manner similar to phenobarbital (PB). This was evidenced in the mode of action studies by induction of CYP2B activity and UDP-glucuronosyltransferase activity toward thyroxine (T4-UGT), and proliferation of smooth endoplasmic reticulum (SER). Therefore, the liver hypertrophy caused by S-2200 TG was judged to be an adaptive response via CAR mediated enzyme induction and not adverse. A similar mode of action also appears plausible in mouse and dog, and would theoretically operate in humans, as demonstrated by CYP2B induction.

At higher doses S-2200 TG induced adverse effects on the liver as evidenced by functional changes or additional pathological findings to the hypertrophy (multiple liver related blood biochemistry findings, hepatocyte vacuolation, degeneration). However, the adverse effects occurred in a dose related manner and there was a threshold at a relatively high exposure level. Most importantly, S-2200 TG did not induce liver tumours in the rat or mouse, reducing concern for the human risk assessment.

Secondly, data was obtained indicating that S-2200 TG increased T4-UGT activity, indirectly perturbing the hypothalamus-pituitary-thyroid hormone axis, and thereby inducing thyroid follicular-cell hypertrophy in rats, in a manner similar to PB, a CAR activator. The relevance of the rat thyroid abnormality to human health was assessed by using the 2008 IPCS Human Relevance Framework. The postulated mode of action (MOA) for possible induction of thyroid follicular-cell hypertrophy in rats was tested against the Bradford Hill criteria, and was found to satisfy the conditions of dose and temporal concordance, biological plausibility, coherence,

strength, consistency, and specificity that fit with a well-established MOA for thyroid follicular-cell hypertrophy. Although the postulated MOA could theoretically operate in humans, marked quantitative differences in the inherent susceptibility for thyroid abnormality, especially tumour induction, to thyroid hormone imbalance in rats is not relevant to humans. Therefore, even though liver and thyroid hypertrophy were induced by S-2200 TG in experimental animals, the findings from a MOA analysis allow the conclusion that S-2200 TG does not pose a practical hazard to humans.

Comment of the RMS:

The RMS agrees with the conclusions drawn in this position paper.

B.6.8.1.2 Ovary issues

Reference:	Interpretation of higher incidence of ovarian sex-cord stromal tumour in female rats treated with S-2200TG in a 2-year carcinogenicity study
Author(s), year:	██████████ 2012
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0069
Guideline(s):	Not applicable, position paper
GLP:	Not applicable, position paper
Deviations:	Not applicable, position paper
Validity:	Yes

Reference:	Up dated interpretation of higher incidence of ovarian sex-cord stromal tumour in female rats treated with S-2200TG in a 2-year carcinogenicity study
Author(s), year:	██████████ 2013
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0075
Guideline(s):	Not applicable, position paper
GLP:	Not applicable, position paper
Deviations:	Not applicable, position paper
Validity:	Yes

The unchanged position paper is included into Appendix 2 of chapter B.6 Toxicology and metabolism of this DAR.

Executive Summary:

S-2200 TG was not genotoxic in a battery of *in vitro* and *in vivo* assays. The tumourigenic potential of S-2200 TG was studied in male and female rats and mice in standard bioassays under the guidelines of Good Laboratory Practice and the test protocols designated by authorities.

An increased incidence of ovary sex-cord stromal tumour (SCST) was observed in female rats, exceeding the historical control range for this strain of rats. Therefore, a causal relationship between S-2200 TG administration and the ovary tumour induction was not ruled out. In the mouse study, the number of tumours in any tissue did not increase by exposure to S-2200 TG. Therefore, one tumour type (benign) in one sex (female) of one species (the rat) occurred in one study. Four and six cases of benign ovarian SCST occurred in female rats exposed to 7000 and 15000 ppm (475 and 1016 mg/kg/day) S-2200 TG, respectively. These were dose levels at which body weight gain was reduced by > 20%, indicating that the maximum tolerated dose was exceeded. It must be highlighted that also the current controls (two cases of benign ovarian SCST) exceeded the historical control range for this strain of rats.

Sex-cord stromal hyperplasia is quite common in aged Wistar rats, and the animals used in the 2-year study with S-2200 TG appear to be derived from a susceptible batch. The incidences of sex-cord stromal proliferative lesions were well within historical controls for all groups, and there was no statistical difference between groups for hyperplasia, tumours, or hyperplasia plus tumours. Higher survival rates in the two

higher groups may contribute to the higher number of ovarian tumours. The known modes of action via endocrine imbalance are unlikely, evidenced by the lack of interaction with the estrogen receptor and steroidogenesis by *in vitro* assays, no direct ovarian toxicity, and no reproductive abnormality. Furthermore, there was no accumulation or persistence of S-2200 and its metabolites in the ovary. Thus, the sex-cord stromal lesions are unlikely to be direct effects of treatment with S-2200 TG.

Based on these considerations, the increased incidence of the SCST observed in the 2-year rat study is not toxicologically significant. Therefore, the overall conclusion is that the data do not suggest a carcinogenic effect of S-2200 TG and thus its classification is not warranted.

Comment of the RMS:

The RMS agrees with the conclusions drawn in this position paper.

Reference:	<i>In vitro</i> Steroidogenesis Assay of S-2200TG in H295R Cells
Author(s), year:	Kubo, H.; 2012
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0065
Guideline(s):	OECD 456
GLP:	No
Deviations:	none
Validity:	Yes

Material and methods:

Test Material:	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G (Batch specification 01)
Purity:	93.4%
Stability of test compound:	Expiry date: June 14 th 2014 (after completion of treatment)
Control Materials:	
Negative:	Solvent, DMSO
Positive:	Forskolin (10µM) for induction Prochloraz (1µM) for repression
Test organism:	Human adrenocortical NCI-H295R cells (ATCC, Cat. No. CRL-2128)
Test concentrations:	Based on the solubility and the potential interference with the hormone measurement systems, the maximum concentrations of S-2200TG for the initial run was set to 100 µM. In run 1, 100 µM of S-2200TG was cytotoxic (≤ 80% viability). Therefore the maximum concentration for subsequent runs was 30 µM. (Run 1) 10 nM, 100 nM, 1 µM, 10 µM, 100 µM (Run 2) 300 nM, 1 µM, 3 µM, 10 µM, 30 µM (Run 3) 300 nM, 1 µM, 3 µM, 10 µM, 30 µM (Run 4) 3 µM, 10 µM, 30 µM

Test procedure

In order to evaluate the effects of S-2200 TG on androgen and estrogen production, H295R cells cultured in 24-well plates were incubated with S-2200 TG in triplicate for 48 hours. For the evaluation of testosterone (T) and estradiol (E2) productions, four (Run 1, 2, 3 and 4) and three (Run 1, 2 and 3) independent experiments

were performed, respectively. Dimethylsulfoxide (DMSO) was used as the vehicle at a final concentration of 0.1%.

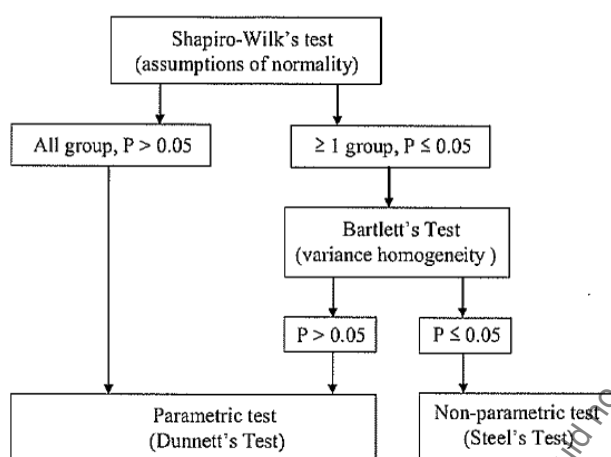
Positive controls (forskolin, a known inducer, and prochloraz, a known inhibitor) were evaluated concurrently with each run to confirm the changes in T and E2 levels in the assay.

T and E2 levels were measured using ELISA systems.

Cytotoxicity was evaluated after removal of the culture medium containing S-2200 TG using “Cell Counting Kit-8” (DOJINDO LABORATORIES).

Statistics:

To evaluate the relative increase or decrease in chemically altered hormone production, test chemical data were normalised to the mean solvent control values in the test plates. Furthermore, the results were normalised to cell viability when the cytotoxicity was less than 20%. The statistical analysis was carried according to the following procedure:



Differences were considered significant at $p \leq 0.05$.

Evaluation criteria:

According to the criteria laid down in the guideline, a chemical is judged to be positive if the induction is statistically different ($p \leq 0.05$) from the solvent control at two adjacent concentrations in at least two independent runs. A chemical is judged to be negative following two independent negative runs, or in three runs, comprising two negative runs and one equivocal (fold change at one concentration is statistically significantly different from the solvent control) or positive run. Results at concentrations exceeding the limits of solubility or at cytotoxic concentrations were not included in the interpretation of results.

Findings:

10 μM of forskolin increased the levels of T and E2 more than 1.5-fold and 7.5-fold respectively, compared to the solvent control. 1 μM of prochloraz decreased both the levels of T and E2 less than 0.5-fold compared to the solvent control. These results indicated that present assay systems were validated by the positive control experiments, as required by the guideline.

In Run 1, S-2200 TG showed cytotoxicity to H295R cells at 100 μM . Therefore, the maximum S-2200 TG concentration was set to 30 μM in subsequent runs. No clear changes in T and E2 levels were observed in the assays (Run 1 through 4).

Table B.6.8.1-7: Summary of cell viability (relative to solvent control), T production (relative to solvent control), and E2 production (relative to solvent control)

Concentration	Run 1			Run 2			Run 3			Run 4	
	T	E2	% Cell viability	T	E2	% Cell viability	T	E2	% Cell viability	T	% Cell viability
Solvent control	1.00	1.00	100.0	1.00	1.00	100.0	1.00	1.00	100.0	1.00	100.0
S-2200TG											
10 nM	1.03	1.17	106.2								
100 nM	1.02	1.11	107.2								
300 nM				1.00	1.03	109.1	1.02	1.07	97.4		
1 µM	1.00	1.10	102.6	1.01	1.14	107.1	1.01	1.09	94.5		
3 µM				0.92	1.21	99.0	0.96	1.06	96.7	1.04	95.5
10 µM	0.93	1.05	101.3	0.95	1.12	96.8	1.00	1.21*	97.3	0.97	102.6
30 µM				0.82*	1.09	98.2	1.01	1.03	82.8	0.98	81.1
100 µM			71.6								
Forskolin 10 µM	1.54	>15.07 ^a	130.6	2.25	16.14	123.9	1.90	>12.50 ^a	113.5	3.48	114.0
Prochloraz 1 µM	0.08	<0.24 ^a	100.6	0.10	<0.26 ^a	106.8	0.10	0.36	91.7	0.17	90.8

^a: Measured OD value was out of the linear range of the standard curve. Fold-change was calculated from the Limit of quantification (31.25 and 500 pg/mL) normalised by cell viability.

* P ≤ 0.05

Conclusions:

Testosterone production

Through Run 1 to 4, no significant differences in T production from the solvent control at two adjacent concentrations were detected. Judging from these results, it is concluded that S-2200 TG does not influence testosterone production in H295R cells at concentrations up to 30 µM.

Estradiol production

No significant differences in E2 production were observed in Run 1 or 2. Significant increases in E2 production was only found at 10 µM in Run 3, however significant increases were not detected at 3 or 30 µM. Judging from these results, all three Runs are considered negative. Therefore it is concluded that S 2200 does not influence E2 production in H295R cells at concentrations up to 30 µM.

The positive controls produced satisfactory results (i.e. induction 1.54-3.48 for T and > 12.50-16.14 for E2; inhibition: 0.08-0.17 for T and < 0.24-0.36 for E2), thus confirming the validity of the study.

Reference:	Evaluation of Effects of S-2200 TG and its Metabolites on Human Estrogen Receptor alpha and Human Androgen Receptor Using <i>in vitro</i> Reporter Gene Assays
Author(s), year:	Suzuki, N.; 2012
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0066
Guideline(s):	OECD 455, Draft OECD Guideline 'Stably Transfected Human Androgen Receptor-α Transcriptional Activation Assay for Detection of Androgenic Agonist/Antagonist Activity of Chemicals', Version 2010, Nov 25
GLP:	No
Deviations:	Both guidelines were combined and adapted to detect estrogen and androgen

	receptor agonistic and antagonistic activities of the test substances
Validity:	Yes

Material and methods:

Test Materials:

S-2200 Technical Grade (S-2200 TG)

Lot/Batch:	ST-0811G (Batch specification 01)
Purity:	93.4%

5-COOH-S-2200

Lot/Batch:	252-001-55-2
Purity:	99.7%

4-OH-S-2200

Lot/Batch:	CTS08026
Purity:	99.9%

5-CH₂OH-S-2200

Lot/Batch:	CTS08030
Purity:	99.9%

5-CA-S-2200-NHM

Lot/Batch:	CTS09005
Purity:	94.9%

Control Materials:

Negative:	Solvent, DMSO
Positive:	17 β -Estradiol (E2) (purity > 97%) for hER α activation 4-Hydroxytamoxifen (HTM) (purity > 98%) for hER α inhibition Dihydrotestosterone (DHT) (purity > 99%) for hAR activation Hydroxyflutamide (HFL) (purity > 98%) for hAR inhibition

Test organisms:

For human estrogen receptor α (hER α) assays:

hER α -HeLa-9903 (Stably transformed cell line derived from Human uterine cervix carcinoma HeLa cells)

For human androgen receptor (hAR) assays:

hAR-HeLa 4-11 (Stably transformed cell line derived from Human uterine cervix carcinoma HeLa cells)

Test concentrations:

Test chemicals were evaluated by cytotoxicity assays (100 pM -100 μ M)

Based on the results of the cytotoxicity assays, the maximum concentrations of the test substances in the receptor activation assays were:

S-2200 TG	hER α : 6 μ M	hAR: 10 μ M
5-COOH-S-2200	hER α : 100 μ M	hAR: 100 μ M

4-OH-S-2200	hERα: 10 µM	hAR: 10 µM
5-CH ₂ OH-S-2200	hERα: 10 µM	hAR: 100 µM
5-CA-S-2200-NHM	hERα: 100 µM	hAR: 100 µM

Cytotoxicity assays:

To determine the appropriate concentration ranges of test substances, cytotoxicity assays were carried out using hERα-HeLa-9903 and hAR-HeLa 4-11 cells. For detection of cell viability, 96 well microplates were prepared by adding test substances dissolved in DMSO at various concentrations.

hERα-HeLa-9903 or hAR-HeLa 4-11 cells were homogeneously suspended in DMEM (phenol free) containing 10% charcoal- treated FBS and plated to each well (n = 6).

After cell culturing at 37°C under 5% CO₂, the viability of the cells was measured by the CellTiter Glo Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's instructions. If cell viability was reduced by more than 20%, the concentrations were regarded as cytotoxic. The data at or above cytotoxic concentrations were excluded from the evaluation according to OECD TG 455.

Reporter gene assays to evaluate agonist and antagonist activities:

In agonist assays using hERα and hAR, positive controls (1 nM of E2 and DHT, respectively) and a vehicle control (DMSO) were analysed in each study.

hERα-HeLa-9903 or hAR-HeLa 4-11 cells were homogeneously suspended in DMEM (phenol free) containing 10% charcoal- treated FBS and plated to each well of 96 well plates (n = 6), and were exposed to serially diluted test substances in DMSO.

In antagonist assays using hERα and hAR, positive controls (HTM and HFL, respectively) and a vehicle control were analysed in each study. hERα-HeLa-9903 or hAR-HeLa 4-11 cells were plated in a manner similar to the agonist assays, and were exposed to serially diluted test chemicals in DMSO with typical hormones (100 pM of E2 and 100 pM of DHT, respectively).

After cell culturing for about 24 (for hERα) or 48 hours (for hAR) at 37°C under 5% CO₂, the medium was removed, and 50 µl of Steady-Glo Luciferase Assay System (Promega) was added to each well.

Cells were lysed by shaking for 30 minutes at room temperature in the dark, and the luciferase activity was measured using 96 wells format luminometer (TopCount NXT, Hewlett-Packard Company, Palo Alto, CA, USA). The luminescence data was converted into Excel format.

Data acceptance criteria:

In agonist assays, a study is judged to be acceptable when more than 4-fold or 10-fold of relative activity was observed at a concentration of 1 nM of E2 (for hERα) or DHT (for hAR), against the vehicle control according to the OECD TG 455 (for hERα) and/or our historical data (for hAR).

In antagonist assays, a study is judged to be acceptable when the value of more than 50% inhibition was observed at a concentration of 1 µM of HTM (for hERα) or HFL (for hAR), against the vehicle control according to our historical data.

Evaluation criteria:

In agonist assays, data analysis was performed for evaluation of test substances according to the OECD TG455 as follows:

- a : mean values of a vehicle control (DMSO)
- b : mean values of positive controls (1 nM of E2 or DHT)
- c : mean values of test substances
- B (relative activity of positive controls): b/a
- C (relative activity of test substances): c/a

PC10: (B-1)/10+1

Judgment:

Positive: PC10 ≤ C Negative: PC10 > C

In antagonist assays, data analysis was performed according to a draft report of OECD guideline for the stably transfected human androgen receptor transcriptional assay for detection of androgenic agonist/antagonist activity of chemicals (Version 2010 Nov.25) as follows:

a : mean values of a vehicle control (DMSO)

b : mean values of positive controls (HTM or HFL)

c : mean values of test substances

C (relative activity of test substances): c/a ×100

Percent inhibition (positive controls): 100-B

Percent inhibition (test substances: 100-C

Judgment:

Positive: Percent inhibition ≥ 30 Negative: Percent inhibition < 30

Findings:

Cytotoxicity assays:

The results are summarized in the following tables. As S-2200 TG was cytotoxic at 1 µM, a further assay was performed with concentrations between 1 and 10 µM S-2200 TG.

Table B.6.8.1-8: hERα-HeLa-9903 viability (mean % relative to vehicle control, n = 6), first assay

Concentration	S-2200 TG	5-COOH-S-2200	4-OH-S-2200	5-CH ₂ OH-S-2200	5-CA-S-2200-NHM
0	100	100	100	100	100
100 pM	102	108	108	113	112
1 nM	110	112	115	119	116
10 nM	110	111	114	118	117
100 nM	109	112	114	118	118
1 µM	104	112	115	119	116
10 µM	59*	110	94	114	115
100 µM	29*	98	36*	72*	92

*Concentration excluded from analysis as cell viability was reduced ≥ 20% compared to solvent controls

Table B.6.8.1-9: hERα-HeLa-9903 viability (mean % relative to vehicle control, n=6), second assay

Concentration	S-2200 TG
0	100
1 µM	107
2 µM	104
4 µM	95
6 µM	81
8 µM	78*
10 µM	58*

*Concentration excluded from analysis as cell viability was reduced ≥ 20% compared to solvent controls

Table B.6.8.1-10: hAR-HeLa 4-11 viability (mean % relative to vehicle control, n=6), second assay

Concentration	S-2200 TG	5-COOH-S-2200	4-OH-S-2200	5-CH ₂ OH-S-2200	5-CA-S-2200-NHM
0	100	100	100	100	100
100 pM	103	102	101	103	102
1 nM	105	103	104	103	104
10 nM	104	102	102	104	102
100 nM	105	102	103	104	102
1 µM	104	102	103	104	103
10 µM	97	102	100	104	102
100 µM	34*	99	70*	96	98

*Concentration excluded from analysis as cell viability was reduced $\geq 20\%$ compared to solvent controls

Agonist and Antagonist Assays for hER α :

The relative activity of a typical agonist E2 (1 nM) was more than 4-fold in both assays. This result satisfied the acceptance criteria.

The relative activities of S-2200 TG, 5-COOH-S-2200, 4-OH-S-2200, 5-CH₂OH-S-2200 and 5-CA-NHM-S-2200 did not exceed the PC10 values.

It was thus concluded that S-2200 TG, 5-COOH-S-2200, 4-OH-S-2200, 5-CH₂OH-S-2200 and 5-CA-S-2200-NHM had no agonistic activities towards hER α .

Table B.1.8.1-11: Agonistic activities (mean ratio of activity to vehicle control, n = 6) of S-2200 TG and its metabolites to hER α , first assay

Concentration	Positive control E2	S-2200TG	5-COOH-S-2200	4-OH-S-2200	5-CH ₂ OH-S-2200	5-CA-S-2200-NHM
0	1.00	1.00	1.00	1.00	1.00	1.00
10 pM	2.20*	NT	NT	NT	NT	NT
100 pM	4.16*	0.99	1.05	1.07	0.93	1.11
1 nM	6.30*	1.04	1.00	0.97	0.93	0.98
10 nM	5.96*	1.13	1.04	0.96	0.97	0.94
100 nM	4.99*	1.02	0.95	1.00	0.98	1.17
1 µM	4.78*	1.01	1.09	1.09	0.90	1.09
10 µM	NT	NT	1.01	1.14	0.87	1.27
100 µM	NT	NT	1.13	NT	NT	0.94

*Positive effect was observed (criterion: relative activity exceeds the PC10 value of 1.53)

NT = Not tested. E2 = 17 β -estradiol

Table B.1.8.1-12: Agonistic activities (mean ratio of activity to vehicle control, n = 6) of S-2200 TG and its metabolites to hER α , second assay

Concentration of S-2200 TG	mean ratio of activity to control	Concentration of positive control E2	mean ratio of activity to control
0 µM	1.00	0	1.00
1 µM	1.08	10 pM	1.82*
2 µM	1.11	100 pM	4.13*
4 µM	1.17	1 nM	5.46*
6 µM	1.18	10 nM	5.22*
8 µM	NT	100 nM	5.63*
10 µM	NT	1 µM	4.77*

		10 µM	NT
--	--	--------------	----

*Positive effect was observed (criterion: relative activity exceeds the PC10 value of 1.45)

NT = Not tested. E2 = 17β-estradiol

For measurement of antagonistic activities, the cells were exposed to the various chemicals and the typical activator E2 at 100 pM.

Since more than 50% inhibition value was observed at a concentration of 1 µM of HTM, the assay system was validated.

The inhibition caused by S-2200 TG, 5-COOH-S-2200, 4-OH-S-2200, 5-CH₂OH-S-2200 and 5-CA-NHM did not exceed 30% of the inhibition caused by 1 µM HTM in both assays.

Therefore, it was concluded that S-2200 TG, 5-COOH-S-2200, 4-OH-S-2200, 5-CH₂OH-S-2200 and 5-CA-S-2200-NHM had no antagonistic activities towards hERα.

Table B.1.8.1-13: Antagonistic activities (mean % inhibition relative to vehicle control, n = 6) of S-2200 TG and its metabolites to hERα, first assay

Concentration	Positive control HTM	S-2200TG	5-COOH-S-2200	4-OH-S-2200	5-CH ₂ OH-S-2200	5-CA-S-2200-NHM
0	0	0	0	0	0	0
10 pM	2	NT	NT	NT	NT	NT
100 pM	-34	-2	-16	-17	-22	-17
1 nM	-28	-17	-10	-14	-10	-41
10 nM	42*	-11	-11	-2	3	-50
100 nM	52*	-8	-6	-20	-20	-43
1 µM	59*	-19	9	4	-19	-47
10 µM	72*	NT	-17	0	1	-28
100 µM	NT	NT	14	NT	NT	-5

*Positive effect was observed (criterion: antagonistic activity is equal to or exceeds 30% of 1 µM HTM)

NT = Not tested. HTM = 4 Hydroxytamoxifen

Table B.1.8.1-14: Antagonistic activities (mean % inhibition relative to vehicle control, n = 6) of S-2200 TG and its metabolites to hERα, second assay

Concentration of S-2200TG	mean ratio of activity to control	Concentration of positive control HTM	mean ratio of activity to control
0 µM	0	0	0
1 µM	1	10 pM	-5
2 µM	-10	100 pM	-27
4 µM	1	1 nM	-33
6 µM	-8	10 nM	48*
8 µM	NT	100 nM	62*
10 µM	NT	1 µM	62*
		10 µM	72*

*Positive effect was observed (criterion: antagonistic activity is equal to or exceeds 30% of 1 µM HTM)

NT = Not tested. HTM = 4 Hydroxytamoxifen

Agonist and Antagonist Assays for hAR:

More than 10-fold induction of relative activity was observed at 1 nM of DHT, thus the acceptance criteria were satisfied.

The relative activities of S-2200 TG, 5-COOH-S-2200, 4-OH-S-2200, 5-CH₂OH-S-2200 and 5-CAS-2200-NHM did not exceed PC10 values.

It was, therefore, concluded that S-2200 TG, 5-COOH-S-2200, 4-OH-S-2200, 5-CH₂OH-S-2200 and 5-CAS-2200-NHM had no agonistic activities to hAR.

Table B.1.8.1-15: Agonistic activities (mean ratio of activity to vehicle control, n=6) of S-2200 TG and its metabolites to hAR

Concentration	Positive control DHT	S-2200 TG	5-COOH-S-2200	4-OH-S-2200	5-CH ₂ OH-S-2200	5-CAS-2200-NHM
0	1.00	1.00	1.00	1.00	1.00	1.00
10 pM	1.86	NT	NT	NT	NT	NT
100 pM	13.67*	0.95	0.96	0.97	0.95	0.98
1 nM	24.89*	0.94	0.96	0.95	0.94	0.95
10 nM	26.10*	0.91	0.94	0.93	0.93	0.91
100 nM	25.82*	0.89	0.94	0.90	0.90	0.90
1 µM	27.25*	0.93	0.91	0.93	0.92	0.93
10 µM	24.15*	1.01	0.90	1.00	0.92	0.93
100 µM	NT	NT	0.99	NT	0.81	1.01

* - Positive effect was observed (criterion: relative activity exceeds the PC10 value of 3.39)

NT = Not tested. DHT = Dihydrotestosterone

For measurement of antagonistic activities, the cells were exposed to the various chemicals and the typical activator DHT at 100 pM.

Since more than 50% inhibition of activation was observed at 1 µM of HFL, the assay system was validated. The percent inhibition values of S-2200 TG, 5-COOH-S-2200, 4-OH-S-2200, 5-CH₂OH-S-2200 and 5-CAS-2200-NHM did not exceed 30% of the inhibition caused by 1 µM HFL.

It was, therefore, concluded that S-2200 TG, 5-COOH-S-2200, 4-OH-S-2200, 5-CH₂OH-S-2200 and 5-CAS-2200-NHM had no antagonistic activities to hAR.

Table B.1.8.1-16: Antagonistic activities (mean % inhibition relative to vehicle control, n=6) of S-2200 TG and its metabolites to hAR

Concentration	Positive control HFL	S-2200 TG	5-COOH-S-2200	4-OH-S-2200	5-CH ₂ OH-S-2200	5-CAS-2200-NHM
0	0	0	0	0	0	0
10 pM	6	NT	NT	NT	NT	NT
100 pM	12	8	7	8	8	7
1 nM	14	9	8	13	12	12
10 nM	31*	13	11	11	15	13
100 nM	69*	12	10	13	16	12
1 µM	88*	12	11	12	15	13
10 µM	79*	16	8	12	9	11
100 µM	NT	NT	13	NT	15	11

Conclusions:

S-2200 TG and its metabolites (5-COOH-S-2200, 4-OH-S-2200, 5-CH₂OH-S-2200 and 5-CAS-2200-NHM) did not show agonistic or antagonistic effects on hERα and hAR –induced transcriptional activation under the conditions of the present study.

B.6.8.2 Summary of mechanistic studies

Several *in vivo* and *in vitro* mechanistic studies were performed, and two detailed position papers were submitted dealing with (i) liver and thyroid effects and (ii) ovary issues.

Two *in vivo* studies were performed (one in rats, one in mice) to gain insight on the mechanistic basis of the liver and thyroid effects observed in the main studies in rats. The effects observed were compared to CAR-mediated induction of liver enzymes and subsequent perturbations of thyroid hormones, a well-studied mode of action with no relevance for human risk assessment.

A detailed description of the effects observed in the main and mechanistic studies, as well as a detailed assessment of the mode of action of mandestrobin is provided in a position paper included in this report.

The proposed phenobarbital-like mode of action for mandestrobin tested in the rat mechanistic *in vivo* 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 render it non relevant for humans. Furthermore, no increased tumour rates were observed up to the highest doses tested in the long term/carcinogenicity studies.

Two *in vitro* studies were performed to address possible hormonal effects of mandestrobin and its metabolites. Mandestrobin had no influence on testosterone and estradiol production, and 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.

In a position paper (and its update), the toxicological significance of the slightly higher incidence of benign ovarian sex-cord stromal tumours in the 2-year rat study is discussed based on existing data of mandestrobin, background data of ovarian proliferative change present in elderly rats, and published information. The overall conclusion is that the data do not suggest a carcinogenic effect of mandestrobin and thus classification is not warranted.

Table B.6.8.2-1: Summary of mechanistic studies

Study	Species/ test cells	Results
<i>In vivo</i> studies		
Short-Term Study for Mode of Action Analysis for Rat Liver and Thyroid Findings by S-2200TG –Dose Response, Time-Course and Reversibility [REDACTED] 2012e	Rats (Crj:WI)	Mandestrobin causes increased liver weight with diffuse, hepatocellular hypertrophy, proliferation of liver smooth endoplasmic reticulum, increased liver CYP2B and T4-UGT activity, and transient increase of replicative DNA synthesis in a dose dependent and reversible manner. In the thyroid, mandestrobin causes slight hypertrophy, slight decreases of serum T4 levels and slight increases in TSH in both genders after 14 days. These findings were reversible.
Short-Term Study for Mode of Action Analysis for Mouse Liver Findings by S-2200TG [REDACTED] 2012b	Mice (Crj:CD1(ICR))	7 days treatment with mandestrobin caused slight increases of liver weight, and induction of CYP2B activity.
<i>In vitro</i> studies		

Study	Species/ test cells	Results
<i>In vitro</i> Steroidogenesis Assay of S-2200TG in H295R Cells [REDACTED] 2012	Human adrenocortical NCI-H295R cell line	No influence of mandestrobin on testosterone and estradiol production was observed
Estrogen Receptor alpha and Human Androgen Receptor Using <i>in vitro</i> Reporter Gene Assays [REDACTED] 2012	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 and hAR – induced transcriptional activation
Position papers		
The toxicological relevance of the liver and thyroid alterations observed in rats treated with S-2200TG based on mode of action [REDACTED] 2012	-	Mode of Action analysis for mandestrobin shows a phenobarbital-like mechanism
Interpretation of higher incidence of ovarian sex-cord stromal tumour in female rats treated with S-2200TG in a 2-year carcinogenicity study [REDACTED] 2012	-	The increased incidence of benign ovary sex-cord stromal tumour observed in females in the 2-year rat study is not considered to be a carcinogenic effect of mandestrobin and, therefore, does not trigger classification for carcinogenicity
Up dated interpretation of higher incidence of ovarian sex-cord stromal tumour in female rats treated with S-2200TG in a 2-year carcinogenicity study [REDACTED] 2013		

B.6.8.3 Toxicity studies on metabolites

Mandestrobin has several metabolites for which the following tests were performed and submitted:

Table B.6.8.3-1: Toxicity studies performed with metabolites of Mandestrobin

Metabolite	Occurs in	Type of study
2-COOH-S-2200	Groundwater, rat, hen, goat	Acute oral toxicity
		Ames test
		<i>In vitro</i> chromosomal aberration
		<i>In vitro</i> mammalian gene mutation assay
		<i>In vivo</i> micronucleus assay
5-COOH-S-2200	Soil, groundwater, surface water, rat, crops, rotational crops, hen, goat	Acute oral toxicity
		Ames test
		<i>In vitro</i> chromosomal aberration
		<i>In vitro</i> mammalian gene mutation assay
2-CH ₂ OH-S-2200	Rat, crops, rotational crops, hen, goat	Acute oral toxicity
		Ames test
4-OH-S-2200	Rat, crops, rotational	Acute oral toxicity

	crops, hen, goat	Ames test
De-Xy-S-2200	Rat, crops, rotational	Acute oral toxicity
	crops, hen, goat	Ames test

Metabolite 2-COOH-S-2200

Reference:	Acute Oral Toxicity Study of 2-COOH-S-2200 in Rats.
Author(s), year:	2012a
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0043
Guideline(s):	OECD 423; EC Directive 2004/73/EC, Acute toxic (oral); Japanese MAFF (12-Nousan No. 8147)
GLP:	Yes (Lab certified by National Authority)
Deviations:	None
Validity:	Yes

Material and methods:

<i>Test Material:</i>	2-COOH-S-2200
Lot/Batch:	Lot No.: 317-001-47-1
Purity:	99.0%
Stability of test compound: spanning the study period	Confirmed for the duration of the study by Certificates of Analysis

<i>Test animals:</i>	
Species:	Rat (females only)
Strain:	:Wistar Hannover/Rcc
Age:	8 weeks (at start of study)
Weight at dosing:	First group: 151 – 160 g Second group: 155 – 161 g
Source:	
Diet:	CRF-1 Pellet diet from Oriental Yeast Co, Ltd. Tokyo, Japan; <i>ad libitum</i>

Animals in the first group of three were each given a single gavage dose of 2000 mg/kg bw 2-COOH S-2200 TG in 0.5% aqueous methylcellulose. When no response was observed in the first group, a second group of three animals was dosed at 2000 mg/kg bw. Accuracy of dose formulation was confirmed by analysis. Clinical and mortality observations were made at 10 and 30 minutes, 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/tissues examined macroscopically.

Findings:

No mortality was observed.
There were no clinical signs throughout the observation period.
The body weights were not affected by the administration of the test compound throughout the study period.
There were no findings at the gross pathological examination.

Conclusion:

No mortality occurred after administration of 2000 mg/kg bw (limit test). According to Regulation (EC) No. 1272/2008, classification of metabolite 2-COOH-S-2200 for acute toxicity is not required.

Reference:	Reverse Mutation Test of 2-COOH-S-2200 in Bacterial Systems
Author(s), year:	Kitamoto, S.; 2012a
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0041
Guideline(s):	US EPA OPPTS 870.5100, OECD 471, EU regulation 440/2008 B13/14, Japanese MAFF 12 Nousan 8147
GLP:	Yes (Lab certified by National Authority)
Deviations:	None
Validity:	Yes

Material and methods:

Test Material:	2-COOH-S-2200
Lot/Batch:	Lot No.: 317-001-47-1
Purity:	99.0%
Stability of test compound:	Confirmed for the duration of the study by Certificates of Analysis spanning the study period. Stability was confirmed analytically in the DMSO solvent.
Solvent:	DMSO

Test strains and chemicals used as positive controls:

Without metabolic activation:

<i>S. typhimurium</i> TA100	0.01 µg/plate	2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
<i>S. typhimurium</i> TA1535	0.5 µg/plate	sodium azide
<i>E. coli</i> WP2uvrA	0.01 µg/plate	2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
<i>S. typhimurium</i> TA98	0.1 µg/plate	2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
<i>S. typhimurium</i> TA1537	80 µg/plate	9-aminoacridine

With metabolic activation:

<i>S. typhimurium</i> TA100	1 µg/plate	2-aminoanthracene
<i>S. typhimurium</i> TA1535	2 µg/plate	2-aminoanthracene
<i>E. coli</i> WP2uvrA	10 µg/plate	2-aminoanthracene
<i>S. typhimurium</i> TA98	0.5 µg/plate	2-aminoanthracene
<i>S. typhimurium</i> TA1537	2 µg/plate	2-aminoanthracene

Drug metabolizing enzyme system (S9 mix):

S9-fraction:	Oriental Yeast Co., Ltd. (Tokyo, Japan); Lot No. 11042201 Liver fraction prepared from male Sprague Dawley rats induced by phenobarbital and 5,6-benzoflavone
S9-mix:	10% (v/v) S9-fraction 8 µmol MgCl ₂ 33 µmol KCl 5 µmol glucose-6-phosphate 4 µmol NADPH 4 µmol NADH 100 µmol Na-phosphate buffer (pH 7.4)

2-COOH-S-2200 was evaluated for its mutagenic potential by a reverse mutation test with four strains of *Salmonella typhimurium* (TA100, TA98, TA1535, and TA1537) and one strain of *Escherichia coli* (WP2uvrA).

The test was conducted by the preincubation method in the presence and absence of metabolic activation (rat liver S9 mix).

In a dose-finding assay the doses ranged from 156-5000 µg/plate (Assay I). No precipitation or cytotoxicity was observed.

In the main assays, 2-COOH-S-2200 was tested in triplicate at doses ranging from 156 - 5000 µg/plate with and without S9 mix in all strains (Assay II). There was no statistically significant dose-related increase in the number of revertant colonies to at least twice as many as that of the solvent control with or without S9 mix. Positive controls showed the appropriate increase in the number of revertant colonies.

Based on these results, it is concluded that 2-COOH-S-2200 is not mutagenic under the test conditions.

Findings:

There was no statistically significant dose-related increase in the number of revertant colonies to at least twice as many as that of the solvent control with or without S9 mix. The negative result was replicated (Assay II). Positive controls showed an appropriate increase in the number of revertant colonies.

Table B.6.8.3-2: Results of reverse mutation test in bacterial systems

Substance (µg/plate)	S9- mix	Revertant colonies/plate (mean)									
		TA100		TA1535		WP2uvrA		TA98		TA1537	
		Assay I	Assay II	Assay I	Assay II	Assay I	Assay II	Assay I	Assay II	Assay I	Assay II
0	-	91	100	13	11	22	25	21	26	15	13
156	-	93	103	11	9	21	18	23	22	16	11
313	-	94	100	10	10	23	27	26	20	13	11
625	-	107	112	11	13	22	27	33	23	15	10
1250	-	87	108	12	14	29	18	28	24	15	13
2500	-	97	96	12	15	27	28	22	18	10	11
5000	-	86	94	13	10	23	18	21	22	11	10
PC	-	698	791	429	444	156	172	544	503	1092	1352
0	+	95	90	18	11	25	30	34	32	27	22
156	+	97	94	18	12	31	28	37	31	32	22
313	+	92	92	10	9	39	35	47	37	30	21
625	+	106	87	10	8	32	38	38	35	32	20
1250	+	96	97	12	16	23	37	41	34	30	26
2500	+	110	98	14	14	29	32	39	31	21	19
5000	+	99	102	8	12	29	31	42	35	26	23
PC	+	631	609	184	195	240	251	217	245	137	132

PC – Positive control

Conclusion:

2-COOH-S-2200 is not mutagenic under the conditions tested.

Reference:	<i>In vitro</i> Chromosomal Aberration Test on 2-COOH-S-2200 in Chinese Hamster Lung Cells (CHL/IU)
Author(s), year:	Kitamoto, S.; 2012b
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0046
Guideline(s):	US EPA OPPTS 870.5375, OECD 473, EU regulation 440/2008 B10, Japanese MAFF 12 Nousan 8147
GLP:	Yes (Lab certified by national authority)
Deviations:	No
Validity:	Yes

Material and methods:

<i>Test Material:</i>	2-COOH-S-2200												
Lot/Batch:	Lot No.: 317-001-47-1												
Purity:	99.0%												
Stability of test compound:	Confirmed for the duration of the study by Certificates of Analysis spanning the study period. Stability and homogeneity were confirmed analytically in the DMSO solvent.												
Solvent:	DMSO												
<i>Control Materials:</i>													
Negative:	Solvent (DMSO)												
Positive:													
- without activation (-S9):	Mitomycin C (MMC)												
- with activation (+S9):	Cyclophosphamide (CP)												
<i>Activation:</i>	S9 was derived from liver homogenates of male SD rats induced with phenobarbital and 5,6-benzoflavone, and obtained commercially (Oriental Yeast Co, Tokyo)												
- S9 Mix composition:	<table> <tr> <td>S9 fraction</td><td>30%</td></tr> <tr> <td>MgCl₂</td><td>5 mM</td></tr> <tr> <td>Glucose-6-phosphate</td><td>5 mM</td></tr> <tr> <td>NADPH</td><td>4 mM</td></tr> <tr> <td>KCl</td><td>33 mM</td></tr> <tr> <td>HEPES buffer (pH 7.2)</td><td>4 mM</td></tr> </table>	S9 fraction	30%	MgCl ₂	5 mM	Glucose-6-phosphate	5 mM	NADPH	4 mM	KCl	33 mM	HEPES buffer (pH 7.2)	4 mM
S9 fraction	30%												
MgCl ₂	5 mM												
Glucose-6-phosphate	5 mM												
NADPH	4 mM												
KCl	33 mM												
HEPES buffer (pH 7.2)	4 mM												
<i>Test cells:</i>	<p>CHL IU cells from Dainippon Pharmaceutical Co, Ltd (Osaka, Japan) were obtained on 29 June 1987, and stored frozen in liquid nitrogen. Cells were confirmed not to have mycoplasma contamination. One ampoule of cell stock was cultured for this experiment; the number of cell passage was from 10 to 17 in this study. Doubling time for these cells is approximately 15 hours, a check of stock cells within two months of this study showed a doubling time of 14 hours.</p>												
<i>Culture medium:</i>	Eagle's minimum essential medium (MEM; Nissui Pharmaceutical Co, Ltd, Tokyo, Japan) supplemented with 10% bovine serum (Lot No.: 765960 Life Technologies, Inc., USA) in plastic dishes under a humidified atmosphere of 5% CO ₂ at 37°C												
<i>Test concentrations:</i>													
Preliminary cytotoxicity assays:													
With and without S9 mix:	0, 13.7, 27.3, 54.7, 109, 219, 438, 875, 1750, 3500* µg/mL												
(* - Precipitate seen at this concentration at the end of treatment)													
Chromosomal aberration assay, (6 hours):													
Without S9 mix:	0, 750, 1500, 3000 µg/mL												
With S9 mix:	0, 700, 1400, 2800 µg/mL												

Chromosomal aberration assay, (24 hours, repeat 6 hours):

Without S9 mix: 0, 550, 1100, 2200 µg/mL

With S9 mix: 0, 700, 1400, 2800 µg/mL

The test material was suspended in DMSO. CHL/IU cells from frozen stock were seeded at approximately 3×10^3 cells/mL, 5 mL per 60 mm-diameter culture dish, 3 days prior to each experiment. Duplicate monolayer plates were exposed to a range on concentrations of test compound with or without concurrent S9 mix for either 6 hours (then washed and held in test compound-free culture for 18 hours), or exposed to the test compound for 24 hours.

Cytotoxicity was tested with and without S9 fraction, using test compound at nine concentrations starting at 3500 µg/mL (approximately 10 mM) and decreasing sequentially by a factor of 2. Cells were incubated in test compound for either 6 hours (then washed and held in fresh medium for 18 hours) or for 24 hours. The plates were then trypsinized and the cell suspension counted on a Coulter Counter. Growth rate (% cells in treated group compared to corresponding control) and precipitation of test compound were recorded.

In the assays for chromosomal aberration, cell plates were treated as before but colcemid was added to the cultures to a final concentration of 0.1 µg/mL 1.5 hours prior to harvest. Cells were trypsinized then examined for cell growth and preparation of metaphase spreads. To examine metaphase spreads, cells from the lowest dose at which the growth rate was 50% or lower were fixed and spread on clean glass slides. After aging for at least one day, the slides were stained with Giemsa. The highest three doses that yielded 200 cells for analysis were used. All slides were read blind with positive and negative controls included. To examine structural aberrations, one hundred metaphases with standard karyotype were evaluated from each duplicate culture. Types of chromosome aberrations were recorded as chromatid gaps, chromosome gaps, chromatid breaks, chromatid exchanges, chromosome breaks, chromosome exchanges (including chromosome rings and dicentric chromosomes) and fragmentation. A gap was defined as an achromatic lesion smaller than the width of one chromatid, and with minimum misalignment of the chromatids. When a cell contained more than 9 chromosomal aberrations, the individual aberrations were not enumerated and the cell was scored as bearing 10 aberrations. To examine numerical aberrations, one hundred metaphases were evaluated from each duplicate culture. Polyploid cells and endoreduplicated cells were recorded. Incidence of structurally aberrant cells (excluding gaps) and that of numerically aberrant cells were classified according to the criteria of Ishidate: Negative (-) < 5%, Marginal (±) ≥ 5 – < 10%, and Positive (+) ≥ 10%. The test compound was concluded to induce chromosomal aberrations when both of the following criteria were fulfilled: a) incidence of cells with structural aberrations excluding gaps and/or with numerical aberrations are Marginal or Positive, and b) a dose/response relationship or reproducibility is observed.

Findings:

Preliminary and cytotoxicity assay:

The highest concentration was set at 3500 µg/mL (approximately 10 mM – limit dose concentration in the guideline) in the medium. Nine concentrations separated by a factor of 2 (3500, 1750, 875, 438, 219, 109, 54.7, 27.3 and 13.7 µg/mL) were used. In the cytotoxicity test, the two highest concentrations were neutralized with 1N aqueous sodium hydroxide. Precipitates were seen in the medium at the beginning of treatment at and above concentrations of 1750 µg/mL in the 6 and 24 hours treatments and at the end of treatment at concentration of 3500 µg/mL in the 6 hours treatment.

Marked growth inhibitions were seen both in the presence and absence of S9 mix at 3500 µg/mL after 6 or 24 hours exposure.

Chromosomal aberration assays:

In the 6 hour exposure assay, in the absence of S9, precipitate was seen in the medium at the beginning of treatment at 3000 µg/mL. Slides from 750, 1500 and 3000 µg/mL were analysed. A concentration of 3000 µg/mL caused slightly greater than 50% cell growth inhibition so this concentration and two lower

concentrations were analysed. In the presence of S9, precipitates were seen in the medium at the beginning of treatment at concentrations of 2800 µg/mL. Slides from 700, 1400 and 2800 µg/mL were analysed. The two highest concentrations were neutralized with 1N aqueous sodium hydroxide both in the absence and presence of S9 mix. The test compound induced no increase in the incidence of chromosomally aberrant cells (structural, numerical or polyploid) in any treatment groups with or without S9.

In a 24 hour exposure assay, CHL cells without S9 were treated with 3 doses, starting with 2200 µg/mL and decreasing sequentially by a factor of 2. Slides from 550, 1100 and 2200 µg/mL were analysed, and showed a small increase in chromatid breaks and exchanged (but not in polyploid cells) only at 2200 µg/mL (approximately 6.4 mM). The two highest concentrations were neutralized with 1N aqueous sodium hydroxide in the absence of S9 mix. In a repeat 6 hour exposure assay with S9 mix using exposure from 700, 1400 and 2800 µg/mL, no increase in the incidence of structurally aberrant cells or polyploid cells was observed. The two highest concentrations were neutralized with 1N aqueous sodium hydroxide in the presence of S9 mix.

All the negative control cultures gave values of chromosome aberrations (structural aberration and polyploidy) within the expected range. Positive control chemicals MMC and CP displayed clear and expected Responses in the incidence of cells with structural aberrations.

Table B.6.8.3-3: Results of the *in vitro* Chromosome aberration test

Group	Dose	Rel growth	N	Structural Aberrations										Judgement	Polyploid
				No. of Aberrations								Cells with abs			
				gap	ctb	cte	csb	cse	mul	Tot		(%)			
	(µg/mL)	(%)								+G	-G	+G	-G		(%)
6-Hour Exposure, without S9 (Exp I)															
Control	0	100	200	3	4	0	0	0	0	7	4	3.5	2.0	-	0.0
2-COOH-S-2200	750	85.2	200	3	1	0	0	0	0	4	1	2.0	0.5	-	0.5
	1500	75.1	200	2	2	0	0	0	0	4	2	2.0	1.0	-	0.0
	3000 _p	36.3	200	4	3	3	0	0	0	10	6	4.5	2.5	-	1.0
MMC	0.06	75.0	200	10	57	15	0	0	0	82	72	27.0	25.5	+	0.0
6-Hour Exposure, with S9 (Exp I)															
Control	0	100	200	3	2	0	0	0	0	5	2	2.5	1.0	-	1.0
2-COOH-S-2200	700	94.8	200	6	8	0	0	0	0	14	8	6.5	3.5	-	0.5
	1400	88.3	200	1	3	0	0	0	0	4	3	2.0	1.5	-	0.5
	2800 _p	47.5	200	3	1	0	0	0	0	4	1	2.0	0.5	-	1.0
CP	10	62.8	200	6	56	34	0	0	0	96	90	35.5	34.5	+	0.0
24-Hour Exposure, without S9 (Exp II)															
Control	0	100	200	1	1	0	0	0	0	2	1	1.0	0.5	-	1.0
2-COOH-S-2200	550	85.7	200	2	4	0	0	0	0	6	4	3.0	2.0	-	0.0
	1100	79.5	200	0	1	0	0	0	0	1	1	0.5	0.5	-	1.0
	2200 _p	48.8	200	10	19	4	0	0	0	33	23	15.0	11.0	+	0.0
MMC	0.02	85.9	200	0	27	8	0	0	0	35	35	14.5	14.5	+	1.0
6-Hour Exposure, with S9 (Exp II)															
Control	0	100	200	4	1	0	0	0	0	5	1	2.5	0.5	-	0.5

2-COOH-S-2200	700	91.6	200	2	2	1	0	0	0	5	3	2.5	1.5	-	1.0
	1400	84.1	200	0	1	0	0	0	0	1	1	0.5	0.5	-	1.0
	2800 _p	46.0	200	1	1	3	0	0	0	5	4	2.5	2.0	-	2.0
CP	10	58.6	200	2	58	53	0	0	0	113	111	37.0	36.0	+	0.0

- Negative, + Positive, ± Marginal as determined by criteria of Ishidate
_p – Precipitation observed at the beginning of the experiment
 Rel growth – % of controls, cytotoxicity
 ctb – chromatid break
 cte – chromatid exchange
 csb – chromosome break
 cse – chromosome exchange
 Mul – multiple aberrations (cells with more than 9 aberrations)
 Tot – total aberrations
 +G – aberrations including gaps; -G – aberrations excluding gaps
 Polyploid – polyploid cells and cells with endoreduplication

In conjunction with the results of the *in vivo* mouse micronucleus test, this positive result (at 2200 µg/mL 2-COOH-S-2200, 24-hour exposure, without S9) was re-evaluated after a request for clarification.

If the relative increase in cell count (RICC) is used to estimate cytotoxicity, as currently recommended by Galloway, S., et al. (2011)², the clastogenic effects occur only at a dose level where significant cytotoxicity is observed and are considered an artefact of cytotoxicity as outlined in Kirkland, D (1998)³: The result is a classic example of a “false positive” as outlined in Kirkland, 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 relative increase in cell count (RICC) index is therefore more appropriate, as discussed by Galloway *et al.*. As shown Table B.6.8.3, the %RICC for 2200 µg/mL (where the response was seen) was just 21.2% of control, which is considerably in excess of the top dose toxicity required by the OECD 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. There was also absolutely no dose response. The response seen at 2200 µg/mL (with precipitate: maximum soluble concentrate estimated at ca. 1600 µg/mL) was practically as intense as the positive control, but reduced to zero at 1100 µg/mL. This would be extremely unusual for a classical non-threshold genotoxic carcinogen. It is considered therefore that the result in this assay is an artefact of the high dose level/ “high toxicity” and is of no biological relevance. As such there is no genuine activity that can be usefully clarified by additional *in vivo* testing.

Table B.6.8.3: Results of the *in vitro* Chromosome aberration test

Group	Dose (µg/mL)	Cell number ^a	RCC ^b (%)	RICC ^c (%)
At the start of treatment		3.35 x 10 ⁵		
Control	0	9.54 x 10 ⁵	100	100
2-COOH-S-2200	550	8.17 x 10 ⁵	85.7	77.9
	1100	7.58 x 10 ⁵	79.5	68.3

² Galloway, S., et al. (2011). Workshop summary: Top concentration for *in vitro* mammalian cell genotoxicity assays; and Report from working group on toxicity measures and top concentration for *in vitro* cytogenetics assays (chromosome aberrations and micronucleus) Mutation Research/ 723, 77-83.

³ Kirkland, D. (1998). Chromosome aberration testing in genetic toxicology—past, present and future. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 404(1), 173-185.

	2200	4.66×10^5	48.8	21.2
--	------	--------------------	------	------

^a Cell number is available in raw data

^b RCC (Relative Cell Count) = [(Number of cells in treated cultures) / (Number of cells in control cultures)] x 100

^c RICC (Relative Increase in Cell Count) = [(Increase in number of cells in treated cultures (final – starting)) / (Increase in number of cells in control cultures (final – starting))] x 100

Conclusion:

In a GLP-compliant study conducted according to modern guidelines, 2-COOH-S-2200 has the potential to induce non-biologically relevant chromosomal aberrations at high doses of 2200 µg/mL (equivalent to 6.4 mM) in Chinese hamster lung cells in culture in the absence of metabolic activation at cytotoxic levels.

Reference:	Gene Mutation Assay in Chinese Hamster V79 Cells in vitro (V79/HPRT) with 2-COOH-S-2200
Author(s), year:	██████████ 2011a
Report/Doc. number:	Sumitomo Chemical Co. Ltd. Report No. ROT-0033
Guideline(s):	US EPA OPPTS 870.5300, OECD 476, EU regulation 440/2008 B17
GLP:	Yes (laboratory certified by national authority)
Deviations:	No
Validity:	Yes

Material and methods:

Test Material:	2-COOH-S-2200
Lot/Batch:	Lot No.: 317-001-47-1
Purity:	99.0%
Stability of test compound:	Confirmed for the duration of the study by Certificates of Analysis spanning the study period. Stability and homogeneity were confirmed analytically in the DMSO solvent.
Solvent:	DMSO
Control Materials:	
Negative:	Culture medium and solvent (DMSO)
Positive:	
- Without activation (-S9):	Ethylmethane sulfonate (EMS)
- With activation (+S9):	7,12-dimethylbenz(a)anthracene (DMBA)
Activation:	S9 mix was prepared from phenobarbital/β-naphthoflavone-induced male rat liver (8-12 weeks old) according to standard protocols. Aliquots were frozen and stored at -80°C, then thawed and mixed with standard S9 co-factor solution prior to use (8 mM MgCl ₂ , 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP in 100 mM sodium-phosphate buffer, pH 7.4).
Test cells:	V79 cell stocks are supplied by the Laboratory for Mutagenicity Testing, Technical University, 64287 Darmstadt, Germany. Each batch is screened for mycoplasma, and checked for karyotype stability and spontaneous mutant frequency. Thawed stock cultures are propagated at 37°C in 80 cm ² plastic flasks seeded with 5×10^5 cells.

Culture medium: Minimal essential medium (MEM)-Hanks' supplemented with 10% fetal calf serum and 1% neomycin. For selection of mutant cells, 11 µg/mL 6-thioguanine (6-TG) was added to the medium.

Locus examined: Hypoxanthine-guanine phosphoribosyl transferase (HPRT)

Test concentrations:

Preliminary cytotoxicity (range finding) assay:

Without S9 mix, 4 hours treatment: 0, 27.3, 54.7, 109.4, 218.8, 437.5, 875, 1750, 3500 µg/mL (approximately 10 mM)

Without S9 mix, 24 hours treatment: 0, 27.3, 54.7, 109.4, 218.8, 437.5, 875, 1750, 3500 µg/mL

With S9 mix, 4 hours treatment: 0, 27.3, 54.7, 109.4, 218.8, 437.5, 875, 1750, 3500 µg/mL

Mutation assay I:

Without S9 mix, 4 hours treatment: 0, 109.4, 218.8, 437.5, 875, 1750, 3500^p µg/mL

With S9 mix, 4 hours treatment: 0, 109.4, 218.8, 437.5, 875, 1750, 3500^p µg/mL

Mutation assay II:

Without S9 mix, 24 hours treatment: 0, 109.4, 218.8, 437.5, 875, 1750, 3500^p µg/mL

With S9 mix, 4 hours treatment: 0, 109.4, 218.8, 437.5, 875, 1750, 3500^p µg/mL

p precipitation

Doses were selected on the basis of a cytotoxicity pre-test. No relevant cytotoxic effects indicated by a relative cloning efficiency were noted up to the maximum concentration with or without metabolic activation following 4 and 24 hours treatment. Therefore, the highest dose for mutagenicity testing was set as 3500 µg/mL, equal to approximately 10 mM. Two day (experiment I) or three day (experiment II) old sub-cultivation stock cultures were trypsinized, rinsed, then used to plate at 1.5×10^6 cells (single culture) and 5×10^2 cells (duplicate cultures) in MEM-Hanks' with 10% FCS (complete medium). After 24 hours the medium was replaced with serum-free medium (or, for 24 hour treatment, with complete medium) containing 2-COOH-S-2200, either without S9 mix or with S9 mix (50 µL/mL). Negative and positive controls were treated in parallel. After the designated treatment period, the cultures were rinsed and complete medium returned to the cell flasks. Four days (experiment I) or three days (experiment II) after treatment 1.5×10^6 cells per experimental point were subcultured, and allowed to grow another 7 days. Cells were again recultured and for each duplicate culture, 10 flasks seeded with about $3 - 5 \times 10^5$ cells in medium containing 6-TG. Two additional flasks were seeded with approximately 500 cells each in non-selective medium to determine cell viability. Cultures were incubated for 11 days in experiment I and 8 days in experiment II, and then stained with methylene blue. Stained colonies with more than 50 cells were counted. Evaluation was performed on at least four concentrations per assay, of which the highest concentration was one producing approximately 10-20% cell survival.

Findings:

Preliminary cytotoxicity assay:

No relevant cytotoxic effect occurred up to the maximum concentration with or without metabolic activation following 4 and 24 hours treatment.

Mutation assays:

Negative and positive controls were within the expected historical range. Neither relevant nor reproducible increase in mutant colony numbers/ 10^6 cells was observed in the main experiments up to the maximum

concentration with and without metabolic activation. The induction factor exceeded the threshold of 3.0 in the first culture of the second experiment without metabolic activation at 1750 µg/mL. At the next higher concentration of 3500 µg/mL the induction factor was relatively high, but, this threshold was not reached. The increase of the induction factor was judged as biologically irrelevant as no comparable trend was noted in the parallel culture under identical experimental conditions. Furthermore, the threshold was only slightly exceeded (3.1 compared to a threshold of 3.0) and the absolute values of the mutation frequency remained within the historical range of negative and solvent controls. The induction factor never exceeded the threshold of 3.0 when compared to the concurrent negative control value of 18.1 mutants per 10⁶ cells. The effect is most likely based on precipitation artefacts since precipitation visible to the unaided eye was observed at 3500 µg/mL. Even though no visible precipitation was noted at the next lower concentration of 1750 µg/mL microscopic precipitation may well lead to irreproducible artefacts especially during continuous treatment for 24 hours.

Table B.6.8.3: Results of *In Vitro* Mammalian Gene Mutation (V79-HPRT)

Substance	Dose	Relative cloning efficiency I - survival		Relative cloning efficiency II - viability		Mutant colonies per 10 ⁶ cells	
	(µg/mL)	Culture 1	Culture 2	Culture 1	Culture 2	Culture 1	Culture 2
Experiment I (without S9)							
Negative control	-	100.0	100.0	100.0	100.0	25.8	22.7
Solvent control (DMSO)	0	100.0	100.0	100.0	100.0	31.2	25.3
Positive control (EMS)	150.0	103.6	98.9	84.7	107.0	144.8	130.5
2-COOH-S-2200	109.4	109.7	93.8	#	#	#	#
	218.8	116.8	88.5	102.4	103.3	31.4	45.4
	437.5	112.4	94.3	93.2	99.4	25.8	19.5
	875.0	113.6	97.8	89.2	112.9	24.9	25.3
	1750.0	116.5	89.5	74.7	97.1	22.6	9.5
	3500.0 ^p	101.3	73.1	90.6	115.5	12.0	26.1
Experiment I (with S9)							
Negative control	-	100.0	100.0	100.0	100.0	14.6	31.7
Solvent control (DMSO)	0	100.0	100.0	100.0	100.0	18.1	24.4
Positive control (DMBA)	1.1	52.1	100.7	86.6	95.0	901.5	1297.3
2-COOH-S-2200	109.4	104.5	95.8	#	#	#	#
	218.8	111.7	99.1	93.1	100.2	26.3	22.3
	437.5	101.5	93.5	107.6	92.5	25.5	28.4
	875.0	107.4	97.7	103.1	88.9	24.6	32.1
	1750.0	95.0	93.0	89.4	91.1	26.2	38.8
	3500.0 ^p	93.6	82.0	103.2	88.2	17.5	35.2
Experiment II (without S9)							
Negative control		100.0	100.0	100.0	100.0	18.1	23.1
Solvent control (DMSO)	0	100.0	100.0	100.0	100.0	12.1	24.0
Positive control (EMS)	150.0	94.0	93.3	64.3	94.0	530.8	417.8
2-COOH-S-2200	109.4	100.2	96.2	#	#	#	#

	218.8	98.4	99.3	117.9	99.1	25.8	7.7
	437.5	96.8	96.4	111.4	96.1	17.9	15.3
	875.0	93.6	95.2	112.4	102.4	17.6	18.7
	1750.0	64.7	95.1	57.3	95.2	37.5	27.4
	3500.0 ^p	86.6	12.6	76.0	73.6	35.4	29.2
Experiment II (with S9)							
Negative control	-	100.0	100.0	100.0	100.0	16.3	8.6
Solvent control (DMSO)	0	100.0	100.0	100.0	100.0	11.2	10.8
Positive control (DMBA)	1.1	73.7	65.1	82.1	94.3	852.6	797.6
2-COOH-S-2200	109.4	97.7	98.7	#	#	#	#
	218.8	99.6	97.1	95.0	103.8	10.6	4.4
	437.5	96.8	97.8	93.2	95.6	16.9	6.4
	875.0	98.8	100.8	92.5	98.9	16.7	14.0
	1750.0	99.3	95.9	83.5	100.9	11.8	8.3
	3500.0 ^p	99.5	97.1	108.9	114.3	12.4	8.5

Relative: as % of controls

^p – Precipitate

- Culture was not continued, since five higher concentrations were selected to be evaluated at the end of the experiment

Conclusion:

Under the experimental conditions reported, the 2-COOH-S-2200 did not induce gene mutations at the HPRT locus in V79 cells.

Reference:	Micronucleus Assay of 2-COOH-S-2200 in Mice
Author(s), year:	2012
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0040
Guideline(s):	US EPA OPPTS 870.5395, OECD 474, EU regulation 440/2008 B12, Japanese MAFF 12 Nousan 8147
GLP:	Yes (lab certified by National Authority)
Deviations:	No
Validity:	Yes

Material and methods:

Test Material:	2-COOH-S-2200
Lot/Batch:	Lot No.: 317-001-47-1
Purity:	99.0%
Stability of test compound:	Confirmed for the duration of the study by Certificates of Analysis spanning the study period. Stability and homogeneity were confirmed analytically in the 0.5% aqueous methylcellulose solvent.
Solvent:	0.5% w/v aqueous methylcellulose
Control Materials:	
Negative control:	0.5% aqueous methylcellulose
Positive control:	Mitomycin C

Test animals:

Species:	Mouse
Strain:	BDF1[SPF]
Age:	9 weeks at assignment to dose groups
Weight:	Range-finding: 24.8 to 29.0 g (males) and 21.2 to 22.6 g (females) Micronucleus test: 24.0 to 28.1 g (males)
Source:	██████████
Number of animals per dose:	Range finding: 3/sex/group, 3 groups Micronucleus test: 6 males/group, 4 groups, 5 animals analysed

Dose levels:

Range finding test: 500, 1000, 2000 mg/kg bw administered by single gavage doses (10 mL/kg) based on weights taken on the day of administration
Micronucleus assay: 0, 500, 1000, 2000 mg/kg bw administered by single gavage doses (10 mL/kg) based on weights taken on the day of administration

A range-finding toxicity assay was conducted in which groups of 3 mice per gender per dose received doses of 500, 1000, or 2000 mg/kg bw 2-COOH-S-2200. Mice were observed for clinical signs and mortality 1, 24 and 48 hours post-dosing. Body weights were recorded prior to dosing and 48 hours after dosing. No abnormal sign was observed in any animals, there were no mortalities and no effects on bodyweights. No sex difference in toxicity was discernible.

For the micronucleus assay, groups of 6 male mice per dose group received a single gavage dose of 0, 500, 1000, or 2000 mg/kg and bone marrow smears prepared 24 hours later from 5 animals per group. Another group received 2000 mg/kg and smears were prepared at 48 hours from 5 animals per group. There was 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).

Animals were sacrificed by CO₂ inhalation. Bone marrow was extracted from femurs, and centrifuged to obtain cell pellets. The pellets were resuspended and cells were smeared on clean glass slides, fixed with methanol, and Giemsa stained. Three slides were prepared per animal. Incidence of micronuclei in 2000 polychromatic erythrocytes (PCEs), and incidence of PCEs in 500 erythrocytes (including normochromatic erythrocytes (NCEs), and PCEs) was recorded for each animal.

Findings:

In the preliminary test, there was no mortality or clinical signs of toxicity, or discernible changes in body weight. There were no substantial differences in gender response. Therefore, 2000 mg/kg was chosen as the highest test dose as recommended in the guidelines.

In the main test, there were no signs of toxicity as a result of administration of 2-COOH-S-2200 in treated animals.

There was no statistically significant increase in micronuclei as a result of 2-COOH-S-2200 administration. The positive control showed appropriate increase in micronuclei formation to validate the sensitivity of the assay.

There was no decrease in the PCE/(PCE+NCE) ratio after exposure to 2-COOH-S-2200 or in the positive control.

Table B.6.8.3: Results of the *in vivo* Micronucleus Test

Treatment	Dose (mg/kg)	Sampling time (hr)	Micronucleated PCE (% , mean \pm SD)	PCE ratio (% , mean \pm SD)
Control	0	24	0.29 \pm 0.11	56.6 \pm 4.5
2-COOH-S-2200	500		0.31 \pm 0.24	58.8 \pm 6.3
	1000		0.36 \pm 0.13	55.9 \pm 6.2
	2000		0.46 \pm 0.18	58.2 \pm 2.4
Mitomycin C	0.5		1.51 \pm 0.35*	52.9 \pm 3.0
Control	0	48	0.31 \pm 0.07	49.0 \pm 1.6
S-2200	2000		0.31 \pm 0.20	49.6 \pm 6.5

Abbreviations used:
PCE - polychromatic erythrocytes; NCE - normochromatic erythrocytes
*p \leq 0.025
Micronuclei: 2000 PCE were examined from each animal
PCE ratio: PCE/(PCE+NCE), 500 erythrocytes examined from each animal

2-COOH-S-2200 seemed to induce a weak increase in the number of micronucleated PCEs at the 24 hour sampling timepoint, and thus clarification was demanded from the notifier.

In their reply, the following evidence was presented that the target organ is reached at sufficient levels: In the rat ADME study performed with the parent molecule (Sumitomo Chemical Co. Ltd. Report No. ROM-0033), S-2200 derived radioactivity in bone marrow peaked at 234 (male) and 217 (female) ng equivalents/g at 0.5 hours post-dose (T_{max}) in the 5 mg/kg dose group, and 7455 (male) and 3828 (female) ng equivalents/g (=7.5 and 3.8 mg equivs/kg) at 8 hours post-dose (T_{max}) in the 1000 mg/kg 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 S-2200 derived radioactivity reached the bone marrow at significant and sufficient concentration at low and high doses, respectively, and can thus be considered to evaluate chromosomal damage.

There is no estimated change to the physical chemical properties of parent (K_{ow}, MW, 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. The ADME study with the parent suggests 2-COOH-S-2200 is formed in the gut and liver, 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 (the magnitude of the water solubility increase is approximately equivalent to the unit decrease in log K_{ow}) would potentially increase exposure of the bone marrow: Greater concentrations compared to parent could be carried in the plasma.

Table B.6.8.3: Comparison of the properties of 2-COOH-S-2200 with mandestrobin

Properties	S-2200	2-COOH-S-2200
Molecular Weight (g mol ⁻¹)	313.39	343.38
Aqueous Solubility at 20°C (mg/L)	15.8	607*
Log K _{ow}	3.51/3.44	2.53*†

*Estimated at 25°C using EPISUITE, †new estimate not in dossier, effectively the unionised form

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

Looking at the individual animal data there are only two animals in the entire study with %MNPCE exceeding 0.56% which is the upper boundary of the historical control data. One is in the 500 mg/kg group (0.70%), and the other is in the 2000 group (0.75%). There were none in the intervening 1000 mg/kg group. If this were a genuine positive dose response, the number of animals with %MNPCE exceeding the historical control range would increase in all dose groups, as would the actual %MNPCE in most animals. This is not apparent in this study, and no toxicologically significant result has been obtained in this study.

Overall, it is considered that in the *in vivo* mouse micronucleus assay, bone marrow exposure will have been considerable, a biologically relevant increase in clastogenicity was not observed.

Table B.6.8.3: Results of the *in vivo* Micronucleus Test

Treatment	Dose (mg/kg)	Sampling time (hr)	Individual animal MNPCE (%)				
Control	0	24	0.40	0.35	0.15	0.35	0.20
2-COOH-S-2200	500		0.20	0.35	0.70	0.05	0.25
	1000		0.55	0.20	0.40	0.30	0.35
	2000		0.50	0.35	0.75	0.40	0.30
Mitomycin C	0.5		1.30	1.30	2.00	1.45	1.70

Conclusion:

2-COOH-S-2200 is not clastogenic under the conditions tested.

Metabolite 5-COOH-S-2200

Reference:	Acute Oral Toxicity Study of 5-COOH-S-2200 in Rats
Author(s), year:	██████████ 2012b
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0044
Guideline(s):	OECD 423; Commission Regulation (EC) No. 440/2008, B1.tris, Acute toxicity (oral); Japanese MAFF (12-Nousan No. 8147)
GLP:	Yes (Lab certified by National Authority)
Deviations:	None
Validity:	Yes

Material and methods:

Test Material:	5-COOH-S-2200
Lot/Batch:	Lot No.: 262-005-10-1
Purity:	97.6%
Stability of test compound:	Confirmed for the duration of the study by Certificates of Analysis spanning the study period
Test animals:	
Species:	Rat (females only)
Strain:	██████████:WistarHannover/Rcc
Age:	8 weeks (at start of study)
Weight at dosing:	First group: 148 – 156 g Second group: 154– 160 g Third group: 160-176 g

Source:

Diet:

CRF-1 Pellet diet from Oriental Yeast Co, Ltd. Tokyo, Japan; *ad libitum*

Acute oral toxicity of 5-COOH-S-2200 was evaluated in nine female Slc:WistarHannover/Rcc rats in a single gavage dose at 300 or 2000 mg/kg bw. All animals were observed for clinical signs daily for 14 days post-dosing and weighed periodically throughout the 14 days, then sacrificed and examined macroscopically.

Findings:

All animals treated at 2000 mg/kg bw died; all animals treated at 300 mg/kg bw survived the observation period. Clinical signs included lateral position and bradypnoea observed in animals treated at 2000 mg/kg bw. There were no clinical signs throughout the observation period in animals treated at 300 mg/kg bw. All animals gained weight. Retention of foamy fluid in the trachea was observed in 2 animals in the 2000 mg/kg bw group during the gross pathological examination. "Uncollapse" and pallor in all lobes of the lung were observed in one animal in the 300 mg/kg bw group during the gross pathological examination.

Conclusion:

The estimated oral LD₅₀ values for female rats were determined to be greater than 300 mg/kg bw but less than 2000 mg/kg bw.

According to Regulation (EC) No. 1272/2008, 5-COOH-S-2200 has to be classified as acutely toxic category 4.

Reference:	Reverse Mutation Test of 5-COOH-S-2200 in Bacterial Systems
Author(s), year:	Kitamoto, S.; 2012c
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No.ROT-0042
Guideline(s):	US EPA OPPTS 870.5100, OECD 471, EU regulation 440/2008 B13/14, Japanese MAFF 12 Nousan 8147
GLP:	Yes (Lab certified by National Authority)
Deviations:	None
Validity:	Yes

Material and methods:

Test Material:	5-COOH-S-2200
Lot/Batch:	Lot No.: 262-005-10-1
Purity:	97.6%
Stability of test compound:	Confirmed for the duration of the study by Certificates of Analysis spanning the study period. Stability was confirmed analytically in the DMSO solvent.
Solvent:	DMSO

Test strains and chemicals used as positive controls:

Without metabolic activation:

<i>S. typhimurium</i> TA100	0.01 µg/plate	2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
<i>S. typhimurium</i> TA1535	0.5 µg/plate	sodium azide
<i>E. coli</i> WP2uvrA	0.01 µg/plate	2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
<i>S. typhimurium</i> TA98	0.1 µg/plate	2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide

S. typhimurium TA1537 80 µg/plate 9-aminoacridine

With metabolic activation:

S. typhimurium TA100 1 µg/plate 2-aminoanthracene
S. typhimurium TA1535 2 µg/plate 2-aminoanthracene
E. coli WP2uvrA 10 µg/plate 2-aminoanthracene
S. typhimurium TA98 0.5 µg/plate 2-aminoanthracene
S. typhimurium TA1537 2 µg/plate 2-aminoanthracene

Drug metabolizing enzyme system (S9 mix):

S9-fraction: Oriental Yeast Co., Ltd. (Tokyo, Japan); Lot No. 11042201
 Liver fraction prepared from male Sprague Dawley rats induced by
 phenobarbital and 5,6-benzoflavone
S9-mix: 10% (v/v) S9-fraction
 8 µmol MgCl₂
 33 µmol KCl
 5 µmol glucose-6-phosphate
 4 µmol NADPH
 4 µmol NADH
 100 µmol Na-phosphate buffer (pH 7.4)

5-COOH-S-2200 was evaluated for its mutagenic potential by a reverse mutation test with four strains of *Salmonella typhimurium* (TA100, TA98, TA1535, and TA1537) and one strain of *Escherichia coli* (WP2uvrA). The test was conducted by the preincubation method in the presence and absence of metabolic activation (rat liver S9 mix).

In a dose-finding assay the doses ranged from 156-5000 µg/plate (Assay I). No precipitation or cytotoxicity was observed.

In the main assays, 5-COOH-S-2200 was tested in triplicate at doses ranging from 156 - 5000 µg/plate with and without S9 mix in all strains (Assay II). There was no statistically significant dose-related increase in the number of revertant colonies to at least twice as many as that of the solvent control with or without S9 mix. Positive controls showed the appropriate increase in the number of revertant colonies.

Based on these results, it is concluded that 5-COOH-S-2200 is not mutagenic under the test conditions.

Findings:

There was no statistically significant dose-related increase in the number of revertant colonies to at least twice as many as that of the solvent control with or without S9 mix. The negative result was replicated (Assay II). Positive controls showed an appropriate increase in the number of revertant colonies.

Table B.6.8.3-9: Results of reverse mutation test in bacterial systems

Substance (µg/plate)	S9- mix	Revertant colonies/plate (mean)									
		TA100		TA1535		WP2uvrA		TA98		TA1537	
		Assay I	Assay II	Assay I	Assay II	Assay I	Assay II	Assay I	Assay II	Assay I	Assay II
0	-	94	95	15	13	29	27	23	30	18	16
156	-	92	95	11	11	30	25	22	20	17	16
313	-	84	103	9	9	29	20	19	23	16	14
625	-	104	93	11	13	33	20	24	23	13	16
1250	-	101	98	11	10	32	24	26	24	15	18
2500	-	96	80	14	9	31	24	24	20	12	13
5000	-	103	95	11	10	25	21	24	23	12	17
PC	-	795	763	442	444	161	181	490	533	1108	1242
0	+	97	94	15	10	28	30	38	31	32	24

156	+	100	95	9	13	33	38	34	37	32	29
313	+	104	94	11	15	31	35	41	37	30	28
625	+	107	90	10	12	29	33	37	40	30	29
1250	+	111	99	11	12	37	36	40	36	33	24
2500	+	108	83	13	16	33	33	42	35	19	25
5000	+	94	98	15	12	33	37	41	36	21	27
PC	+	631	617	199	192	288	271	245	225	151	139

PC – Positive control

Conclusion:

5-COOH-S-2200 is not mutagenic under the test conditions.

Reference:

In vitro Chromosomal Aberration Test on 5-COOH-S-2200 in Chinese Hamster Lung Cells (CHL/IU)

Author(s), year: Kitamoto, S.; 2012d

Report/Doc. number: Sumitomo Chemical Co. Ltd. Report No. RQT-0047

Guideline(s): US EPA OPPTS 870.5375, OECD 473, EU regulation 440/2008 B10, Japanese MAFF 12 Nousan 8147

GLP: Yes (Lab certified by national authority)

Deviations: No

Validity: Yes

Material and methods:

Test Material:

5-COOH-S-2200

Lot/Batch: Batch No.: 262-005-10-1

Purity: 97.6%

Stability of test compound: Confirmed for the duration of the study by Certificates of Analysis spanning the study period

Solvent: DMSO

Control Materials:

Negative: Solvent (DMSO)

Positive:

- without activation (-S9): Mitomycin C (MMC)

- with activation (+S9): Cyclophosphamide (CP)

Activation:

S9 was derived from liver homogenates of male SD rats induced with phenobarbital and 5,6-benzoflavone, and obtained commercially (Oriental Yeast Co, Tokyo)

S9 Mix composition:

S9 fraction	30%
MgCl ₂	5 mM
Glucose-6-phosphate	5 mM
NADPH	4 mM
KCl	33 mM
HEPES buffer (pH 7.2)	4 mM

Test cells:

CHL/IU cells from Dainippon Pharmaceutical Co, Ltd (Osaka, Japan) were obtained on 29 June 1987, and stored frozen in liquid nitrogen.

Cells were confirmed not to have mycoplasma contamination. One ampoule of cell stock was cultured for this experiment; the number of cell passage was from 20 to 27 in this study. Doubling time for these cells is approximately 15 hours, a check of stock cells within two months of this study showed a doubling time of 14 hours.

Culture medium:

Eagle's minimum essential medium (MEM; Nissui Pharmaceutical Co, Ltd, Tokyo, Japan) supplemented with 10% bovine serum (Lot No.: 765960 Life Technologies, Inc., USA) in plastic dishes under a humidified atmosphere of 5% CO₂ at 37°C

Test concentrations:

Preliminary cytotoxicity assays:

With and without S9 mix: 0, 13.7, 27.3, 54.7, 109, 219, 438, 875, 1750, 3500* µg/mL (appr. 10mM)

(* - Precipitate seen at this concentration at the end of treatment)

Chromosomal aberration assay, (6 hours):

Without S9 mix: 0, 438, 875, 1750, 3500 µg/mL

With S9 mix: 0, 438, 875, 1750 µg/mL

Chromosomal aberration assay, (24 hours, repeat 6 hours):

Without S9 mix: 0, 219, 438, 875 µg/mL

With S9 mix: 0, 438, 875, 1750 µg/mL

The test material was suspended in DMSO. CHL/0 cells from frozen stock were seeded at approximately 3 x 10³ cells/mL, 5 mL per 60 mm-diameter culture dish, 3 days prior to each experiment. Duplicate monolayer plates were exposed to a range of concentrations of test compound with or without concurrent S9 mix for either 6 hours (then washed and held in test compound-free culture for 18 hours), or exposed to the test compound for 24 hours.

Cytotoxicity was tested with and without S9 fraction, using test compound at nine concentrations starting at 3500 µg/mL (approximately 10 mM) and decreasing sequentially by a factor of 2. Cells were incubated in test compound for either 6 hours (then washed and held in fresh medium for 18 hours) or for 24 hours. The plates were then trypsinized and the cell suspension counted on a Coulter Counter. Growth rate (% cells in treated group compared to corresponding control) and precipitation of test compound were recorded.

In the assays for chromosomal aberration, cell plates were treated as before but colcemid was added to the cultures to a final concentration of 0.1 µg/mL 1.5 hours prior to harvest. Cells were trypsinized then examined for cell growth and preparation of metaphase spreads. To examine metaphase spreads, cells from the lowest dose at which the growth rate was 50% or lower were fixed and spread on clean glass slides.

After aging for at least one day, the slides were stained with Giemsa. The highest three doses that yielded 200 cells for analysis were used. All slides were read blind with positive and negative controls included. To examine structural aberrations, one hundred metaphases with standard karyotype were evaluated from each duplicate culture. Types of chromosome aberrations were recorded as chromatid gaps, chromosome gaps, chromatid breaks, chromatid exchanges, chromosome breaks, chromosome exchanges (including chromosome rings and dicentric chromosomes) and fragmentation. A gap was defined as an achromatic lesion smaller than the width of one chromatid, and with minimum misalignment of the chromatids. When a cell contained more than 9 chromosomal aberrations, the individual aberrations were not enumerated and the cell was scored as bearing 10 aberrations. To examine numerical aberrations, one hundred metaphases were evaluated from each duplicate culture. Polyploid cells and endoreduplicated cells were recorded.

Incidence of structurally aberrant cells (excluding gaps) and that of numerically aberrant cells were classified according to the criteria of Ishidate: Negative (-) < 5%, Marginal (±) ≥ 5 – < 10%, and Positive (+) ≥ 10%. The

test compound was concluded to induce chromosomal aberrations when both of the following criteria were fulfilled: a) incidence of cells with structural aberrations excluding gaps and/or with numerical aberrations are Marginal or Positive, and b) a dose/response relationship or reproducibility is observed.

Findings:

Preliminary and cytotoxicity assay:

The highest concentration was set at 3500 µg/mL (approximately 10 mM – limit dose concentration in the guideline) in the medium. Nine concentrations separated by a factor of 2 (3500, 1750, 875, 438, 219, 109, 54.7, 27.3 and 13.7 µg/mL) were used. In the cytotoxicity test, the two highest concentrations were neutralized with 1N aqueous sodium hydroxide. Precipitates were seen in the medium at the end of treatment at concentration of 3500 µg/mL with or without S9 mix.

Marked growth inhibitions were seen both in the presence and absence of S9 mix after 24 hours exposure. Dose dependent growth inhibition was observed after 6 hours treatment / 18 hours recovery without S9 mix, but the inhibition of growth rate was less than 50%.

Chromosomal aberration assays:

In the 6 hour exposure assay, in the absence of S9 (Experiment I) precipitates were seen in the medium at the beginning of treatment at concentrations of 1750 µg/mL or more and at the end of treatment at a concentration of 3500 µg/mL. Slides from 875, 1750 and 3500 µg/mL were analysed. Slides from 438 µg/mL were not analysed because 200 metaphases were analysable in three highest doses. In the presence of S9 (Experiment I) precipitates were seen in the medium at the beginning of treatment at concentrations of 1750 µg/mL. Slides from 438, 875 and 1750 µg/mL were analysed. A concentration of 1750 µg/mL caused slightly greater than 50% cell growth inhibition so this concentration and two lower concentrations were analysed. The two highest concentrations in the absence of S9 mix and the highest concentration in the presence of S9 mix were neutralized with 1N aqueous sodium hydroxide. The test compound induced no increase in the incidence of cells with structural aberrations or polyploid cells in any treatment groups in the presence or absence of S9 mix.

In a 24 hour exposure assay, the doses for chromosomal aberration test the analysed concentrations were 219, 438 and 875 µg/mL. No precipitate was seen in the medium at any concentrations. The test compound induced no increases in the incidence of structurally aberrant cells or polyploid cells in any treatment group in the absence of S9 mix. In a repeat 6 hour exposure assay with S9 mix, the cells were treated with the test compound in the same manner as in Experiment 1. The highest concentration in the presence of S9 mix was neutralized with 1N aqueous sodium hydroxide. The test compound induced no increases in the incidence of structurally aberrant cells or polyploid cells in any treatment group in the presence of S9 mix.

All the negative control cultures gave values of chromosome aberrations (structural aberrations and polyploidy) within the expected range. Positive control chemicals, MMC (without metabolic activation) and CP (with metabolic activation) showed clear and expected responses in the incidence of cells with structural aberrations.

Table B.6.8.3-10: Results of the *in vitro* Chromosome aberration test

Group	Dose (µg/mL)	Rel growth (%)	N	Structural Aberrations										Ishidate Judgement	Polypl	
				No. of Aberrations								Cells with abs				
				gap	ctb	cte	Csb	cse	mul	Tot		(%)			(%)	
											+G	-G	+G	-G		
6-Hour Exposure, without S9																
Control	0	100	200	0	0	0	0	0	0	0	0	0	0.0	0.0	-	1.0
5- COOH- S-2200	875	74.0	200	1	1	0	0	0	0	2	1	1.0	0.5	-	0.0	
	1750 _p	58.8	200	1	1	0	0	0	0	2	1	1.0	0.5	-	0.0	

	3500 _p	54.3	200	1	5	0	0	0	0	6	5	3.0	2.5	-	0.5
MMC	0.06	83.7	200	1	24	11	0	0	0	36	35	16.0	16.0	+	0.0
6-Hour Exposure, with S9															
Control	0	100	200	4	2	0	0	0	0	6	2	2.5	1.0	-	0.5
5-COOH-S-2200	438	81.3	200	1	1	0	0	0	0	2	1	1.0	0.5		1.0
	875	72.2	200	1	8	0	0	0	0	9	8	3.0	2.5		1.0
	1750 _p	47.2	200	3	4	0	0	0	0	7	4	3.5	2.0	-	2.0
CP	10	70.3	200	8	50	31	2	4	0	95	87	32.0	31.0	+	0.0
24-Hour Exposure, without S9															
Control	0	100	200	1	3	0	0	0	0	4	3	2.0	1.5	-	0.5
5-COOH-S-2200	219	87.6	200	4	4	1	0	0	0	9	5	4.0	2.5	-	2.0
	438	76.3	200	2	7	0	0	0	0	9	7	4.5	3.5	-	1.0
	875	46.6	200	2	4	1	0	0	0	7	5	3.5	2.5	-	1.5
MMC	0.02	86.3	200	7	34	11	0	0	0	52	45	21.5	19.0	+	1.5
6-Hour Exposure, with S9, first repeat															
Control	0	100	200	4	0	0	0	0	0	4	0	2.0	0.0	-	1.0
5-COOH-S-2200	438	87.1	200	1	0	0	0	0	0	1	0	0.5	0.0	-	1.0
	875	76.4	200	1	1	0	0	0	0	2	1	1.0	0.5	-	1.5
	1750 _p	36.4	200	1	3	2	0	0	0	6	5	3.0	2.5	-	2.0
CP	10	73.0	200	0	34	18	0	0	0	52	52	23.0	23.0	+	0.0
- Negative, + Positive, ± Marginal as determined by criteria of Ishidate P – Precipitation observed at the beginning of the experiment Rel growth – % of controls, cytotoxicity ctb – chromatid break cte – chromatid exchange csb – chromosome break cse – chromosome exchange Mul – multiple aberrations (cells with more than 9 aberrations) Tot – total aberrations +G – aberrations including gaps; -G – aberrations excluding gaps PolypI – polyploid cells and cells with endoreduplication															

Conclusion:

In a GLP-compliant study conducted to modern guidelines 5-COOH-S-2200 has no potential to induce chromosomal aberrations in Chinese hamster lung cells in culture under the conditions tested.

Reference:

Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT) with 5-COOH-S-2200
 Author(s), year: Wollny, H-E.; 2011b
 Report/Doc. number: Sumitomo Chemical Co., Ltd. Report No. ROT-0034
 Guideline(s): US EPA OPPTS 870.5300, OECD 476, EU regulation 440/2008 B17
 GLP: Yes (laboratory certified by national authority)
 Deviations: No
 Validity: Yes

Material and methods:

Test Material: 5-COOH-S-2200
 Lot/Batch: Lot No.: 262-005-10-1
 Purity: 97.6%
 Stability of test compound: Confirmed for the duration of the study by Certificates of Analysis

Solvent:	spanning the study period. Stability and homogeneity were confirmed analytically in the DMSO solvent. DMSO
Control Materials:	
Negative:	Culture medium and solvent (DMSO)
Positive:	
- Without activation (-S9):	Ethylmethane sulfonate (EMS)
- With activation (+S9):	7,12-dimethylbenz(a)anthracene (DMBA)
Activation:	S9 mix was prepared from phenobarbital/β-naphthoflavone-induced male rat liver (8-12 weeks old) according to standard protocols. Aliquots were frozen and stored at -80°C, then thawed and mixed with standard S9 co-factor solution prior to use (8 mM MgCl ₂ , 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP in 100 mM sodium-phosphate buffer, pH 7.4).
Test cells:	V79 cell stocks are supplied by the Laboratory for Mutagenicity Testing, Technical University, 64287 Darmstadt, Germany. Each batch is screened for mycoplasma, and checked for karyotype stability and spontaneous mutant frequency. Thawed stock cultures are propagated at 37°C in 80 cm ² plastic flasks seeded with 5 × 10 ⁵ cells.
Culture medium:	Minimal essential medium (MEM)-Hanks' supplemented with 10% fetal calf serum and 1% neomycin. For selection of mutant cells, 11 µg/mL 6-thioguanine (6-TG) was added to the medium.
Locus examined:	Hypoxanthine-guanine phosphoribosyl transferase (HPRT)
Test concentrations:	
Preliminary cytotoxicity (range finding) assay:	
Without S9 mix, 4 hours treatment:	0, 27.3, 54.7, 109.4, 218.8, 437.5, 875, 1750 ^p , 3500 ^p µg/mL (approximately 10 mM)
Without S9 mix, 24 hours treatment:	0, 27.3, 54.7, 109.4, 218.8, 437.5, 875, 1750, 3500 ^p µg/mL
With S9 mix, 4 hours treatment:	0, 27.3, 54.7, 109.4, 218.8, 437.5, 875 ^p , 1750 ^p , 3500 ^p µg/mL
Mutation assay I:	
Without S9 mix, 4 hours treatment:	0, 109.4, 218.8, 437.5, 875, 1750 ^t , 3500 ^p µg/mL
With S9 mix, 4 hours treatment:	0, 109.4, 218.8, 437.5, 875, 1750 ^t , 3500 ^p µg/mL
Mutation assay II:	
Without S9 mix, 24 hours treatment:	0, 109.4, 218.8, 437.5, 875, 1750, 3500 ^p µg/mL
With S9 mix, 4 hours treatment:	0, 109.4, 218.8, 437.5, 875, 1750, 3500 ^p µg/mL
p:	precipitation
t:	turbidity

Doses were selected on the basis of a cytotoxicity pre-test. No relevant cytotoxic effects indicated by a change in relative cloning efficiency were noted up to the maximum concentration with or without metabolic activation following 4 and 24 hours treatment. Therefore, the highest dose for mutagenicity testing was set

as 3500 µg/mL, equal to approximately 10 mM. Two day (experiment I) or three day (experiment II) old sub-cultivation stock cultures were trypsinized, rinsed, then used to plate at 1.5×10^6 cells (single culture) and 5×10^2 cells (duplicate cultures) in MEM-Hanks' with 10% FCS (complete medium). After 24 hours the medium was replaced with serum-free medium (or, for 24 hour treatment, with complete medium) containing 2-COOH-S-2200, either without S9 mix or with S9 mix (50 µL/mL). Negative and positive controls were treated in parallel. After the designated treatment period, the cultures were rinsed and complete medium returned to the cell flasks. Three days (experiment I) or four days (experiment II) after treatment 1.5×10^6 cells per experimental point were subcultured, and allowed to grow another 7 days. Cells were again recultured and for each duplicate culture, 10 flasks seeded with about $3 - 5 \times 10^5$ cells in medium containing 6-TG. Two additional flasks were seeded with approximately 500 cells each in non-selective medium to determine cell viability. Cultures were incubated for 8 days and then stained with methylene blue. Stained colonies with more than 50 cells were counted. Evaluation was performed on four concentrations per assay.

Findings:

Preliminary cytotoxicity assay:

No relevant cytotoxic effect occurred up to the maximum concentration with or without metabolic activation following 4 and 24 hours treatment. Without metabolic activation, relevant cytotoxic effects were observed at 1750 µg/mL in experiment I and at 1750 and 3500 µg/mL in experiment II following 4 and 24 hours of treatment.

Mutation assays:

No relevant cytotoxic effect occurred up to the maximum concentration with metabolic activation. Without metabolic activation, relevant cytotoxic effects were observed at 1750 µg/mL in experiment I and at 1750 and 3500 µg/mL in experiment II following 4 and 24 hours of treatment. Negative and positive controls were within the expected historical range. Neither relevant nor reproducible increase in mutant colony numbers/ 10^6 cells was observed in the main experiments up to the maximum concentration with and without metabolic activation.

Table B.6.8.3-11: Results of *In Vitro* Mammalian Gene Mutation (V79-HPRT)

Substance	Dose	Relative cloning efficiency I – survival		Relative cloning efficiency II - viability		Mutant colonies per 10^6 cells	
	(µg/mL)	Culture 1	Culture 2	Culture 1	Culture 2	Culture 1	Culture 2
Experiment I (without S9)							
Negative control	-	100.0	100.0	100.0	100.0	25.5	29.9
Solvent control (DMSO)	0	100.0	100.0	100.0	100.0	18.7	29.6
Positive control (EMS)	150.0	91.9	99.2	35.1	53.2	359.4	359.2
5-COOH-S-2200	109.4	89.2	99.8	#	#	#	#
	218.8	98.0	97.2	97.4	63.7	13.8	31.4
	437.5	97.7	99.4	101.1	189.6	17.6	14.0
	875.0	98.1	87.1	102.1	179.8	21.3	19.8
	1750.0	35.2	33.4	102.2	191.8	28.2	23.0
	3500.0 ^p	56.2	55.5	108.6	142.2	18.8	14.9
Experiment I (with S9)							
Negative control	-	96.9	96.4	100.0	100.0	12.8	11.4
Solvent control (DMSO)	0	100.0	100.0	100.0	100.0	30.3	25.7
Positive control (DMBA)	1.1	38.2	28.3	66.4	89.9	1147.4	871.5
5-COOH-S-2200	109.4	99.9	97.7	#	#	#	#
	218.8	94.1	85.6	92.0	100.0	17.4	19.6
	437.5	91.2	87.7	98.4	101.8	10.8	17.8

	875.0 ^t	85.3	89.0	102.7	100.1	13.7	19.8
	1750.0 ^t	90.9	89.8	98.6	94.6	15.2	10.7
	3500.0 ^p	89.7	84.8	100.3	100.7	13.8	17.4
Experiment II (without S9)							
Negative control	-	100.0	100.0	100.0	100.0	11.5	27.2
Solvent control (DMSO)	0	100.0	100.0	100.0	100.0	17.2	15.6
Positive control (EMS)	150.0	92.3	106.4	93.2	97.4	420.8	609.0
5-COOH-S-2200	109.4	95.0	95.9	#	#	#	#
	218.8	79.1	93.4	97.3	112.3	13.0	29.4
	437.5	81.5	66.4	99.7	122.1	25.1	9.7
	875.0	60.0	75.2	99.5	105.7	29.8	23.6
	1750.0	35.1	30.0	113.9	107.5	17.2	15.7
	3500.0 ^p	24.6	22.7	85.9	116.2	13.2	29.0
Experiment II (with S9)							
Negative control	-	103.4	97.0	100.0	100.0	13.6	19.6
Solvent control (DMSO)	0	100.0	100.0	100.0	100.0	16.4	19.6
Positive control (DMBA)	1.1	136.6	98.6	79.8	93.9	851.6	698.4
5-COOH-S-2200	109.4	108.5	96.3	#	#	#	#
	218.8	118.0	96.5	85.9	100.3	36.0	19.6
	437.5	101.2	100.0	104.1	61.7	34.4	16.1
	875.0	101.9	96.9	97.2	57.6	25.2	29.8
	1750.0	94.7	96.0	103.3	46.2	12.3	33.2
	3500.0 ^p	105.1	98.1	102.8	58.7	20.0	25.2

Relative: as % of controls

- Culture was not continued, since a minimum of only four analysable concentrations is required

p – Precipitation of the test item visible to the unaided eye at the end of treatment

t – Turbidity observed at the end of treatment

Conclusion:

Under the experimental conditions reported the 5-COOH-S-2200 did not induce gene mutations at the HPRT locus in V79 cells.

Metabolite 2-CH₂OH-S-2200

Reference:	Acute Oral Toxicity Study of 2-CH ₂ OH-S-2200 in Rats
Author(s), year:	2012c
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0053
Guideline(s):	OECD 423, EU regulation 440/2008 B1 Acute oral toxicity – Acute toxic class method, Japanese MAFF 12-Nousan-No.8147
GLP:	Yes (Lab certified by National Authority)
Deviations:	None
Validity:	Yes

Material and methods:

Test Material:	2-CH ₂ OH-S-2200
Lot/Batch:	Lot No.: 118-110407-1
Purity:	100.0%
Stability of test compound: spanning the study period	Confirmed for the duration of the study by Certificates of Analysis

Test animals:

Species:	Rat (females only)
Strain:	WistarHannover/Rcc
Age:	8 weeks (at start of study)
Weight at dosing:	First group: 155 – 160 g Second group: 151 – 161 g
Source:	
Diet:	CRF-1 Pellet diet from Oriental Yeast Co, Ltd. Tokyo, Japan; <i>ad libitum</i>

Animals in the first group of three were each given a single gavage dose of 2000 mg/kg bw 2-CH₂OH-S-2200 in 0.5% aqueous methylcellulose. When no response was observed in the first group, a second group of three animals was dosed at 2000 mg/kg bw. Accuracy of dose formulation was confirmed by analysis. Clinical and mortality observations were made at 10 and 30 minutes, 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/tissues examined macroscopically.

Findings:

No mortality was observed. There were no clinical signs throughout the observation period. The body weights were not affected by the administration of the test compound throughout the study period. There were no findings at the gross pathological examination.

Conclusion:

No mortality occurred after administration of 2000 mg/kg bw (limit test). According to Regulation (EC) No. 1272/2008, classification of 2-CH₂OH-S-2200 for acute toxicity is not required.

Reference:	Reverse Mutation Test of 2-CH ₂ OH-S-2200 in Bacterial Systems
Author(s), year:	Kitamoto, S.; 2012e
Report/Doc. number:	Sumitomo Chemical Co. Ltd. Report No. ROT-0048
Guideline(s):	US EPA OPPTS 870.5100, OECD 471, EU regulation 440/2008 B13/14, Japanese MAFF 12 Nousan 8147
GLP:	Yes (Lab certified by National Authority)
Deviations:	None
Validity:	Yes

Material and methods:

Test Material:	2-CH ₂ OH-S-2200
Lot/Batch:	Lot No.: 118-110407-1
Purity:	100.0%
Stability of test compound:	Confirmed for the duration of the study by Certificates of Analysis spanning the study period. Stability was confirmed analytically in the DMSO solvent.
Solvent:	DMSO

Test strains and chemicals used as positive controls:

Without metabolic activation:

<i>S. typhimurium</i> TA100	0.01 µg/plate	2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
-----------------------------	---------------	---

<i>S. typhimurium</i> TA1535	0.5 µg/plate	sodium azide
<i>E. coli</i> WP2uvrA	0.01 µg/plate	2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
<i>S. typhimurium</i> TA98	0.1 µg/plate	2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
<i>S. typhimurium</i> TA1537	80 µg/plate	9-aminoacridine

With metabolic activation:

<i>S. typhimurium</i> TA100	1 µg/plate	2-aminoanthracene
<i>S. typhimurium</i> TA1535	2 µg/plate	2-aminoanthracene
<i>E. coli</i> WP2uvrA	10 µg/plate	2-aminoanthracene
<i>S. typhimurium</i> TA98	0.5 µg/plate	2-aminoanthracene
<i>S. typhimurium</i> TA1537	2 µg/plate	2-aminoanthracene

Drug metabolizing enzyme system (S9 mix):

<i>S9-fraction:</i>	Oriental Yeast Co., Ltd. (Tokyo, Japan); Lot No. 1107150 Liver fraction prepared from male Sprague Dawley rats induced by phenobarbital and 5,6-benzoflavone
<i>S9-mix:</i>	10% (v/v) S9-fraction 8 µmol MgCl ₂ 33 µmol KCl 5 µmol glucose-6-phosphate 4 µmol NADPH 4 µmol NADH 100 µmol Na-phosphate buffer (pH 7.4)

2-CH₂OH-S-2200 was evaluated for its mutagenic potential by a reverse mutation test with four strains of *Salmonella typhimurium* (TA100, TA98, TA1535, and TA1537) and one strain of *Escherichia coli* (WP2uvrA). The test was conducted by the preincubation method in the presence and absence of metabolic activation (rat liver S9 mix).

In a dose-finding assay the doses ranged from 156 - 5000 µg/plate (Assay I). Precipitation was observed at and above concentrations of 1250 µg/plate. No cytotoxicity was observed.

In the main assays, 2-CH₂OH-S-2200 was tested in triplicate at doses ranging from 156 - 5000 µg/plate with and without S9 mix in all strains (Assay II). There was no statistically significant dose-related increase in the number of revertant colonies to at least twice as many as that of the solvent control with or without S9 mix.

Findings:

There was no statistically significant dose-related increase in the number of revertant colonies to at least twice as many as that of the solvent control with or without S9 mix. The negative result was replicated (Assay II). Positive controls showed an appropriate increase in the number of revertant colonies.

Table B.6.8.3-12: Results of reverse mutation test in bacterial systems

Substance (µg/plate)	S9- mix	Revertant colonies/plate (mean)									
		TA100		TA1535		WP2uvrA		TA98		TA1537	
		Assay I	Assay II	Assay I	Assay II	Assay I	Assay II	Assay I	Assay II	Assay I	Assay II
0	-	103	122	13	10	20	20	14	21	10	7
156	-	113	118	10	10	23	25	18	14	10	7
313	-	106	118	11	12	23	23	18	16	8	8
625	-	97	109	13	11	28	20	17	17	6	10
1250 ^p	-	113	118	15	12	21	21	19	18	9	9
2500 ^p	-	100	118	9	11	20	19	18	18	7	7
5000 ^p	-	93	107	7	12	13	19	15	18	5	7
PC	-	638	705	347	337	103	102	391	398	739	725

0	+	106	110	10	9	26	27	33	34	21	20
156	+	97	99	8	7	23	22	30	31	19	16
313	+	93	103	8	8	26	22	34	33	17	20
625	+	103	105	14	9	29	25	36	30	16	17
1250 ^p	+	97	109	10	9	31	20	31	31	25	16
2500 ^p	+	98	107	11	6	25	26	24	26	14	14
5000 ^p	+	94	100	7	8	21	21	24	26	15	15
PC	+	598	605	193	206	442	427	238	213	108	128

p precipitation
PC – Positive control

Conclusion:

2-CH₂OH-S-2200 is not mutagenic under the conditions tested.

Metabolite 4-OH-S-2200

Reference:	Acute Oral Toxicity Study of 4-OH-S-2200 in Rats.
Author(s), year:	██████████ 2012d
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0054
Guideline(s):	OECD 423; EC Directive 2004/73/EC, Acute toxic (oral); Japanese MAFF (12-Nousan No. 8147)
GLP:	Yes (Lab certified by National Authority)
Deviations:	None
Validity:	Yes

Material and methods:

Test Material:	4-OH-S-2200
Lot/Batch:	Lot No.: 089-110514-1
Purity:	99.9%
Stability of test compound:	Confirmed for the duration of the study by Certificates of Analysis spanning the study period

Test animals:

Species:	Rat (females only)
Strain:	██████████:WistarHannover/Rcc
Age:	8 weeks (at start of study)
Weight at dosing:	First group: 149 – 153 g Second group: 144 – 155 g
Source:	██
Diet:	CRF-1 Pellet diet from Oriental Yeast Co, Ltd. Tokyo, Japan; <i>ad libitum</i>

Animals in the first group of three were each given a single gavage dose of 2000 mg/kg bw 4-OH-S-2200 in 0.5% aqueous methylcellulose. When no response was observed in the first group, a second group of three animals was dosed at 2000 mg/kg bw. Accuracy of dose formulation was confirmed by analysis. Clinical and mortality observations were made at 10 and 30 minutes, 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/tissues examined macroscopically.

Findings:

No mortality was observed. There were no clinical signs throughout the observation period.
The body weights were not affected by the administration of the test compound throughout the study period.
There were no findings at the gross pathological examination.

Conclusion:

No mortality occurred after administration of 2000 mg/kg bw (limit test). According to Regulation (EC) No. 1272/2008, classification of 4-OH-S-2200 for acute toxicity is not required.

Reference:	Reverse Mutation Test of 4-OH-S-2200 in Bacterial Systems
Author(s), year:	Kitamoto, S.; 2012f
Report/Doc. number:	Sumitomo Chemical Co. Ltd. Report No. ROT-0049
Guideline(s):	US EPA OPPTS 870.5100, OECD 471, EU regulation 440/2008 B13/14, Japanese MAFF 12 Nousan 8147
GLP:	Yes (Lab certified by National Authority)
Deviations:	None
Validity:	Yes

Material and methods:

Test Material:	4-OH-S-2200
Lot/Batch:	Lot No.: 089-110514-1
Purity:	99.9%
Stability of test compound:	Confirmed for the duration of the study by certificates of analysis spanning the study period. Stability was confirmed analytically in the DMSO solvent.
Solvent:	DMSO

Test strains and chemicals used as positive controls:

Without metabolic activation:

<i>S. typhimurium</i> TA100	0.01 µg/plate	2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
<i>S. typhimurium</i> TA1535	0.5 µg/plate	sodium azide
<i>E. coli</i> WP2uvrA	0.01 µg/plate	2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
<i>S. typhimurium</i> TA98	0.1 µg/plate	2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
<i>S. typhimurium</i> TA1537	80 µg/plate	9-aminoacridine

With metabolic activation:

<i>S. typhimurium</i> TA100	1 µg/plate	2-aminoanthracene
<i>S. typhimurium</i> TA1535	2 µg/plate	2-aminoanthracene
<i>E. coli</i> WP2uvrA	10 µg/plate	2-aminoanthracene
<i>S. typhimurium</i> TA98	0.5 µg/plate	2-aminoanthracene
<i>S. typhimurium</i> TA1537	2 µg/plate	2-aminoanthracene

Drug metabolizing enzyme system (S9 mix):

S9-fraction:	Oriental Yeast Co., Ltd. (Tokyo, Japan); Lot No. 1107150 Liver fraction prepared from male Sprague Dawley rats induced by phenobarbital and 5,6-benzoflavone
S9-mix:	10% (v/v) S9-fraction

8 µmol MgCl₂
33 µmol KCl
5 µmol glucose-6-phosphate
4 µmol NADPH
4 µmol NADH
100 µmol Na-phosphate buffer (pH 7.4)

4-OH-S-2200 was evaluated for its mutagenic potential by a reverse mutation test with four strains of *Salmonella typhimurium* (TA100, TA98, TA1535, and TA1537) and one strain of *Escherichia coli* (WP2uvrA). The test was conducted by the preincubation method in the presence and absence of metabolic activation (rat liver S9 mix).

In a dose-finding assay the doses ranged from 156 - 5000 µg/plate (Assay I). Precipitation was observed at and above concentrations of 1250 µg/plate. No cytotoxicity was observed.

In the main assays, 4-OH-S-2200 was tested in triplicate at doses ranging from 156 - 5000 µg/plate with and without S9 mix in all strains (Assay II).

Findings:

There was no statistically significant dose-related increase in the number of revertant colonies to at least twice as many as that of the solvent control with or without S9 mix. The negative result was replicated (Assay II). Positive controls showed an appropriate increase in the number of revertant colonies.

Table B.6.8.3-13: Results of reverse mutation test in bacterial systems

Substance (µg/plate)	S9- mix	Revertant colonies/plate (mean)									
		TA100		TA1535		WP2uvrA		TA98		TA1537	
		Assay I	Assay II	Assay I	Assay II	Assay I	Assay II	Assay I	Assay II	Assay I	Assay II
0	-	83	93	6	7	18	19	18	22	7	9
156	-	75	103	8	9	13	15	22	15	8	6
313	-	96	95	8	8	15	17	15	24	12	9
625	-	94	87	9	9	17	12	19	25	11	9
1250 ^p	-	97	100	6	9	17	18	21	19	7	6
2500 ^p	-	91	94	6	6	19	18	17	18	6	8
5000 ^p	-	87	88	8	8	18	15	16	16	5	7
PC	-	511	570	247	279	104	100	346	348	448	437
0	+	74	74	7	9	18	18	32	34	11	14
156	+	93	99	10	8	26	21	33	34	7	4
313	+	105	88	9	7	24	26	38	36	8	10
625	+	96	88	7	10	27	25	35	33	7	8
1250 ^p	+	102	95	7	10	18	20	29	35	6	8
2500 ^p	+	102	92	7	7	21	18	28	32	8	8
5000 ^p	+	99	91	5	8	19	20	33	29	7	10
PC	+	482	514	160	161	305	323	193	196	83	110

p – precipitation
PC – Positive control

Conclusion:

4-OH-S-2200 is not mutagenic under the conditions tested.

Metabolite De-Xy-S-2200

Reference:	Acute Oral Toxicity Study of De-Xy-S-2200 in Rats
Author(s), year:	██████████ 2011
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0031
Guideline(s):	OECD 423; EC Directive 2004/73/EC, Acute toxic (oral); Japanese MAFF (12-Nousan No. 8147)
GLP:	Yes (Lab certified by National Authority)
Deviations:	None
Validity:	Yes

Material and methods:

<i>Test Material:</i>	De-Xy-S-2200
Lot/Batch:	Lot No.: 089-100702-1
Purity:	100.0%
Stability of test compound:	Confirmed for the duration of the study by Certificates of Analysis spanning the study period

Test animals:

Species:	Rat (females only)
Strain:	██████████:WistarHannover/Rcc
Age:	8 weeks (at start of study)
Weight at dosing:	First group: 155 – 157 g Second group: 149 – 165 g
Source:	██
Diet:	CRF-1 Pellet diet from Oriental Yeast Co, Ltd. Tokyo, Japan; <i>ad libitum</i>

Animals in the first group of three were each given a single gavage dose of 2000 mg/kg bw S-2200 TG in 0.5% aqueous methylcellulose. When no response was observed in the first group, a second group of three animals was dosed at 2000 mg/kg bw. Accuracy of dose formulation was confirmed by analysis. Clinical and mortality observations were made at 10 and 30 minutes, 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/tissues examined macroscopically.

Findings:

No mortality was observed.

All animals showed a decrease of spontaneous activity from 30 minutes after administration. This finding disappeared on day 3. In 4 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 3 and 2 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.

The body weights were not affected by the administration of the test compound throughout the study period. There were no findings at the gross pathological examination.

Conclusion:

No mortality occurred after administration of 2000 mg/kg bw (limit test). According to Regulation (EC) No.

1272/2008, classification of De-Xy-S-2200 for acute toxicity is not required.

Reference:	Reverse Mutation Test of De-Xy-S-2200 in Bacterial Systems
Author(s), year:	Kitamoto, S.; 2011
Report/Doc. number:	Sumitomo Chemical Co. Ltd. Report No. ROT-0038
Guideline(s):	US EPA OPPTS 870.5100, OECD 471, EU regulation 440/2008 B13/14, Japanese MAFF 12 Nousan 8147
GLP:	Yes (Lab certified by National Authority)
Deviations:	None
Validity:	Yes

Material and methods:

Test Material:	De-Xy-S-2200
Lot/Batch:	Lot No.: 089-100702-1
Purity:	100.0%
Stability of test compound:	Confirmed for the duration of the study by Certificates of Analysis spanning the study period. Stability was confirmed analytically in the DMSO solvent.
Solvent:	DMSO

Test strains and chemicals used as positive controls:

Without metabolic activation:

<i>S. typhimurium</i> TA100	0.01 µg/plate	2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
<i>S. typhimurium</i> TA1535	0.5 µg/plate	sodium azide
<i>E. coli</i> WP2uvrA	0.01 µg/plate	2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
<i>S. typhimurium</i> TA98	0.1 µg/plate	2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
<i>S. typhimurium</i> TA1537	80 µg/plate	9-aminoacridine

With metabolic activation:

<i>S. typhimurium</i> TA100	1 µg/plate	2-aminoanthracene
<i>S. typhimurium</i> TA1535	2 µg/plate	2-aminoanthracene
<i>E. coli</i> WP2uvrA	10 µg/plate	2-aminoanthracene
<i>S. typhimurium</i> TA98	0.5 µg/plate	2-aminoanthracene
<i>S. typhimurium</i> TA1537	2 µg/plate	2-aminoanthracene

Drug metabolizing enzyme system (S9 mix):

S9-fraction:	Oriental Yeast Co., Ltd. (Tokyo, Japan); Lot No. 11042201 Liver fraction prepared from male Sprague Dawley rats induced by phenobarbital and 5,6-benzoflavone
S9-mix:	10% (v/v) S9-fraction 8 µmol MgCl ₂ 33 µmol KCl 5 µmol glucose-6-phosphate 4 µmol NADPH 4 µmol NADH 100 µmol Na-phosphate buffer (pH 7.4)

De-Xy-S-2200 was evaluated for its mutagenic potential by a reverse mutation test with four strains of *Salmonella typhimurium* (TA100, TA98, TA1535, and TA1537) and one strain of *Escherichia coli* (WP2uvrA).

The test was conducted by the preincubation method in the presence and absence of metabolic activation (rat liver S9 mix).

In a dose-finding assay the doses ranged from 156 - 5000 µg/plate (Assay I). No precipitation or cytotoxicity was observed.

In the main assays, De-Xy-S-2200 was tested in triplicate at doses ranging from 156 - 5000 µg/plate with and without S9 mix in all strains (Assay II).

Findings:

There was no statistically significant dose-related increase in the number of revertant colonies to at least twice as many as that of the solvent control with or without S9 mix. The negative result was replicated (Assay II). Positive controls showed an appropriate increase in the number of revertant colonies.

Table B.6.8.3-14: Results of reverse mutation test in bacterial systems

Substance (µg/plate)	S9- mix	Revertant colonies/plate (mean)									
		TA100		TA1535		WP2uvrA		TA98		TA1537	
		Assay I	Assay II	Assay I	Assay II	Assay I	Assay II	Assay I	Assay II	Assay I	Assay II
0	-	99	96	12	9	17	21	23	19	6	9
156	-	106	100	10	9	20	20	21	16	6	6
313	-	90	96	11	8	20	20	22	18	6	10
625	-	111	106	6	8	19	28	19	16	7	6
1250	-	94	98	11	6	20	22	21	20	4	7
2500	-	92	99	8	8	25	19	19	20	6	11
5000	-	101	98	9	10	22	22	21	21	5	7
PC	-	703	749	420	408	131	132	479	427	861	1059
0	+	101	104	6	10	27	26	35	36	19	24
156	+	103	100	5	12	27	28	35	35	13	23
313	+	94	86	6	12	27	24	38	37	17	25
625	+	101	92	8	11	24	24	31	34	17	19
1250	+	86	95	7	10	31	20	30	35	21	25
2500	+	102	102	7	7	27	27	32	36	20	21
5000	+	107	99	7	11	26	26	29	34	21	21
PC	+	602	565	188	191	220	243	213	216	130	125

PC – Positive control

Conclusion:

De-Xy-S-2200 is not mutagenic under the conditions tested.

B.6.8.4 Summary of toxicological studies on metabolites

The toxicological properties of the mandestrobin metabolites 2-COOH-S-2200, 5-COOH-S-2200, 2-CH₂OH-S-2200, 4-OH-S-2200, and De-Xy-S-2200 have been investigated as summarized in the table below.

Acute toxicity testing showed weak oral toxicity for 5-COOH-S-2200.

Genotoxicity testing revealed no evidence for mutagenic potential for any of the metabolites tested.

Table B.6.8.4-1: Results of toxicity studies with metabolites of Mandestrobin

Metabolite	Type of study	Test system	Results
2-COOH-S-2200	Acute oral toxicity	Rat	LD ₅₀ > 2000 mg/kg bw
	Ames test	<i>S. typhimurium</i> , <i>E. coli</i>	Not mutagenic
	<i>In vitro</i> chromosomal aberration	Chinese hamster lung cells (CHL/IU)	induces non-biologically relevant chromosomal aberrations at high dose of 2200 µg/mL (equivalent to 6.4 mM) without metabolic activation at the cytotoxic level
	<i>In vitro</i> mammalian gene mutation assay	Chinese hamster V79 cells	Not mutagenic
	<i>In vivo</i> micronucleus assay	Mouse	Not clastogenic
5-COOH-S-2200	Acute oral toxicity	Rat	LD ₅₀ > 300 - < 2000 mg/kg bw
	Ames test	<i>S. typhimurium</i> , <i>E. coli</i>	Not mutagenic
	<i>In vitro</i> chromosomal aberration	Chinese hamster lung cells (CHL/IU)	Not clastogenic
	<i>In vitro</i> mammalian gene mutation assay	Chinese hamster V79 cells	Not mutagenic
2-CH ₂ OH-S-2200	Acute oral toxicity	Rat	LD ₅₀ > 2000 mg/kg bw
	Ames test	<i>S. typhimurium</i> , <i>E. coli</i>	Not mutagenic
4-OH-S-2200	Acute oral toxicity	Rat	LD ₅₀ > 2000 mg/kg bw
	Ames test	<i>S. typhimurium</i> , <i>E. coli</i>	Not mutagenic
De-Xy-S-2200	Acute oral toxicity	Rat	LD ₅₀ > 2000 mg/kg bw
	Ames test	<i>S. typhimurium</i> , <i>E. coli</i>	Not mutagenic

B.6.8.5 Assessment of the relevance of metabolites in groundwater

Two metabolites of mandestrobin, 2-COOH-S-2200 and 5-COOH-S-2200, were predicted to occur in groundwater at annual average concentrations exceeding 0.1 µg/L. Therefore, these metabolites are subject to further assessment in accordance with the “Guidance document on the assessment of the relevance of metabolites in groundwater of substances regulated under Council Directive 91/414/EEC” (Sanco/221/2000-rev.10-final, 25 February 2003).

Step 1: Exclusion of degradation products of no concern

The metabolites 2-COOH-S-2200 and 5-COOH-S-2200 do not meet the criteria of degradation products of no concern (as outlined in the guidance document). Therefore, additional information is required.

Step 2: Quantification of potential groundwater contamination

Predicted environmental concentrations in groundwater (PEC_{GW}) of S-2200 and its metabolites 2-COOH-S-2200 and 5-COOH-S-2200 were calculated for the intended use in winter oil seed rape according to FOCUS guidance using FOCUS PEARL 4.4.4.

For the metabolite 2-COOH-S-2200 the calculated PEC_{GW} values exceeded 0.1 µg/L in all relevant FOCUS scenarios and reached up to 0.4094 µg/L (Hamburg). For the metabolite 5-COOH-S-2200 the calculated PEC_{GW} values exceeded 0.1 µg/L in three out of six relevant FOCUS scenarios and reached up to 0.1734 µg/L (Hamburg). Therefore, a relevance assessment for the metabolites 2-COOH-S-2200 and 5-COOH-S-2200 is required.

(for details see chapter B.8.10)

Step 3: Hazard assessment: Identification of relevant metabolites

a. Stage 1 of Step 3: Screening for biological activity

The metabolites 2-COOH-S-2200 and 5-COOH-S-2200 were tested for their biological activity. None of the metabolites showed herbicidal, insecticidal or fungicidal activity.

(for details see chapters B.9.9.1 and B.9.11)

b. Stage 2 of Step 3: Screening for genotoxicity

Genotoxicity testing revealed no genotoxic potential for any of the metabolites:

Metabolite 2-COOH-S-2200 was not mutagenic in a bacterial reverse mutation test (Ames test in *S. typhimurium* and *E. coli*) and in an *in vitro* mammalian gene mutation assay (Chinese hamster V79 cells). In an *in vitro* chromosomal aberration test in Chinese hamster lung cells (CHL/IU), 2-COOH-S-2200 induced chromosomal aberrations only at the high dose of 2200 µg/mL (equivalent to 6.4 mM) without metabolic activation after 24 hours exposure at cytotoxic concentrations. However, the metabolite was negative in an *in vivo* micronucleus assay in the mouse and is therefore, overall, not considered to be clastogenic.

Metabolite 5-COOH-S-2200 was not mutagenic in a bacterial reverse mutation test (Ames test in *S. typhimurium* and *E. coli*) and in an *in vitro* mammalian gene mutation assay (Chinese hamster V79 cells) and not clastogenic in an *in vitro* chromosomal aberration test in Chinese hamster lung cells (CHL/IU).

(for details see chapter B.6.8.3 Toxicity studies on metabolites)

c. Stage 3 of Step 3: Screening for toxicity

Acute toxicity testing was performed with the metabolites 2-COOH-S-2200 and 5-COOH-S-2200 resulting in LD_{50} values of greater than 2000 mg/kg bw and > 300 - < 2000 mg/kg bw, respectively. According to Directive 67/548/EEC, the respective classification of the metabolites 2-COOH-S-2200 and 5-COOH-S-2200 is no classification and **Xn; R22** ("Harmful if swallowed").

Furthermore, the classification of the parent active substance mandestrobin is used as a starting point for toxicity screening. According to Regulation (EC) No. 1272/2008 as well as to Directive 67/548/EEC, mandestrobin requires no classification for acute, chronic, reproductive toxicity or carcinogenicity.

According to the guidance document, both metabolites are considered as "non-relevant metabolites" and are subject to an exposure and/or risk assessment as outlined in the steps below.

Step 4: Exposure assessment – threshold of concern approach

Following the TTC concept (toxicological threshold of concern), for substances of unknown structure the Scientific Committee on Plants proposed a toxicological threshold of concern of 1.5 µg/person/day (or 0.02 µg/kg bw/day). Assuming a consumption of 2 L of water per day, the acceptable exposure level relates to an acceptable estimated upper limit for the concentration of a metabolite of 0.75 µg/L.

For both metabolites, 2-COOH-S-2200 and 5-COOH-S-2200, the predicted environmental concentrations in groundwater are below 0.75 µg/L. There are no indications of potential exposure for consumers via other sources but drinking water. Therefore, the exposure of consumers to the non-relevant metabolites 2-COOH-S-2200 and 5-COOH-S-2200 via drinking water is considered acceptable.

Step 5: Refined risk assessment for non-relevant metabolites

Not required.

B.6.9 Medical data and information (Annex IIA 5.9)

B.6.9.1 Report on medical surveillance on manufacturing plant personnel

Reference:	Statement from S-2200 Manufacturer
Author(s), year:	Nishioka, K.; 2012
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0074
Guideline(s):	None applicable
GLP:	Not applicable
Deviations:	Not applicable
Validity:	Not applicable

A formal statement from the manufacturer is presented, that members of staff involved in the synthesis and development of S-2200 are routinely monitored and that no indication of S-2200-related ill-health have been detected by, or reported to, medical staff.

B.6.9.2 Report on clinical cases and poisoning incidents

No poisoning incidents or clinical cases have been reported.

B.6.9.3 Observations on exposure of the general population and epidemiological studies

S-2200 is a new chemical currently in development. There has been no exposure of the general population or epidemiology study.

B.6.9.4 Clinical signs and symptoms of poisoning and details of clinical tests

There is no experience of poisoning in man. From animal studies, a short exposure to S-2200 would not be expected to cause any specific symptoms. No diagnostic clinical test is appropriate.

B.6.9.5 First aid measures

No specific first aid measures are recommended to treat S-2200 contaminated patients. In case of a symptomatic patient, it is strongly recommended that other potential causes of the symptoms be addressed.

B.6.9.6 Therapeutic regimes

No specific therapeutic measures are recommended to treat S-2200 contaminated patients. In case of a symptomatic patient, it is strongly recommended that other potential causes of the symptoms be addressed.

B.6.9.7 Expected effects and duration of poisoning as a function of the type, level and duration of exposure or ingestion

No symptoms would be expected as a result of S-2200 poisoning. S-2200 is rapidly absorbed and eliminated; even in the absence of treatment.

B.6.9.8 Expected effects and duration of poisoning as a function of varying time periods between exposure or ingestion and commencement of treatment

It is expected that no reaction to S-2200 would be detectable, or harmful. No duration can be attributed.

B.6.10 Summary of mammalian toxicity and overall evaluation (Annex IIA 5.11)

Adsorption, distribution, metabolism, excretion

Adsorption and Excretion

Following a single oral high (1000 mg/kg bw) or low (5 mg/kg bw) dose administration of racemic [^{14}C]S-2200, labelled at either the benzyl or phenoxy rings, radioactivity was rapidly absorbed (more rapid at low than high doses). At the low dose, absorption of S-2200, calculated from the radioactivity recovered in urine and bile, was greater than 90% of the administered dose. At the high dose, systemic exposure (AUC) to S-2200 derived radioactivity was sub-proportional, indicating saturation of absorption processes following administration. Secondary absorption phases following administration of 1000 mg/kg doses of S-2200 indicated that systemic exposure at high concentrations may have resulted from one or more of the following: (a) enterohepatic recirculation, (b) differential absorption during transit through the gastro-intestinal tract, or (c) transient solubility of the S-2200. There were no significant gender differences in the pharmacokinetics of S-2200 at 5 or 1000 mg/kg bodyweight.

Clearance from the plasma was almost complete by 120 hours post-administration after a single oral dose of 5 or 1000 mg/kg bodyweight. Faecal elimination, via the bile, was the primary route of elimination of radioactivity. However, renal elimination was also important for the excretion of metabolites. More than 70% of radioactivity was eliminated within 48 hours after single oral administration. There did not appear to be any gender-, dose- or radiolabel-related differences in either the rates or routes of excretion.

In an investigation of ADME of the two stereo isomers of the active substance, coded S-2200 *R*-isomer and S-2200 *S*-isomer, both were absorbed immediately after administration. S-2200 *R*-isomer derived radioactivity was rapidly excreted after single oral administration, whereas excretion of radiolabel from S-2200 *S*-isomer was less rapid (likely due to enterohepatic recirculation). In this study, urinary excretion was greater in female than male rats for both isomers. Excretion into expired air was negligible.

Following the repeated daily administration of [benzyl- ^{14}C]S-2200 at 5 mg/kg bw for 14 days, there were no differences compared to single dose of 5 mg/kg bw. Faeces were again the primary route of excretion.

Distribution

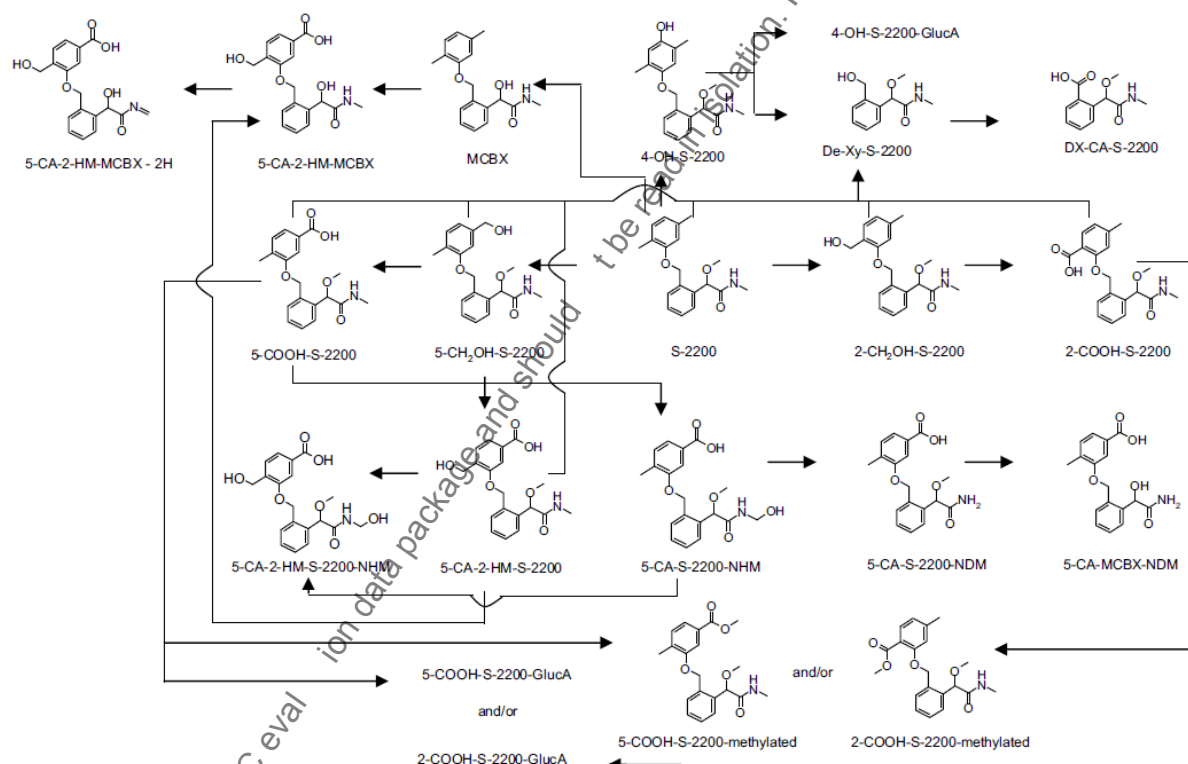
Following oral exposure, S-2200 is widely distributed throughout the body. After single oral administration there was no major difference in distribution between high and low doses (1000 and 5 mg/kg bw, respectively) or between sexes. The major tissue residues were seen in the gastrointestinal tract, and in liver and kidney, as well as in uterus and ovaries at 168 hours after dosing. Tissue distribution was similar in animals administered [benzyl- ^{14}C]S-2200 or [phenoxy- ^{14}C]S-2200 indicating that the core of the molecule was stable during systemic exposure. Furthermore, a similar distribution of radioactivity into tissues was observed following 1, 6, 10 and 14 daily doses of 5 mg/kg bw. There was no evidence of accumulation into tissues.

Metabolism

S-2200 was extensively metabolised to numerous metabolites. Unchanged parent was found in faeces only at < 0.2% of administered dose after a single low dose of 5 mg/kg bw and at < 6% after single high dose of 1000 mg/kg bw. The primary routes of metabolism were by (i) oxidation and subsequent conjugation with glucuronic acid, (ii) demethylation with subsequent oxidation, or (iii) oxidation with subsequent demethylation. Metabolite fractions in plasma, liver and kidney were identified. Residues were in general

Following repeated administration of radiolabelled S-2200 at 5 mg/kg bw for 14 days, metabolism was similar to that after single dose, however, kidney profiles exhibited both gender and repeat dose differences, in the type and number of metabolites observed.

B.6.9.8-1 Proposed metabolic pathway in rats



Acute toxicity

All conducted studies are compliant with the EU and OECD testing guidelines and Good Laboratory Practice (GLP).

Mandestrobins are of very low acute toxicity in the rat by the oral and dermal route and by inhalation. It is not a skin irritant and induced only mild eye irritation in the rabbit. Mandestrobins did not cause skin sensitisation in test animals. Therefore, no classification and labelling is required for acute toxicity, skin and eye irritation, or sensitisation.

Short term toxicity

A series of studies was carried out to investigate the effects of orally administered mandestrobin in rats (one 90-day study), mice (one 90-day study) and dogs (one 90-day and one 1-year study) following repeated exposure via the oral route over subchronic periods. In addition, effects after repeated exposure via the dermal route were also investigated in the rat (one 28-day study). Furthermore, a 28 day immunotoxicity study in rats was performed.

In the 90-days rat study, the NOAEL was set at 4000 ppm (282.6 ♂ and 320.1 ♀ mg/kg bw/day) based on liver weight increase of more than or close to 20%, hepatocellular hypertrophy, increased cholesterol, and thyroid follicular cell hypertrophy at 10000 ppm.

The mechanistic basis for increases in liver weight, hepatocellular hypertrophy, and follicular cell hypertrophy in the thyroid gland observed from 4000 ppm is considered to be liver enzyme induction (considered an adaptive effect) and increased catabolism of thyroid hormones in a manner similar to the observed effects induced by phenobarbital (see also position paper Yamada 2012a and study Asano 2012e). These effects are not considered relevant for human risk assessment.

In a 90-days toxicity study in the mouse (0, 1750, 3500 and 7000 ppm S-2200 TG), liver weight increases were noted in all treated groups in both sexes. Because an adaptive mechanism is ascribable for the increase in liver weights rather than a pathological effect, the increases were considered non-adverse, particularly in the absence of any biochemical or histological markers of liver pathology.

In the absence of other treatment-related adverse findings, the NOAEL is considered to be 7000 ppm (807.3 mg/kg bw/day for males and 1111.2 mg/kg bw/day for females, respectively).

In the 90-days study in dogs the NOAEL is proposed at 4000 ppm (90.9 mg/kg bw/day for male and 102.7 mg/kg bw/day for female animals), based on increased liver weight, histopathological changes in the liver (pigmentation and centrilobular degeneration), and increased alkaline phosphatase levels.

In the 1-year dog study the NOAEL for males is proposed at 800 ppm (19.2 mg/kg bw/day), and the NOAEL for females at 4000 ppm (92.0 mg/kg bw/day). Mandestrobin administration of 8000 ppm was associated with increased absolute and relative liver weights, hepatocyte hypertrophy, hepatocyte pigment and disturbances to clinical biochemistry parameters (increased alkaline phosphatase, γ -glutamyltransferase and triglycerides) in both genders. At 4000 ppm, male animals showed increased ALP-activity, hepatocyte hypertrophy, and pigmentation. The livers of female animals showed similar changes at the next higher dose level (8000 ppm).

In the dermal 28-days study in rats, no toxicological change related to administration of the test compound was observed in any tested parameter. Therefore, the NOAEL is concluded to be 1000 mg/kg bw/day (the highest dose tested) for both males and females.

In an oral range finding study and in an immunotoxicity study, no treatment related effects were observed up to the highest dose tested. The NOAEL for immunotoxicity was therefore set at 15000 ppm (equivalent to 1419 mg/kg bw/day).

Genotoxicity

Mandestrobin was tested in a standard battery of genotoxicity and mutagenicity tests *in vitro* and *in vivo*. There was no indication of induction of gene mutation either in the presence or absence of metabolic activation in both the bacterial reverse mutation and the mammalian gene mutation tests (HPRT). A negative response for chromosomal aberrations was observed *in vitro* in the Chinese hamster lung cells (CHL/IU). A mouse micronucleus test *in vivo* gave clear negative results. It can be concluded that mandestrobin has no genotoxic potential.

Long term toxicity/carcinogenicity

Groups of 70 male and 70 female Wistar rats were offered S-2200 TG in the diet at concentrations of 0 (control), 400, 2000, 7000, and 15000 ppm. After 52 weeks, satellite groups of 20 males and 20 females were used for interim sacrifice and the remaining survivors sacrificed after 104 weeks of treatment.

Due to the magnitudes of the decreased body weight and body weight gain (at 15000 ppm) and toxicological alterations in the liver including increased liver weights in combination with a higher degree of hepatocellular hypertrophy and hepatocyte vacuolation (both at ≥ 7000 ppm) and increased blood biochemistry parameters (total cholesterol and gamma-glutamyltransferase in males offered 15000 ppm), the No Observed Adverse Effect Level (NOAEL) for males was considered to be 2000 ppm (105.1 mg/kg bw/day).

For females, body weight and body weight gain was significantly decreased at ≥ 2000 ppm following 104 weeks of treatment. Toxicological alterations in the liver in females included increased liver weights (at ≥ 2000 ppm), a higher degree of hepatocellular hypertrophy and hepatocyte vacuolation (at ≥ 2000 ppm and at ≥ 7000 ppm, respectively) and increased blood biochemistry parameters (increased total cholesterol and increased gamma glutamyltransferase at ≥ 7000 ppm and at ≥ 15000 ppm, respectively). Therefore, the NOAEL for females was considered to be 400 ppm (26.7 mg/kg bw/day) for this study following 104 weeks of treatment.

Regarding the carcinogenic potential of S-2200 TG, no increase of neoplastic findings exceeding the historical control range was observed in any organ of treated animals, with exception of benign sex-cord stromal tumours in the ovary. Four and six cases occurred in female rats of the carcinogenicity cohort dosed with 7000 ppm and 15000 ppm, respectively, at dose levels where body weight gain was reduced by more than 20%, indicating that the maximum tolerated dose was exceeded. S-2200 TG is not genotoxic. The incidence of only one benign tumour type was increased. Sex-cord stromal hyperplasia does occur in aged Wistar rats, and the animals used in this study appear to be derived from a susceptible batch. The incidences of sex-cord stromal proliferative lesions were within historical controls for all groups, and there was no statistically significant difference between groups for hyperplasia or tumours. Higher survival rates in animals offered 7000 and 15000 ppm may have contributed to the higher numbers of tumours. The increased incidence of sex-cord stromal tumour in the ovary at 7000 ppm and 15000 ppm exceeded the Covance, Harrogate historical control range (0-3.1%) for this strain of rat. However, it must be highlighted that the concurrent control animals (incidence 2/50 = 4%) also exceeded the historical control range for sex-cord stromal tumours.

Since there is no interaction of the test article with the oestrogen receptor and steroidogenesis as evidenced by *in vitro* assays, no direct ovarian toxicity and no reproductive abnormalities, a mode of action via endocrine imbalance is considered to be unlikely. Furthermore, there was no accumulation or persistence of S-2200 TG or its metabolites in the ovary. In the corresponding mouse carcinogenicity bioassay, the number of tumours in any tissue was not increased by exposure to S-2200 TG. Thus, based on this evidence, the sex-cord stromal lesions are considered unlikely to be toxicologically relevant.

Overall, according to Regulation (EC) No. 1272/2008 and in consideration of the *Guidance on the Application of CLP Criteria, Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, version 2.0 April 2012*, no classification and labelling as carcinogenic substance is proposed for the active substance mandestrobin.

Groups of 51 male and 51 female CD-1 mice were given S-2200 TG in the diet at concentrations of 0 (controls), 700, 2000, and 7000 ppm for 78 weeks. Satellite groups of 12 mice per sex per dose were reared up to 52 weeks for interim sacrifice.

Treatment with S-2200 TG was well-tolerated. In the absence of adverse effects, the NOAEL for this study was considered to be 7000 ppm (823.9 mg/kg bw/day for males and 994.0 mg/kg bw/day for females), the

top dose tested, following 78 weeks of treatment. There were no effects on survival/mortality or on the incidence or morphology of tumours to indicate any oncogenic potential.

Reproductive toxicity

S-2200 TG was examined in a two-generation reproductive toxicity study in the rat and in teratogenicity studies in the rat and rabbit.

In the 2-generation study (doses of 1000, 3000, and 10000 ppm via diet), S-2200 TG showed no evidence of an effect on fertility or reproductive function, resulting in a NOAEL for the reproductive effects at the highest dose level tested in this study of 10000 ppm (559 mg/kg bw/day).

Regarding the general toxicological effects on parental animals, suppressed body weight gain and reduced food consumption were noted in males and in females in the 10000 ppm group in both F0 and F1 generations.

Pathological examination revealed treatment-related changes in the liver of both sexes. At necropsy, dark brownish change and enlargement of the liver were noted in F0 and F1 females in the 10000 ppm group. Liver weights increased in males at ≥ 3000 ppm and in females at ≥ 1000 ppm in both generations. In the histopathological examination the following findings were observed: Brown pigment in the bile duct/periportal area (in F0 animals at 10000 ppm and in F1 animals at ≥ 3000 ppm), focal periductular inflammatory cell infiltration (in F0 males and females and F1 males at 10000 ppm and in F1 females at ≥ 3000 ppm), and brown pigment deposition in the peribulbar hepatocyte and proliferation of the bile duct (in F0 and F1 females in the 10000 ppm group). Diffuse hypertrophy of the hepatocyte was also observed in males at ≥ 3000 ppm and in females at ≥ 1000 ppm in both generations. In the 1000 ppm group, only hepatocellular hypertrophy and increased liver weights were observed in F0 and F1 females, without any other change. Therefore, the changes in the liver observed in females in the 1000 ppm group were considered to be an adaptive change, and of no toxicological significance.

Furthermore, increases in the thyroid weights were observed in F0 males at ≥ 3000 ppm and in F1 males at 10000 ppm, and hypertrophy of the follicular cells of the thyroid was observed in some F0 males in the 10000 ppm group, which was considered to be secondary to the increased hormonal turnover and the changes in the liver.

Treatment-related hypertrophy of cortical cells in the fascicular zone was observed in the adrenals in some F1 females in the 10000 ppm group. In addition, decreases were observed in ovary weights in F0 and F1 females and uterus weights in F0 females in the 10000 ppm group, however, in absence of any histopathological changes.

Furthermore, greenish change in the cortex of the kidney was observed in some F0 females at necropsy in the 10000 ppm group, with increased organ weights but without any histopathological changes. Therefore, the changes in the kidney observed in F0 females were considered to be of no toxicological significance. Based on the adverse liver effects observed at dose levels of 3000 ppm and above, the parental NOAEL is considered to be 1000 ppm (43 mg/kg bw/d).

Regarding the effects on offspring, postnatal body weight gain was suppressed in both sexes of F1 and F2 offspring in the 10000 ppm group, probably due to undernourishment of their dams.

In addition, lower spleen weights at weaning were noted in F1 males in the 3000 ppm group, in which no change was found in postnatal body weight gain, and in all F1 and F2 animals at 10000 ppm. The lower spleen weights of F1 animals at weaning completely recovered to the control level at adult in both sexes even in the 10000 ppm group, suggesting a transient retardation in growth. Moreover, both the absolute and relative spleen weights of F1 and F2 animals were within the historical control range in the test facility, indicating slight changes.

A slight delay in sexual maturation was found in both sexes (mean difference from the control group: 1.5 days for vaginal opening in F1 females and 1.6 days for preputial separation in F1 males) in the 10000 ppm group, which was considered to be related to the growth retardation.

Under the conditions of this study, the NOAEL for effects on offspring is considered to be 1000 ppm (56 mg/kg bw/d).

In a gavage developmental toxicity study, administration of 0, 100, 300, and 1000 mg/kg bw/day from Days 6 to 19 of gestation to pregnant rats elicited no treatment-related adverse effects up to the highest dose level tested. Therefore, both the maternal and the foetal NOAEL were set at 1000 mg/kg bw/day

Administration of S-2200 TG by oral gavage to pregnant rabbits at doses of 0, 100, 300 or 1000 mg/kg bw/day from Days 7 to 28 of pregnancy elicited no systemic toxicity to maternal female rabbits. There was no evidence of embryotoxicity or developmental effects at any dose level tested. Under the conditions of this study, both the maternal and the foetal NOAEL were set at 1000 mg/kg bw/day.

Based on the results of one 2-generation reproduction toxicity study in the rat and two developmental toxicity studies in rat and rabbit, no classification and labelling is triggered for mandestrobin.

Neurotoxicity

The potential for mandestrobin to cause neurotoxicity was thoroughly assessed in acute and subchronic neurotoxicity studies.

In a range-finding assay designed to identify the time of peak effect after an acute oral limit dose (2000 mg/kg bw), no reaction was seen, so no peak effect was evident. In the subsequent definitive acute neurotoxicity study, investigations intended for the “time of peak effect” were therefore conducted at the latest timepoint compatible with guideline, i.e. 8 hours post-dose.

The only treatment-related finding in the definitive acute neurotoxicity study was a decrease in mean locomotor activity, seen at the top dose (2000 mg/kg bw) at 8 hours post-dose. In the absence of any other specific neurological findings, this is probably attributable to transient systemic toxicity rather than typical neurotoxicity. The NOAEL, set on a highly precautionary basis assuming a decrease in locomotor activity in absence of any other neurological change might be meaningful, was 1000 mg/kg bw.

In a 90-day subchronic neurotoxicity study at doses of up to 15000 ppm, no evidence of specific neurotoxicity was found. The NOAEL for specific neurotoxicity was therefore approximately 1024 mg/kg bw/day (the highest dose level tested). On the basis of body weight gain retardation at the top dose in males only, the NOAEL for systemic toxicity was 338 mg/kg bw/day.

Mechanistic studies

Several *in vivo* and *in vitro* mechanistic studies were performed, and two detailed position papers were submitted dealing with (i) liver and thyroid effects and (ii) ovary issues.

Two *in vivo* studies were performed (one in rats, one in mice) to gain insight on the mechanistic basis of the liver and thyroid effects observed in the main studies in rats. The effects observed were compared to CAR-mediated induction of liver enzymes and subsequent perturbations of thyroid hormones, a well-studied mode of action with no relevance for human risk assessment.

A detailed description of the effects observed in the main and mechanistic studies, as well as a detailed assessment of the mode of action of mandestrobin is provided in a position paper included in this report.

The proposed phenobarbital-like mode of action for mandestrobin tested in the rat mechanistic *in vivo* 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 render it non relevant for humans. Furthermore, no increased tumour rates were observed up to the highest doses tested in the long term/carcinogenicity studies.

Two *in vitro* studies were performed to address possible hormonal effects of mandestrobin and its metabolites. Mandestrobin had no influence on testosterone and estradiol production, and 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.

In a position paper (and its update), the toxicological significance of the slightly higher incidence of benign ovarian sex-cord stromal tumours in the 2-year rat study is discussed based on existing data of mandestrobin, background data of ovarian proliferative change present in elderly rats, and published information. The overall conclusion is that the data do not suggest a carcinogenic effect of mandestrobin and thus classification is not warranted.

Toxicological studies on metabolites

The toxicological properties of the mandestrobin metabolites 2-COOH-S-2200, 5-COOH-S-2200, 2-CH₂OH-S-2200, 4-OH-S-2200, and De-Xy-S-2200 have been investigated.

Acute toxicity testing showed weak oral toxicity for 5-COOH-S-2200, resulting in a proposal for classification and labelling as acutely toxic after oral administration category 4.

Genotoxicity testing revealed no evidence for mutagenic potential for any of the metabolites tested.

Medical data

A formal statement from the manufacturer is presented, that members of staff involved in the synthesis and development of S-2200 are routinely monitored and that no indication of S-2200-related ill-health have been detected by, or reported to, medical staff.

B.6.10.1 Proposal for ADI

The estimation of the Acceptable Daily Intake (ADI) is based on the most relevant no-observed adverse effect level (NOAEL) observed in studies with respect to subchronic and chronic toxicity, carcinogenicity and reproductive toxicity. Given the results from all studies considered to be relevant for setting an ADI (see Table B.6.10.1-1), the dog was the most sensitive species with liver effects observed in male dogs after 1 year of treatment at 92 mg/kg bw/day (hepatocyte hypertrophy, pigmentation, and increased alkaline phosphatase levels). The NOAEL in this study was set at 19.2 mg/kg bw/day for male dogs.

No risk for carcinogenicity, neurotoxicity, developmental or reproductive toxicity was identified, and therefore the conventional safety factor of 100 is considered appropriate.

The proposed ADI is 0.19 mg/kg bw/day (safety factor 100) based on the 1 year toxicity study in dogs.

Table B.6.10.1-1: Summary of toxicity studies considered relevant for setting the ADI

Study	Dose levels	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Effects observed at the LOAEL
Dog, 1-year (oral)	0, 200, 800, 4000, 8000 ppm	♂ 19.2 ♀ 92.0	♂ 92.0 ♀ 225.7	♂ hepatocyte hypertrophy, pigmentation ↑ alkaline phosphatase levels
2012a	♂: 0, 4.3, 19.2, 92.0, 180.7 mg/kg bw/day ♀: 0, 4.5, 20.4, 92.0, 225.7 mg/kg bw/day			♀ ↑ rel liver weight hepatocyte hypertrophy, pigmentation ↑ alkaline phosphatase levels

Study	Dose levels	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Effects observed at the LOAEL
Rat, 104 weeks (oral via diet) 2012b	0, 400, 2000, 7000, 15000 ppm ♂: 0, 21.0, 105.1, 375.6 and 804.3 mg/kg bw/day ♀: 0, 26.7, 135.2, 475.0 and 1016.2 mg/kg bw/day	♂ 105.1 ♀ 26.7	♂ 375.6 ♀ 135.2	♂ ↑ liver weight ↑ hepatocellular hypertrophy ↑ hepatocyte vacuolation ♀ ↓ body weight and bw gain ↑ liver weight ↑ hepatocellular hypertrophy
Mouse, 78 weeks (oral via diet) 2012c	0, 700, 2000, 7000 ppm ♂: 0, 82.5, 238.8 and 823.9 mg/kg bw/day ♀: 0, 99.2, 280.3 and 994.0 mg/kg bw/day	♂ 823.9 ♀ 994.0	♂ - ♀ -	No adverse effects of treatment at the highest dose tested
Rat, dose range- finding study for two-generation reproduction toxicity study 2010	0, 5000, 10000, 20000 ppm equivalent to: 0, 244 – 782, 499 – 1429, 1033 – 2441 mg/kg bw/d (rounded)	Parental: ♂ - (lowest dose tested was a LOAEL) ♀ 317 (5000 ppm)	Parental: ♂ 244 (5000 ppm) ♀ 667 (10000 ppm)	♂ ↑ liver weight > 20% ♀ ↓ body weight and bw gain and food consumption ↑ liver weight ↑ hepatocellular hypertrophy Brown pigment in bile duct/perilobular hepatocytes, Inflammatory cell infiltration in periductular region ↓ vacuolation in the interstitial gland in the ovary ↓ uterus weight atrophy of the uterus

Study	Dose levels	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Effects observed at the LOAEL
Rat, two-generation reproduction toxicity study 2012	0, 1000, 3000, 10000 ppm equivalent to: 0, 43 – 163, 132 - 511, 452 – 1688 mg/kg bw/d (rounded)	Offspring: 317 (5000 ppm)	Offspring: 668 (10000 ppm)	↓ body weight and body weight gain ↓ spleen weight
		Reproduction: 1229 (20000 ppm)	Reproduction: -	No effects at highest dose tested
		Parental: 43 (1000 ppm)	Parental: 132 (3000 ppm)	♂ ♀ Liver: Increased liver weight and diffuse hepatocellular hypertrophy, brown pigment in bile duct/periportal area, focal periductular inflammatory cell infiltration Thyroid: Increased thyroid weight (males)
		Offspring: 56 (1000 ppm)	Offspring: 166 (3000 ppm)	♂ ↓ spleen weights at weaning
		Reproduction: 559 (10000 ppm)	Reproduction: -	No effects at highest dose tested

B.6.10.2 Proposal for ARfD

For the determination of the Acute Reference Dose (ARfD), results from oral studies with acute or short term exposure are considered to be the most relevant. Mandestrobin is of low acute toxicity. No developmental effects independent of maternal toxicity were observed at high dose levels. No clinical effects other than those consistent with a phenobarbital-like mode of action were observed early in repeat dose studies. There were no indications of neurotoxic effects, and no hormonal or biochemical alterations that could conceivably be elicited by a single dose.

Since no risk can be foreseen from acute exposure or intake, derivation of an Acute Reference Dose is not necessary.

Table B.6.10.2-1: Summary of toxicity studies considered relevant for setting the ARfD

Study	Dose levels	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Effects observed at the LOAEL
Rat, 28-days (dermal) 2011	0, 100, 300, 1000 mg /kg bw/day	1000	-	No treatment related effects observed at the highest tested dose level

<p>Rat, 28-days (oral), range finding study for immunotox and neurotox studies</p> <p>██████████ 2011a</p>	<p>0, 1500, 5000, 15000 ppm</p> <p>♂: 0, 132, 430, 1200 mg/kg bw/day</p> <p>♀: 0, 135, 436, 1340 mg/kg bw/day</p>	<p>♂ 1200</p> <p>♀ 1340</p>	-	<p>No treatment related effects observed at the highest tested dose level</p>
<p>Rat, 28-days (oral), immunotox study</p> <p>██████████ 2011b</p>	<p>0, 1500, 5000, 15000 ppm</p> <p>♀: 0, 147, 471, 1419 mg/kg bw/day</p>	<p>♀ 1419</p>	-	<p>No treatment related effects observed at the highest tested dose level</p>
<p>Rat, 90-days (oral)</p> <p>██████████ 2011a</p>	<p>0, 800, 4000, 10000, 20000 ppm</p> <p>♂: 0, 54, 282.6, 742.7, 1544.6 mg/kg bw/day</p> <p>♀: 0, 61.6, 320.1, 788.5, 1886.5 mg/kg bw/day</p>	<p>♂ 282.6</p> <p>♀ 320.1</p>	<p>♂ 742.7</p> <p>♀ 788.5</p>	<p>↑ absolute and relative liver weight</p> <p>Hepatocellular hypertrophy</p> <p>Follicular cell hypertrophy in the thyroid</p> <p>↑ Cholesterol levels</p>
<p>Mouse, 90-days (oral)</p> <p>██████████ 2011b</p>	<p>0, 1750, 3500, 7000 ppm</p> <p>♂: 0, 204.1, 404.9, 807.3 mg/kg bw/day</p> <p>♀: 0, 251.8, 529.1, 1111.2 mg/kg bw/day</p>	<p>♂ 807.3</p> <p>♀ 1111.2</p>	-	<p>No treatment related effects observed at the highest tested dose level</p>

Dog, 90-days (oral)	0, 4000, 12000, 40000 ppm	♂ 90.9 ♀ 102.7	♂ 267.8 ♀ 304.4	↑ liver weight Pigmentation of the liver Centrilobular degeneration ↑ alkaline phosphatase levels
2012d	♂: 0, 90.9, 267.8, 933.1 mg/kg bw/day ♀: 0, 102.7, 304.4, 820.4 mg/kg bw/day			
Rat, 90-day oral dietary neurotoxicity study	0, 1500, 5000, 15000 ppm	Neurotoxicity: ♂ 1024 ♀ 1223	Neurotoxicity: -	No neurotoxicity at highest dose tested
2012	♂: 0, 99, 338, 1024 mg/kg bw/day ♀: 0, 122, 415, 1223 mg/kg bw/day	Systemic tox.: ♂ 338 ♀ 1223	Systemic tox: ♂ 1024 ♀ -	Systemic toxicity: ♂: ↓ body weight, body weight gain and food consumption ♀: no adverse effects at the highest dose tested
Rat, dose range-finding study for two-generation reproduction toxicity study	0, 5000, 10000, 20000 ppm equivalent to: 0, 244 – 782, 499 – 1429, 1033 – 2441 mg/kg bw/day (rounded)	Parental: ♂ - (lowest dose tested was a LOAEL) ♀ 317 (5000 ppm)	Parental: ♂ 244 (5000 ppm) ♀ 667 (10000 ppm)	♂ ↑ liver weight > 20% ♀ ↓ body weight and bw gain and food consumption ↑ liver weight ↑ hepatocellular hypertrophy Brown pigment in bile duct/perilobular hepatocytes, Inflammatory cell infiltration in periductular region ↓ vacuolation in the interstitial gland in the ovary ↓ uterus weight atrophy of the uterus
2010		Offspring: 317 (5000 ppm)	Offspring: 668 (10000 ppm)	↓ body weight and body weight gain ↓ spleen weight
		Reproduction: 1229 (20000 ppm)	Reproduction: -	No effects at highest dose tested

Rat, two-generation reproduction toxicity study 2012	0, 1000, 3000, 10000 ppm equivalent to: 0, 43 – 163, 132 - 511, 452 – 1688 mg/kg bw/day (rounded)	Parental: 43 (1000 ppm)	Parental: 132 (3000 ppm)	♂ ♀ Liver: Increased liver weight and diffuse hepatocellular hypertrophy, brown pigment in bile duct/periportal area, focal periductular inflammatory cell infiltration ♂ Thyroid: Increased thyroid weight (males)
		Offspring: 56 (1000 ppm)	Offspring: 166 (3000 ppm)	♂ ↓ spleen weights at weaning
		Reproduction: 559 (10000 ppm)	Reproduction: -	No effects at highest dose tested
Rat, developmental toxicity range finding study 2009a	0, 250, 500, 1000 mg/kg bw/day	Maternal: 1000	-	No treatment-related effects at highest dose tested
		Foetal: 1000	-	No treatment-related effects at highest dose tested
Rat, developmental toxicity study 2012a	0, 100, 300, 1000 mg/kg bw/day	Maternal: 1000	-	No treatment-related effects at highest dose tested
		Foetal: 1000	-	No treatment-related effects at highest dose tested
Rabbit, developmental toxicity range finding study 2009b	0, 250, 500, 1000 mg/kg bw/day	Maternal: 1000	-	No treatment-related effects at highest dose tested
		Foetal: 1000	-	No treatment-related effects at highest dose tested
Rabbit, developmental toxicity study 2012b	0, 100, 300, 1000 mg/kg bw/day	Maternal: 1000	-	No treatment-related effects at highest dose tested
		Foetal: 1000	-	No treatment-related effects at highest dose tested

B.6.10.3 Proposal for drinking water limit

According to Directive 98/83/EC a drinking water limit of 0.1 µg mandestrobin/L is established.

B.6.10.4 Proposal for AOEL

According to the principles of Annex II to Regulation 1107/2009/EEC, the proposed Acceptable Operator Exposure Level (AOEL) should be established on the basis of the highest dose at which no adverse effect is observed in relevant studies in the most sensitive species. The AOEL is usually based on the NOAEL of an oral short term study (typically a 90 day study, occasionally a 1 year study), provided that the critical end points of the substance (e.g. reproductive, developmental toxicity, or neurotoxicity) are covered.

The most sensitive species was the dog, with the NOAEL of 4000 ppm or 90.9 mg/kg bw/day in male dogs as the lowest NOAEL derived from a short term study. The effects observed at the LOAEL in this study are increased liver weight, pigmentation of the liver, centrilobular degeneration and increased alkaline phosphatase activity.

Since enteral resorption is estimated to be about 90%, no correction factor is necessary. The standard safety factor of 100 is considered appropriate, and therefore the proposed AOEL is 0.91 mg/kg bw/day (safety factor 100, rounded) based on the NOAEL for male animals in the 90 day toxicity study in dogs.

Table B.6.10.4-1: Summary of toxicity studies considered relevant for setting the AOEL

Study	Dose levels	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Effects observed at the LOAEL
Rat, 90-days (oral) 2011a	0, 800, 4000, 10000, 20000 ppm ♂: 0, 54, 282.6, 742.7, 1544.6 mg/kg bw/day ♀: 0, 61.6, 320.1, 788.5, 1886.5 mg/kg bw/day	♂ 282.6 ♀ 320.1	♂ 742.7 ♀ 788.5	↑ absolute and relative liver weight Hepatocellular hypertrophy Follicular cell hypertrophy in the thyroid ↑ Cholesterol levels
Mouse, 90-days (oral) 2011b	0, 1750, 3500, 7000 ppm ♂: 0, 204.1, 404.9, 807.3 mg/kg bw/day ♀: 0, 251.8, 529.1, 1111.2 mg/kg bw/day	♂ 807.3 ♀ 1111.2	-	No treatment related effects observed at the highest tested dose level

Dog, 90-days (oral) 2012d	0, 4000, 12000, 40000 ppm ♂: 0, 90.9, 267.8, 933.1 mg/kg bw/day ♀: 0, 102.7, 304.4, 820.4 mg/kg bw/day	♂ 90.9 ♀ 102.7	♂ 267.8 ♀ 304.4	↑ liver weight Pigmentation of the liver Centrilobular degeneration ↑ alkaline phosphatase levels
Dog, 1-year (oral) 2012a	0, 200, 800, 4000, 8000 ppm ♂: 0, 4.3, 19.2, 92.0, 180.7 mg/kg bw/day ♀: 0, 4.5, 20.4, 92.0, 225.7 mg/kg bw/day	♂ 19.2 ♀ 92.0	♂ 92.0 ♀ 225.7	♂ hepatocyte hypertrophy, pigmentation ↑ alkaline phosphatase levels ↑ rel liver weight hepatocyte hypertrophy, pigmentation ↑ alkaline phosphatase levels
Rat, dose range- finding study for two-generation reproduction toxicity study 2010	0, 5000, 10000, 20000 ppm equivalent to: 0, 244 – 782, 499 – 1429, 1033 – 2441 mg/kg bw/day (rounded)	Parental: ♂ - (lowest dose tested was a LOAEL) ♀ 317 (5000 ppm)	Parental: ♂ 244 (5000 ppm) ♀ 667 (10000 ppm)	♂ ↑ liver weight > 20% ♀ ↓ body weight and bw gain and food consumption ↑ liver weight ↑ hepatocellular hypertrophy Brown pigment in bile duct/perilobular hepatocytes, Inflammatory cell infiltration in periductular region ↓ vacuolation in the interstitial gland in the ovary ↓ uterus weight atrophy of the uterus

		Offspring: 317 (5000 ppm)	Offspring: 668 (10000 ppm)	↓ body weight and body weight gain ↓ spleen weight
		Reproduction: 1229 (20000 ppm)	Reproduction: -	No effects at highest dose tested
Rat, two-generation reproduction toxicity study 2012	0, 1000, 3000, 10000 ppm equivalent to: 0, 43 – 163, 132 - 511, 452 – 1688 mg/kg bw/day (rounded)	Parental: 43 (1000 ppm)	Parental: 132 (3000 ppm)	♂ ♀ Liver: Increased liver weight and diffuse hepatocellular hypertrophy, brown pigment in bile duct/periportal area, focal periductular inflammatory cell infiltration ♂ Thyroid: Increased thyroid weight (males)
		Offspring: 56 (1000 ppm)	Offspring: 166 (3000 ppm)	♂ ↓ spleen weights at weaning
		Reproduction: 559 (10000 ppm)	Reproduction: -	No effects at highest dose tested
Rat, developmental toxicity range finding study 2009a	0, 250, 500, 1000 mg/kg bw/day	Maternal: 1000	-	No treatment-related effects at highest dose tested
		Foetal: 1000	-	No treatment-related effects at highest dose tested
Rat, developmental toxicity study 2012a	0, 100, 300, 1000 mg/kg bw/day	Maternal: 1000	-	No treatment-related effects at highest dose tested
		Foetal: 1000	-	No treatment-related effects at highest dose tested
Rabbit, developmental toxicity range finding study 2009b	0, 250, 500, 1000 mg/kg bw/day	Maternal: 1000	-	No treatment-related effects at highest dose tested
		Foetal: 1000	-	No treatment-related effects at highest dose tested
Rabbit, developmental toxicity study 2012b	0, 100, 300, 1000 mg/kg bw/day	Maternal: 1000	-	No treatment-related effects at highest dose tested
		Foetal: 1000	-	No treatment-related effects at highest dose tested

Rat, 90-day oral dietary neurotoxicity study 2012	0, 1500, 5000, 15000 ppm	Neurotoxicity: ♂ 1024	Neurotoxicity: -	No neurotoxicity at highest dose tested
	♂: 0, 99, 338, 1024 mg/kg bw/day ♀: 0, 122, 415, 1223 mg/kg bw/day	♀ 1223 Systemic tox.: ♂ 338 ♀ 1223	Systemic tox.: ♂ 1024 ♀ -	Systemic toxicity: ♂: ↓ body weight, body weight gain and food consumption ♀: no adverse effects at the highest dose tested

B.6.11 Acute toxicity of the preparations (Annex IIIA 7.1)

B.6.11.1 Acute oral toxicity

Reference:	Acute oral toxicity study of S-2200 25SC in rats
Author(s), year:	2011a
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0029
Guideline(s):	JMAFF 12 Nousan No 8147 (2011), OECD 423 "acute toxic class method" (2001), 2004/73/EC Part B.1 tris (2004)
GLP:	Yes
Deviations:	No
Validity:	Yes

Material and methods:

Test Material:	S-2200 25SC
Lot/Batch:	C09-5F101G
Purity:	24.96% active substance
Vehicle:	Distilled water
Test animals:	
Species:	Rat (females only)
Strain:	Sprague Dawley [CD(SD)]
Age at dosing:	8 weeks
Weight at dosing:	195 to 203 g
Source:	
Diet:	Pelleted diet CRF-1 (Oriental Yeast Co., Ltd.), <i>ad libitum</i>

The acute oral toxicity of S-2200 25SC in female Sprague Dawley rats was evaluated in two dose steps/groups with 3 animals in each group. The starting dose level (dose step 1) was set at 2000 mg/kg. Thereafter, in accordance with the Acute Toxic Class Method, and the absence of mortality in the first group, the second dose level (dose step 2) was also set at 2000 mg/kg.

Dose volume was set at 10 mL/kg body weight, and the specified amount of the test suspension was administered once orally by gavage. The animals were fasted overnight (approximately 16 hours) before administration. The body weight on the day of administration (study day 0) was used to calculate the volume of dosing suspension. Food was re-supplied after the clinical observation which was conducted 4 hours after administration. The observation period was for 14 days after administration.

The animals were observed for clinical signs (such as abnormalities of external appearance, nutritional condition, posture, behaviour and excretions) frequently after administration (from immediately to 5 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 4 and 6 hours after administration), and once daily thereafter for 14 days.

Body weight was measured on study days 0 (immediately before administration), 1, 3, 7 and 14. All animals were sacrificed after the 14-day observation period, and external appearance and organs/tissues in the cranial, thoracic and abdominal cavities were examined macroscopically.

The approximate LD₅₀ value was estimated based on mortality during the 14-day period after administration. For body weight, mean values with standard deviations were calculated on each day of measurement. Body weight gain during the observation period was calculated from the body weight on the day of administration and day 14 after administration, and the mean values, with standard deviations were calculated in the same manner.

Findings:

There were no mortality at either dose step and no clinical signs in any animal during the observation period. All animals in the first and second dose step showed normal body weight development. There were no abnormalities in the external appearance, or in organs/tissues in the cranial, thoracic or abdominal regions in any animal.

Conclusion:

The oral LD₅₀ of S-2200 25SC in female rats was greater than 2000 mg/kg bw in this acute oral toxicity study. Therefore no classification and labelling for acute oral toxicity is required.

B.6.11.2 Acute percutaneous (dermal) toxicity

Reference:	Acute dermal toxicity study of S-2200 25SC in rats
Author(s), year:	██████████ 2011b
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No ROT-0030
Guideline(s):	JMAFF 12 Nousan No. 8147 (2011), OECD 402 (1987), 92/69/EEC Part B.3 (1992)
GLP:	Yes
Deviations:	No
Validity:	Yes

Material and methods:

Test Material:	S-2200 25SC
Lot/Batch:	C09-5F101G
Purity:	24.96% active substance
Vehicle:	None (test material was used as supplied)

Test animals:	
Species:	Rat
Strain:	Sprague Dawley ██████████ CD(SD)]
Age:	8 weeks
Weight at dosing:	254 – 259 g (males) and 225-232 g (females)
Source:	██
Diet:	Pelleted diet CRF-1 (Oriental Yeast Co., Ltd.), <i>ad libitum</i>

The test material was administered to 5 male and 5 female rats. Because the acute dermal toxicity of the test substance was expected to be low, 2000 mg/kg was selected as the dose level. Dorsal fur (approximately 30 cm²: 5 cm x 6 cm) was clipped using an electric clipper on the day before administration. The requisite amount of the test substance was put on a lint sheet (approximately 20 cm²: 4 cm x 5 cm) and applied to the fur-clipped dorsal area. The lint sheet was covered and fixed with an elastic bandage. The body weight on the day of administration (study day 0) was used to calculate the volume of administration. After the 24-hour application period, the lint sheet and the elastic bandage were removed and the application site was wiped with water and absorbent gauze. The animals were observed for 14 days after application.

The animals were observed for clinical signs (such as abnormalities of external appearance, nutritional condition, posture, behaviour and excretions) frequently after administration (from immediately to 5 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 4 and 6 hours after administration), and once daily thereafter for 14 days.

Body weight was measured on study days 0 (immediately before administration), 3, 7 and 14.

All animals were sacrificed after the 14-day observation period, and external appearance including application site and organs/tissues in the cranial, thoracic and abdominal cavities were examined macroscopically.

The approximate LD₅₀ value was estimated based on mortality during the 14-day period after administration. For body weight, the mean values with standard deviations were calculated on each day of measurement for males and females. Mean body weight gains (and standard deviations) during the observation period were calculated from the body weight on the day of administration and day 14 after administration.

Findings:

There were no mortality and no clinical signs in any animal during the observation period. There were no signs of skin irritation such as erythema, eschar or oedema at the application site. All animals showed normal body weight development during the observation period. No abnormalities were observed at necropsy.

Conclusion:

The dermal LD₅₀ of S-2200 25SC in rats was greater than 2000 mg/kg bw in this acute dermal toxicity study. Therefore no classification and labelling for acute dermal toxicity is required.

B.6.11.3 Acute inhalation toxicity to rats

Reference:	S-2200 25SC: An acute inhalation toxicity study in Sprague-Dawley rats
Author(s), year:	██████████ 2011
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No ROT-0032
Guideline(s):	JMAFF 12 Nousan No. 8147, OECD 403, 92/69/EEC method B.2
GLP:	Yes
Deviations:	There were no methodological deficiencies; however the achieved concentration of 4.37 mg/L fell short of the limit concentration (5.0 mg/L) above which non-classification can be determined with certainty.
Validity:	Yes

Material and methods:

Test Material:	S-2200 25SC
Lot/Batch:	C09-5F101G

Test animals:

Species:	Rat
Strain:	Sprague-Dawley (SD:CD(SD))
Age:	Approximately 8 weeks at start of treatment
Weight at dosing:	297-328 g (males) and 206-230 g (females)
Source:	[REDACTED]
Diet:	Teklad Certified Rodent Diet (W) #8728C ad libitum

During the acclimation period, 5 male and 5 female rats were assigned to the dose group by block randomization based on body weights. Animals were exposed (nose-only) for 4 hours to an aerosol of the test article. The target dose concentration of 5 mg/L of S-2200 25SC was selected based on the data obtained from the previous acute inhalation study of S-2200 TG in rats (Sumitomo Chemical Co., Ltd. Report No. ROT-0020). In this study, there were no deaths and no treatment-related clinical signs during the exposure period (4 hours at a mean aerosol concentration of 4964 mg/m³ of S-2200 Technical Grade) and the 14-day observation period.

The aerosol was produced by metering the flow of the test article to one clinical nebulizer (MicroMist®, Hudson RCI). The aerosol produced was discharged through a 40-mm diameter tube into a flow-past inhalation exposure system. The airflow rate through the exposure system was monitored and recorded manually during the aerosol generation. Airflow to the exposure system was controlled by the absolute volume of air (Medical grade air, NQ 5710-500/2000, by Kaeser SM-11/ASD 401 Air compressors) supplying the aerosol generators using variable area flow meters (variable area-glass float, Model GF-6541, Gilmont). Control of the aerosol exhaust flow from the animal exposure system was achieved using an exhaust valve, and the overall balance of airflows in the exposure system was monitored using pressure gauges. The system (with 12 open ports) provided a minimum of 1 L/min atmosphere to each animal exposure port and was balanced to ensure a slight positive pressure at the site of the animal exposure. This ensured that there was no dilution of the generated aerosol. An equal delivery of aerosol to each exposure position was achieved by employing a distribution network that was identical for each individual exposure position attached to the system.

Nine aerosol concentration filter (GF/A glass microfibre filters, Fisher Scientific) samples were collected during exposure of Group 1 animals. Each sample was weighed for gravimetric determination of the aerosol concentration and all samples were transferred to ITR's Analytical Laboratory for chemical determination of S-2200 content using a validated method.

The distribution of particle size in the generated aerosols was determined three times during exposure of Group 1 by collecting substrates samples into a 7-Stage Mercer Cascade Impactor. All the stages of the impactors were transferred to ITR's Analytical Laboratory for chemical determination of S-2200 content using a validated method and, based on these results, the Mass Median Aerodynamic Diameter (MMAD) and the Geometric Standard Deviation (GSD) were calculated.

Mortality, clinical observations and body weights:

Mortality checks were performed at least once daily.

Cage-side clinical signs (ill health, behavioural changes etc.) were recorded at least once daily during the acclimation period. Detailed clinical examination (DCE) was performed for all rats on the day of dosing at

prior to the start of exposure. Clinical signs were recorded for each rat during exposure at 1, 2, 3 and 4 hours (without removing each rat from exposure system) from the start of exposure. Following completion of the 4-hour exposure, DCE was performed for each rat immediately after releasing from the restraint tube and at 1 hour post-exposure. Furthermore, DCE was performed daily throughout the observation period and prior to necropsy.

Body weights were recorded for all animals once prior to group assignment, on the day of dosing (Day 0) prior to dosing, on Days 1, 3, 7 and 14 of the observation period, and prior to necropsy. Body weight gain was calculated between the measuring occasions.

Necropsy examination:

The animals were sacrificed upon completion of the 14-day observation period following an overnight period without food. These animals were anesthetized with sodium pentobarbital followed by exsanguination, and necropsied. Gross pathology consisted of an external examination, including identification of all clinically recorded lesions, as well as a detailed internal examination with particular attention to any changes in the respiratory tract. For all animals the lungs were dissected, trimmed free of fat and weighed.

On completion of the gross pathology examination, tissues and organs were retained for possible histopathology examination (if necessary, to be included in a protocol amendment) using neutral buffered 10% formalin for fixation and preservation unless otherwise indicated.

Statistics:

Numerical data obtained during the conduct of the study were subjected to calculation of group means and standard deviations.

Findings:

Achieved test atmosphere concentrations:

The mean gravimetrically achieved aerosol concentration of S-2200 25SC was 4.370 mg/L. The mean chemically determined concentration of S-2200 TG was 2.163 mg/L, and the corresponding theoretical aerosol concentration based on chemical determination was 8.667 mg/L. The mean mass median aerodynamic diameter (MMAD) was 3.8 µm and the geometric standard deviation (GSD) was 2.01 µm.

Mortality, clinical observations and body weights:

There was no mortality in the study.

There were no test article-related clinical signs during the exposure (at 1, 2, 3 and 4 hours from the start of exposure) or immediately after completion of 4-hour exposure. However, it was noted that the activity level of all rats was decreased with their eyes partially closed for most of the rats following 1 hour from completion of 4-hour exposure. This adverse effect was no longer observed on the next day (Day 1).

During the observation period, one female (1501A) started to show a sign of slight hair loss on the neck/head/dorsal thorax areas from Day 2 onwards. The severity on the neck area progressed to a moderate level as of Day 8. Another female (1505A) also showed a sign of very slight hair loss on the dorsal thorax as of Day 8, but the sign was no longer evident on Day 15 (before necropsy). Males 1003A and 1004A also had hair loss on the forepaws starting on Days 6 and 8, respectively. Hairloss only manifested in 4/10 animals treated with S-2200 25SC and this effect could also have been from the stress of dosing for a long duration.

All other clinical signs such as wet fur, pasty material on nose/muzzle/head, dry material on pinna, scales on tail, stained fur, mucoid materials in cage tray, reduced faecal output, abnormal sound (respiration) and skin discoloration on paws were considered incidental in origin or transient and commonly observed in rats following inhalation exposure.

There was a slight body weight loss (2-10%) in the majority of the treated animals on Day 1. However, this loss was considered to be due to the long duration of tube restraint (4 hours) during which the rats were deprived of food and water. Body weight gains were noted on Day 3 in all animals. Thus, this slight bodyweight loss was not considered an effect of S-2200 25SC.

Necropsy examination:

There was no evidence of test article-related findings at necropsy. Findings in the kidneys, Peyer's patch, skin and subcutis, and thymus were considered to be incidental or procedure-related. As there were no test article-related macroscopic findings, histopathological examination was not performed for this study.

Conclusion:

In this acute inhalation toxicity study, the 4-hour inhalation LC₅₀ was determined to be above 4.37 mg/L, the maximum attainable concentration, for male and female rats. Therefore no classification and labelling for acute inhalation toxicity is required.

B.6.11.4 Skin irritation

Reference:	A Skin Irritation Study of S-2200 25SC in Rabbits
Author(s), year:	██████████ 2011a
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No ROT-0026
Guideline(s):	JMAFF 12 Nousan No 8147 (2008), OECD 404 (2002), Regulation (EC) No. 440/2008 method B.4 (2008)
GLP:	Yes
Deviations:	No
Validity:	Yes

Material and methods:

<i>Test Material:</i>	S-2200 25SC
Lot/Batch:	C09-5F101G
Purity:	24.96% active substance
Vehicle:	None (test material was used as supplied)

<i>Test animals:</i>	
Species:	Rabbit
Strain:	Japanese White
Age:	18 weeks
Weight at dosing:	3.20 to 3.41 kg
Source:	████████████████████
Diet:	RC4 pelleted diet, Oriental Yeast Co., Ltd <i>ad libitum</i>

On the day prior to application of the test article, fur was clipped from the dorsal area of the rabbits using an electric clipper and the skin condition was observed. Three of five female Japanese White rabbits available, with no clinical signs and smooth skin in good condition, were chosen on the day of application of the test article. The dorsal area of each animal was divided into 2 sections right and left of the median line. 0.5 mL of test article was spread uniformly on a 2.5 x 2.5 cm square lint patch, which was lined with oil-paper. The lint patch was then applied to the application site of the test article on the animal. Only a 2.5 x 2.5 cm square lint patch which was lined with oil paper was applied to the control site. The lint patches were covered with an elastic bandage and then with polythene film tape. The duration of application was 4 hours, and during that

period the animals were held in a restrainer in order to keep them in a fixed position. After the 4 hour application period, the lint patches were removed and the application site was wiped with absorbent cotton soaked with water for injection. The application sites were observed for erythema/eschar formation and oedema formation at 1, 24, 48 and 72 hours after removal of the test article. Skin reactions were scored according to the Draize scale.

Findings:

No skin reactions were observed in any animal at any observation time of 1, 24, 48 and 72 hours after removal of the test article. The skin irritation scores (mean values after 24, 48 and 72 hours) were 0 for both erythema and oedema.

Conclusion:

Under the conditions of this study, S-2200 25 SC was not irritating to the skin of rabbits. In accordance with the criteria specified in Regulation (EC) No. 1272/2008, no classification and labelling is required for skin irritation.

B.6.11.5 Eye irritation

Reference:	An Eye Irritation Study of S-2200 25SC in Rabbits
Author(s), year:	██████████ 2011b
Report/Doc. number:	Sumitomo Chemical Co., Ltd., Report No : ROT-0027
Guideline(s):	JMAFF 12 Nousan No 8147 (2008), OECD 405 (2002), Regulation (EC) No. 440/2008 method B.5 (2008)
GLP:	Yes
Deviations:	No
Validity:	Yes

Material and methods:

Test Material:	S-2200 25SC
Lot/Batch:	C09-5F101G
Purity:	24.96% active substance
Vehicle:	None (test material was used as supplied)

Test animals:	
Species:	Rabbit
Strain:	Japanese White
Age:	15-16 weeks
Weight at dosing:	2.34 – 2.83 kg
Source:	████████████████████
Diet:	RC4 pelleted diet, Oriental Yeast Co., Ltd. <i>ad libitum</i>

Rabbits' eyes were examined with an ophthalmoscope on each day before application of the test article. At the time of examination, the cornea was also examined, using 2% fluorescein sodium aqueous solution, for the presence or absence of abnormalities. Three animals with no ocular abnormalities, showing no clinical signs and regular body weight gain, were chosen for each group. Two test groups (3 animals per group), an unwashed eye group and a washed eye group, were formed. In the unwashed group, 0.1 mL of the test article was applied into the conjunctival sac of the left eye after gently pulling the lower eyelid away from the eyeball. Then both eyelids were held together for about 1 second in order to prevent loss of the test article.

The untreated right eye served as the control. Since irritation reactions were observed in the unwashed eye group, the washed eye group were added. The rabbits of the washed group were treated with 0.1 mL of the test article in the same manner of the unwashed group, but 30 seconds after instillation, the treated eye was washed for 30 seconds with 100 mL of water for injection. The right eye was washed in the same manner as the left eye and this served as a washed control. The cornea, iris and conjunctiva were examined macroscopically and by an ophthalmoscope at 1, 24, 48 and 72 hours after application. The grade of eye irritation was scored according to the Draize scale.

Findings:

At 1 hour after application, conjunctival redness (score 1) and chemosis (score 1) were observed in all and in 2/3 animals of the unwashed eye group, respectively. In the washed eye group, at 1 hour after application, conjunctival redness (score 1) was observed in all animals. At 24 hours after application, these conjunctival changes were no longer observed in either group, and there were no irritation reactions in any animal at 24, 48 and 72 hours after application in any animal. No corneal, iridal or other ocular changes were observed in any animal during the observation period. At 24 hours after application, no fluorescein staining was observed in the cornea of any animal.

The scores of eye irritation test are given in Table B.6.11.5-1.

Table B.6.11.5-1: Eye irritation scores

		Rabbit No.	1 hour	24 hours	48 hours	72 hours	Mean/Rabbit (24+48+72 hours)
Unwashed group							
Conjunctivae	Chemosis	1101f	1	0	0	0	0.00
		1102f	0	0	0	0	0.00
		1103f	1	0	0	0	0.00
	Redness	1101f	1	0	0	0	0.00
		1102f	1	0	0	0	0.00
		1103f	1	0	0	0	0.00
Iris	Congestion	1101f	0	0	0	0	0.00
		1102f	0	0	0	0	0.00
		1103f	0	0	0	0	0.00
Cornea	Opacity	1101f	0	0	0	0	0.00
		1102f	0	0	0	0	0.00
		1103f	0	0	0	0	0.00
Washed group							
Conjunctivae	Chemosis	2101f	0	0	0	0	0.00
		2102f	0	0	0	0	0.00
		2103f	0	0	0	0	0.00
	Redness	2101f	1	0	0	0	0.00
		2102f	1	0	0	0	0.00
		2103f	1	0	0	0	0.00
Iris	Congestion	2101f	0	0	0	0	0.00
		2102f	0	0	0	0	0.00
		2103f	0	0	0	0	0.00

		Rabbit No.	1 hour	24 hours	48 hours	72 hours	Mean/Rabbit (24+48+72 hours)
Cornea	Opacity	2101f	0	0	0	0	0.00
		2102f	0	0	0	0	0.00
		2103f	0	0	0	0	0.00

Conclusion:

Under the conditions of this study, S-2200 25 SC provoked only very slight and transient eye irritation at one hour after application into the conjunctival sac of the eyes of rabbits. In accordance with the criteria specified in Regulation (EC) No. 1272/2008, no classification and labelling is required for eye irritation.

B.6.11.6 Skin sensitisation

Reference:	A Skin Sensitization Study of S-2200 25SC in Guinea Pigs (Buehler Test)
Author(s), year:	██████████ 2011c
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0028
Guideline(s):	JMAFF 12 Nousan No 8147 (2008), OECD 406 (1992), Regulation (EC) No. 440/2008 method B.6 (2008)
GLP:	Yes
Deviations:	No
Validity:	Yes

Material and methods:

Test Material:	S-2200 25SC
Lot/Batch:	C09-5F101G
Purity:	24.96% active substance
Vehicle:	None (test material was used as supplied)
Negative control:	Water for injection
Positive control:	Dinitrochlorobenzene (DNCB)

Test animals:	
Species:	Guinea pig
Strain:	Hartley (██████ Hartley)
Age:	6 weeks
Weight at dosing:	344 to 423 g (females)
Source:	██
Diet:	RC4 pelleted diet, Oriental Yeast Co., Ltd. <i>ad libitum</i>

Based on the results of a dose-finding study using four animals, undiluted test substance (100%) which was judged to be the maximum non-irritating concentration was selected for induction and challenge in the main study.

Induction: In the main study, on the day before the start of induction, the left abdominal flank area of 30 animals was clipped free of hair and shaved with an electric shaver (approximately 5 cm × 5 cm); 20 animals were designated for treatment with the test compound (test group), and 10 animals were used for the control group.

On the next day, 0.2 mL of the undiluted test article was spread on a patch of diameter 2.5 cm. The patch was applied to the left flank of the 20 animals of the test group and covered with polyethylene film tape for occlusive application. 6 hours after application, the patch was removed and the induction site was wiped with absorbent cotton soaked with water for injection. The negative control group received a patch with 0.2 mL water for injection only applied to the animals in the same manner as for the test group. The above occlusive application was carried out three times on Days 0, 7 and 14. Fur clipping and shaving was carried out on the days before each induction.

Challenge: On day 27, the fur of the right abdominal flank (5 x 5 cm) of each animal in both groups was clipped and shaved. On the next day, 0.2 mL of the undiluted test article was spread on a patch of diameter 2.5 cm and applied to the right flank. The challenge site was covered with polyethylene film tape for occlusive application. The patch was removed at 6 hours after application and the challenge site was wiped with absorbent cotton soaked with water for injection.

Observations for skin reactions, erythema and oedema were made at 24, and 48 hours after removal of the patch (after each induction and challenge exposure) and graded according to the criteria of Magnusson and Kligman.

Daily clinical observations were performed from Days 0 to 30. Body weights were measured on Days 0, 14, 28 and 30.

A positive control group was not provided in this study; however skin sensitisation studies of DNCB (1-chloro-2,4-dinitrobenzene), a known sensitizer, have been conducted periodically to confirm the sensitivity of the Buehler test in this guinea pig strain.

Findings:

In the test group as well as in the control group, no skin reactions were observed in any animal challenged with undiluted test article at either observation time of 24 or 48 hours after removal of challenge exposure. Mean score of each observation time was 0, and the positive reaction rate was 0%.

No skin reactions were observed during induction with the undiluted test article in the test group.

There were no abnormalities in clinical signs or body weight of any animal in any group.

Positive control studies produced a sensitization rate of 100%, confirming the sensitivity of the test system.

Conclusion:

Under the conditions of this Buehler test, S-2200 25 SC did not show any skin sensitising potential in the treated animals. In accordance with the criteria specified in Regulation (EC) No. 1272/2008, no classification and labelling is required.

B.6.11.7 Summary of toxicity studies with the formulation

In a series of guideline and GLP compliant studies, S-2200 25SC showed no evidence of acute toxicity by oral and dermal routes or via inhalation (at the maximum attainable concentration of 4.37 mg/L), and was not a skin or eye irritant, or a skin sensitizer.

Table B.6.11.7-1: Summary of acute toxicity data for the formulation “S-2200 25SC”

Type of study	Vehicle	Results	Reference (Report No.)
Acute oral toxicity in the rat (OECD 423, Acute Toxic Class)	Distilled water	LD ₅₀ > 2000 mg/kg	██████ 2011a (ROT-0029)
Acute dermal toxicity in the Rat (OECD 402)	None	LD ₅₀ > 2000 mg/kg	██████ 2011b (ROT-0030)
Acute inhalation toxicity study in the Rat (OECD 403)	Sterile water	LC ₅₀ > 4.37 mg/L air (maximum attainable concentration)	██████; 2011 (ROT-0032)
Skin irritation study in the Rabbit (OECD 404)	None	Not irritating	██████ 2011a (ROT-0026)
Eye irritation study in the Rabbit (OECD 405)	None	Not irritating	██████ 2011b (ROT-0027)
Skin sensitization study in the Guinea pig – Buehler method (OECD 406)	None	Not sensitising	██████ 2011c (ROT-0028)

B.6.12 Dermal absorption (Annex IIIA 7.6)

B.6.12.1 Dermal absorption, *in vivo* in the rat

No *in vivo* study was conducted, not mandatory.

B.6.12.2 Comparative dermal absorption, *in vitro* using rat and human skin

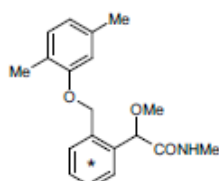
Reference:	S-2200 25SC: <i>In Vitro</i> Absorption through Human Epidermis using [¹⁴ C]S-2200
Author(s), year:	Hadfield, N.; 2011
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROM-0024
Guideline(s):	OECD 428 (2004)
GLP:	Yes
Deviations:	No
Validity:	Yes

Material and methods

Test Material:

[benzyl-¹⁴C]S-2200

(*RS*)-2-methoxy-*N*-methyl-2-[α-(2,5-xylyloxy)-*o*-tolyl]acetamide;
labelled uniformly at the benzyl position.



* : Labelled position

Lot/Batch:

C09-5F101G

Purity:

From CoA and analyses dated January 2008:

Chemical purity 98.0%; radiochemical purity 99.9%, specific activity 14.9 MBq/mg
TLC analysis of the stock solution at the test laboratory showed radiochemical purity to > 95%
Stability of the test compound: TLC analysis of the dose preparations confirmed that the radiochemical purity of [¹⁴C]S-2200 when formulated as the formulation concentrate and 1/5000 aqueous spray dilution was greater than 95% for a period of 48 hours post preparation. Stability was therefore confirmed for a period of time greater than that used in the study

Non-labelled S-2200

Lot/Batch: 081103G

Purity: 100%

Stability of the test compound: Expiry November 18, 2011 stored refrigerated. S-2200 was used within the expiry date

S-2200 25 SC (blank formulation)

Lot/Batch: C10-7F101

Stability of the test compound: Not specified stored at room temperature

Vehicle/ positive control: None for the concentrate, water for the in-use dilution

Test device/ skin membranes: Human skin samples were obtained from a tissue bank. The skin samples were immersed in water at 60°C for 40-45 seconds and the epidermis teased away from the dermis.
Each membrane was given an identifying number and stored frozen, at approximately - 20°C, on aluminium foil until required for use.

Diffusion cells: Static diffusion cells with exposed membrane area of 2.54 cm² and receptor chamber volume of approximately 4.5 mL. Discs approximately 3.3 cm diameter of prepared skin membrane were mounted, dermal side down, in diffusion cells held together with individually numbered clamps and placed in a water bath maintained at 32°C ± 1°C.

Assembly of the diffusion cells

Cells were selected such that each application was represented by six intact membranes from three different subjects.

The receptor chambers of the cells containing small magnetic stirrer bars were filled with a recorded volume of receptor fluid (50% ethanol in water) and placed in a water bath maintained at a temperature of 32°C ± 1°C. S-2200 is soluble in water at 15.8 mg/L and this choice of receptor fluid ensures that the S-2200 can freely partition into the receptor fluid from the skin membrane and never reaches a concentration that would limit its diffusion.

Measurement of membrane integrity

Membrane integrity was determined by measurement of the electrical resistance across the skin membrane. Membranes with a measured resistance of < 10 kΩ were regarded as having a lower integrity than normal and not used for exposure to the test materials.

Formulation concentrate

249.1 mg of unlabelled S-2200 was added to a vial, followed by 528 µL of radiolabelled S-2200 stock solution, providing 0.7 mg of S-2200 and 10.43 MBq radioactivity. A further 1.5 mL of acetonitrile was added in order to dissolve the mixture, which was then blown to dryness under a stream of nitrogen gas overnight. When dry, the vial was weighed to confirm the removal of the acetonitrile and 795.9 mg of the blank formulation was added and the weight recorded. Thirty glass beads (2 mm) were then added to the vial and the preparation was vortexed for 2 minutes to ensure a homogeneous preparation was achieved.

1/5000 v/v Aqueous spray strength dilution

39.9 µL of radiolabelled S-2200 stock solution, providing 0.053 mg of S-2200 and 0.79 MBq radioactivity was added to a vial. This was then blown to dryness under a stream of nitrogen and stored overnight at room temperature. When dry, the vial was weighed to confirm the removal of the acetonitrile. Subsequently 999.8 mg of water containing 0.162 mg of the blank formulation was added to the vial and the weight recorded. Thirty glass beads (2 mm) were then added to the vial and the preparation was vortexed for 2 minutes to ensure a homogeneous preparation was achieved.

Radioactivity content of the dose preparations

The radioactivity content of each formulated [¹⁴C]S-2200 preparation was determined by analysing sub-samples of solvent dilutions by LSC.

Homogeneity of the dose preparations

Homogeneity of each formulated [¹⁴C]S-2200 preparation was confirmed by analysing replicate sub-samples of solvent dilutions by LSC immediately after preparation.

Radiochemical purity and stability

A sample of each formulated [¹⁴C]S-2200 preparation was analysed to determine the radiochemical purity of the formulated S-2200 (TLC).

Application and measurement of dermal penetration

The formulation was applied to the skin membranes as the product concentrate and as a 1/5000 v/v aqueous spray strength dilution. The applications were left unoccluded for the duration of the experiment (24 hours). The actual dose rates achieved are summarised in the following table:

Table B.6.12.2-1: Actual dose rates of active substance

Group	Concentrate	Spray dilution
Actual application rate	10 µL/cm ² ≡ 2500 µg ai/cm ²	10 µL/cm ² ≡ 0.5 µg ai/cm ²
Total volume/amount applied	25.4 µL	25.4 µL
Concentration	250 g a.i./L	0.05 g a.i./L

A pre-treatment sample (0.5 mL) was taken from each receptor chamber for analysis by LSC prior to dosing. An equal volume of fresh receptor fluid was added to each receptor chamber to replace the volume removed.

Samples of the receptor fluid (0.5 mL) were taken using an autosampler at 1, 2, 3, 4, 6, 8, 10, 12, 16, 20 and 24 hours after application for analysis by LSC. The volume of fluid in the receptor chamber was maintained

by the replacement of a volume of receptor fluid, equal to the sample volume immediately after each sample was taken.

After the 6 hour sample had been taken the skin was then washed by gently swabbing with a series of 3 natural sponges (approximately 1 cm³) pre-wetted with 3% Teepol[®] L in water to remove excess test product. Decontamination was shown to be complete following assessment of residual radioactivity levels on the skin surface with a Geiger counter. Another 2 sponges, pre-wetted with water, were used to further swab the surface. All the sponges were combined and digested in Soluene 350[®]. The digests were made up to a recorded volume and a sample taken for analysis.

After the final receptor fluid sample had been taken at the end of the experimental period, the remaining fluid in the receptor chamber was discarded and the chamber rinsed with fresh receptor fluid which was also discarded.

The donor chamber was carefully removed and the underside wiped with one sponge pre-wetted with 3% Teepol L[®] in water. The donor chamber was extracted using acetonitrile and a sample analysed for S-2200 by LSC.

The epidermal surface of the skin was decontaminated by gently swabbing the application site with three natural sponges pre-wetted with 3% Teepol L[®] in water. Decontamination was shown to be complete following assessment of residual radioactivity levels on the skin surface with a Geiger counter. Another 2 sponges, pre-wetted with water, were used to further swab the surface. The sponges were digested in Soluene 350[®] and made up to a recorded volume. A sample was taken for analysis.

The surface of the skin was allowed to dry naturally.

To assess penetration through human stratum corneum, successive layers of the skin surface were removed by the repeated application of adhesive tape (e.g. Scotch 3M Magic Tape, 1.9 cm wide), to a maximum of 5 strips. A strip of adhesive tape was pressed onto the skin surface and then carefully peeled off to remove the stratum corneum. The adhesive strips were soaked individually in acetonitrile to extract any test material. The extracts were sequentially numbered and analysed by LSC.

The remaining epidermis was carefully removed from the receptor chamber, digested in Soluene 350[®], and a sample of the digest analysed by LSC.

Quantification of Total Radioactivity

All samples, except for the samples associated with the barrier integrity assessment, were counted for 6 minutes (or to 0.5% SD) using a liquid scintillation analyser (Packard 3100-TR).

Findings:

Dose levels achieved and homogeneity of the dose preparations

Analysis of the dose preparations confirmed that the dose levels achieved were 249 g S-2200/L and 0.04 g S-2200/L for the formulation concentrate and 1/5000 v/v aqueous spray strength dilution, respectively.

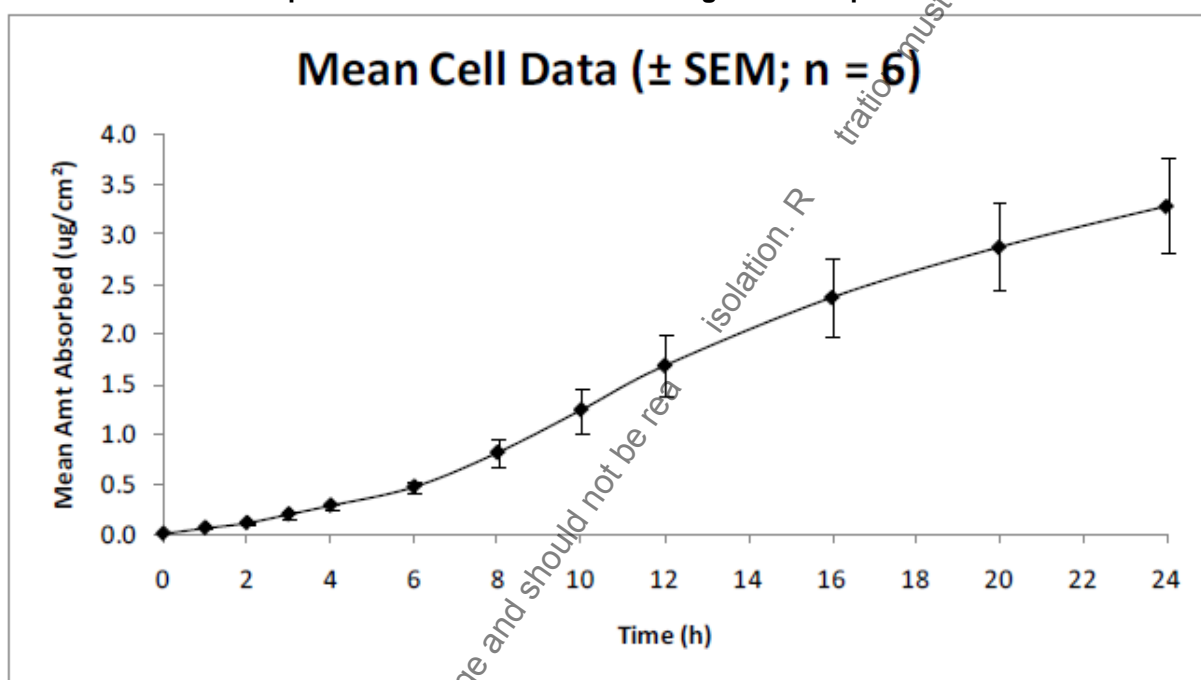
The dose preparations were considered to be homogeneous. For the 249 g/L dose preparation, the dose data showed a 1.97% deviation between the replicates. For the 0.04 g/L dose preparation, the dose data showed a 0.56% deviation between the replicates.

Formulation concentrate

The mean total recovery of the applied test material was 109.7%. The vast majority of the applied S-2200 (mean 109%) was washed off the skin at 6 hours, with a further 0.491% washed off at 24 hours. A small proportion of the dose applied was recovered from the stratum corneum and remaining epidermis (0.003% and 0.007%, respectively).

For the formulation concentrate absorption up to 12 hours was 52% of that at 24 hours (< 75%). According to 2012 EFSA guidance where this is the case, the dermal absorption value may be considered to be a summation of receptor fluid + receptor chamber washes + skin sample (excluding tape strips 1 and 2) = **0.1%** (value rounded in accordance with the EFSA guidance document).

Table B.6.12.2-2: Absorption from the concentrate through human epidermis

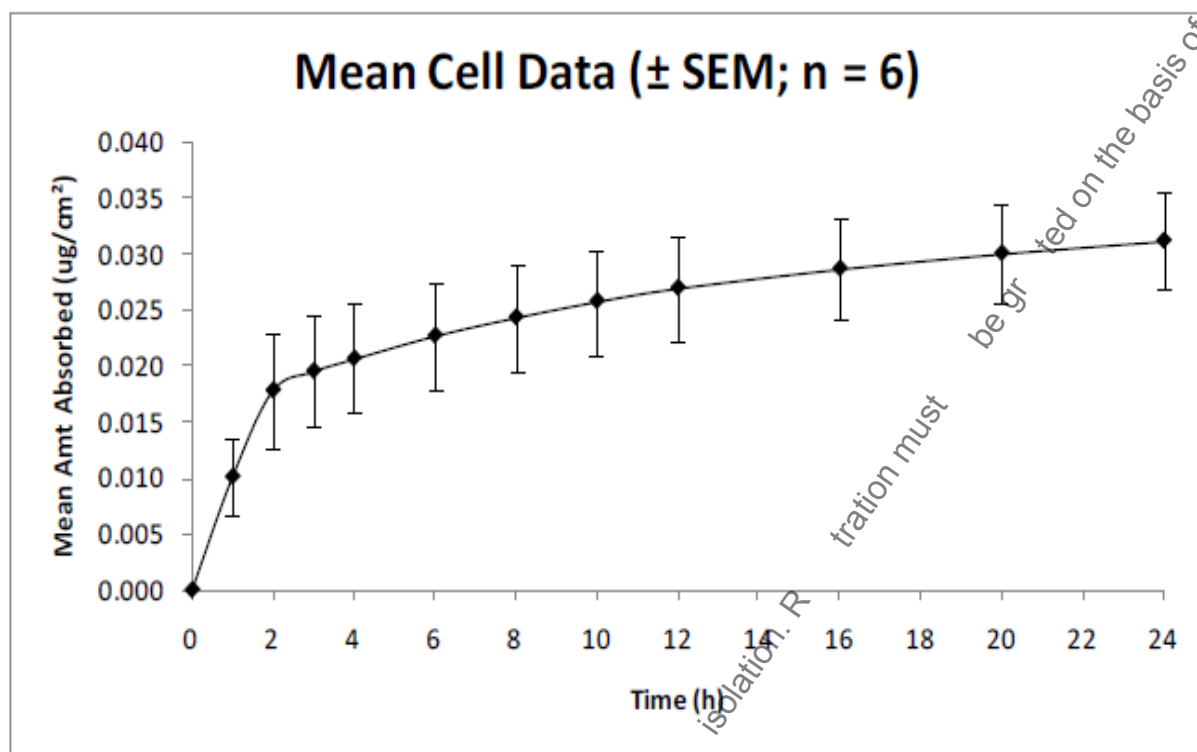


1/5000 v/v Aqueous spray strength dilution

The mean total recovery of the applied test material was 108.2%. The vast majority of the applied S-2200 (mean 91.7%) was washed off the skin at 6 hours, with a further 7.73% washed off at 24 hours. A small proportion of the dose applied was recovered from the stratum corneum and remaining epidermis (0.193% and 0.213%, respectively).

For the in-use dilution, absorption up to 12 hours was 87% of that at 24 hours (> 75%). According to 2012 EFSA guidance where this is the case, the dermal absorption value may be considered to be a summation of receptor fluid + receptor chamber washes + skin sample (excluding all tape strips) = **8%** (value rounded in accordance with the EFSA guidance document).

Table B.6.12.2-3: Absorption from the 1/5000 v/v dilution through human epidermis



The distribution of radioactivity in the different compartments is summarized in the following table. In several cases, when the detected amount of radioactivity is very low, the standard deviation exceeds 25% of the mean. According to EFSA guidance, in such cases one standard deviation should be added to the mean and the calculations should be performed using mean + 1 standard deviation. Doing so does not change the outcome of 0.1% dermal absorption for the concentrate and 8% for the dilution.

Table B.6.12.2-4: Distribution of radioactivity (% of applied dose) at 24 h post dose following topical application of the concentrate or in-use dilution of [14 C]S-2200 25SC in to human split thickness skin

	Concentrate (250 g a.i./L)		In use dilution (0.05 g a.i./L)	
	Mean (n = 6)	SEM	Mean (n = 6)	SEM
Donor Chamber wash	0.266	0.080	0.115	0.056
Skin Wash 6 h	109	1.24	91.7	2.72
Skin Wash 24 h	0.491	0.220	7.73	1.72
Stratum corneum tape strips:				
Tape strip 1	0.001*	0.0002	0.089	0.037
Tape strip 2	0.0005*	0.0001	0.044*	0.017
Tape strip 3	0.0005*	0.0002	0.026*	0.017
Tape strip 4	0.0007*	0.0003	0.020*	0.009
Tape strip 5	0.0004*	0.0002	0.027*	0.004
Tape strips 1-5	0.003*	0.0007	0.193	0.074
Remaining Exposed Skin	0.007	0.002	0.213	0.063
Receptor Fluid	0.132	0.019	8.27	1.15

Mass Balance	109.7	1.28	108.2	4.10
---------------------	-------	------	-------	------

* Values between the mean LOD and mean LOQ have been flagged with * and used as positive values in the means, SEMs or totals.
Remaining Exposed Skin = Epidermal tissue remaining after tape stripping. SEM = standard error of the mean.

Flux

Formulation concentrate:

The mean amount absorbed over the entire 24 hour exposure period was 3.28 µg/cm², corresponding to 0.132% of the applied dose.

Absorption of S-2200 was fastest between 6-12 hours (0.203 µg/cm²/h). The mean absorption rate slowed to 0.132 µg/cm²/h between 12-24 hours. Between 0-6 hours the mean absorption rate was 0.079 µg/cm²/h. Between 0-24 hours, the absorption rate was, on average, 0.149 µg/cm²/h.

1/5000 v/v Aqueous spray strength dilution:

The mean amount absorbed over the entire 24 hour exposure period was 0.031 µg/cm², corresponding to 8.27% of the applied dose.

Absorption of S-2200 was fastest between 0-2 hours (0.009 µg/cm²/h). The mean absorption rate slowed to 0.0009 µg/cm²/h between 2-12 hours. Between 12-24 hours the mean absorption rate slowed further to 0.0004 µg/cm²/h and between 0-24 hours, the absorption rate, on average, was 0.0009 µg/cm²/h.

Figure B.6.12.2-5: Flux (µg or ng equiv./cm²/h) of [¹⁴C]S-2200 into receptor fluid following topical application of the concentrate suspension or in-use dilution of [¹⁴C]S-2200 25SC in to human split thickness skin

Concentrate (250 g a.i./L)			In use dilution (0.05 g a.i./L)		
µg equiv./cm ² /h			ng equiv./cm ² /h		
Time (h)	Mean (n = 6)	SEM	Time (h)	Mean (n = 6)	SEM
0 – 6	0.079	0.010	0 – 2	0.009	0.003
6 – 12	0.203	0.044	2 – 12	0.0009	0.00006
12 – 24	0.132	0.017	12 – 24	0.0004	0.00006
0 – 24	0.149	0.023	0 – 24	0.0009	0.00007

Conclusion:

The results obtained in this study demonstrate that the absorption of S-2200 from the S-2200 25SC formulation concentrate and its 1/5000 w/v dilution through human epidermis is at a low rate for the concentrate, although higher for the in-use dilution. The vast majority of the applied dose was removed by gentle skin washing after 6 hours. Low proportions of the dose were associated with the skin at the end of the 24 hour experimental period.

The extent of S-2200 absorption through human skin from the formulation S-2200 25SC was quantified at 0.1% for the concentrate and amounted to 8% of the applied dose for the aqueous dilution after 24 hours.

B.6.12.3 Summary on dermal absorption

An *in vitro* dermal absorption study with human skin has been conducted to determine levels of absorption associated with exposure to S-2200 25SC concentrate and diluted product. The dermal absorption values for mandestrobin contained in the formulation S-2200 25SC are 0.1% for the concentrate and 8% for the dilution.

B.6.13 Toxicological data on non-active substances (Annex IIIA 7.9)

B.6.13.1 Material safety data sheet for each formulant

For each formulant, safety data sheets have been submitted.

B.6.13.2 Available toxicological data for each formulant

Additional data are currently not available.

B.6.14 Exposure data (Annex IIIA 7.3, 7.4 and 7.5)

The plant protection product S-2200 25SC containing 250 g/L mandestrobin is intended to be used as a fungicide on outdoor crops of winter oilseed rape.

The product is applied once per season during flowering (BBCH 63-67).

The formulation is a suspension concentrate, supplied in 1, 5 (wide-necked) and 10 litre (wide-necked) high density polyethylene (HDPE) containers.

Crop	Application rate			Application method
	Product (L/ha)	a.s. (kg/ha)	Water (L/ha)	
Winter oilseed rape (North and South EU)	0.8	0.2	100	Tractor-mounted field crop (boom) sprayer

B.6.14.1 Operator exposure (Annex IIIA 7.3)

Estimates of exposure for operators applying S-2200 25SC for the intended use have been undertaken using the following predictive models:

- The German model for the protection of operators (BBA 1992)
Uniform principles for assuring the protection of operators' health when using plant protection products (uniform principles for operator protection), Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft, Heft 277, Berlin 1992 (in German)
- UK-POEM [Predictive Operator Exposure Model (POEM), UK MAFF, 1992]

Estimations were compared to the following end points; values for dermal absorption are derived from a study conducted with S-2200 25SC formulation (see B.6.12.2).

End point	Value
Dermal absorption	0.1% (concentrate) 8% (spray dilution)
Systemic AOEL (short-term)	0.91 mg/kg bw/day

The operator exposure was estimated assuming that no personal protective equipment (PPE) is used:

Table B.6.14.1.1-1: Estimations of operator exposure in relation to the AOEL – no protective clothing and equipment used

Operator exposure model	Operator Exposure	
	mg/kg bw/day	% of AOEL
GERMAN MODEL	0.0096	1
UK MODEL	0.1145	13

Without PPE, the calculated operator exposure is 1% and 13% of the AOEL when using the German model and the UK-POEM model, respectively. The corresponding spread sheets/calculations are presented in Appendix 1.

B.6.14.1.1 Estimation of operator exposure assuming personal protective equipment is used

For completeness, estimations of operator exposure with protective clothing (gloves during mixing/loading and application in both models) were performed.

Table B.6.14.1-1: Estimations of operator exposure in relation to the AOEL – protective clothing and equipment used (gloves during mixing/loading and application)

Operator exposure model	Operator Exposure	
	mg/kg bw/day	% of AOEL
GERMAN MODEL	0.0077	0.8
UK MODEL	0.0193	2

B.6.14.1.2 Measurement of operator exposure – (Mixer/Loader/Applicator)

Model calculations do not indicate a risk for operators. No studies on operator exposure were provided.

B.6.14.1.3 Summary on operator exposure

For the intended use in winter oilseed rape, a safe use is given in both operator exposure models (German model, UK POEM) even without using PPE.

B.6.14.2 Bystander exposure (Annex IIIA 7.4)

B.6.14.2.1 Estimation of bystander exposure

As currently no agreed model for resident exposure exists, calculations are considered indicative. Bystander and resident exposure were calculated according to the following models:

- Martin, S., Westphal, D., Erdtmann-Vourliotis, M., Dechet, F., Schulze-Rosario, C., Stauber, F., Wicke, H., Chester, G. (2008) Guidance for Exposure and Risk Evaluation for Bystanders and Residents to Plant Protection Products during and after Application J.Verbr.Lebensm. 3(2008) 272-281
- Lloyd, G.A, and Bell, G.J. (1983) "Hydraulic nozzles: comparative spray drift study" (MAFF/ADAS)

For exposure estimates according to *Martin et al.*, detailed calculations are presented in Appendix 1. Briefly, a drift deposit of 0.29% (10 m distance, professional application on field crops) was used for the calculation.

Table B.6.14.2-1: Estimations of bystander/resident exposure in relation to the AOEL

Bystander and resident exposure model according to <i>Martin et al.</i>	Systemic exposure	
	mg/kg bw/day	% of AOEL
Bystanders		
Adults	0.000078	0.01
Children	0.000062	0.01
Residents		
Adults	0.00028	0.03
Children	0.00053	0.06

The following calculations for bystander exposure were conducted according to *Lloyd and Bell*:

$$\text{Systemic exposure} = (\text{PDE} \times \text{SC} \times \% \text{DA} + \text{PIE} \times \text{SC} \times 100\%) / \text{BW}$$

PDE = potential dermal exposure (mL spray dermal) = 0.1 mL spray/person dermal for field crops

PIE = potential inhalation exposure (mL spray inhaled) = 0.006 mL spray/person inhaled for field crops

SC = concentration of active substance in spray (mg a.s./mL spray dilution) = 2 mg a.s. / mL water

%DA = percentage dermal absorption = 8%

BW = bodyweight (60 kg)

$$\text{Systemic exposure} = (0.1 \text{ mL spray/person} \times 2 \text{ mg a.s./mL water} \times 8\% + 0.006 \text{ mL spray/person} \times 2 \text{ mg a.s./mL water} \times 100\%) / 60 \text{ kg} = 0.000467 \text{ (0.05\% of the AOEL)}$$

B.6.14.2.2 Measurement of bystander exposure

Estimated exposure for bystanders and residents is very low, no studies were conducted.

B.6.14.2.3 Summary on bystander exposure

No risk for bystanders (adults and children) was estimated with both models (*Martin et al.*, *Lloyd and Bell*).

B.6.14.3 Worker exposure (Annex IIIA 7.5)

Winter oilseed rape is mechanically harvested. The re-entry scenario for workers is therefore those who re-enter crops treated with S-2200 25SC to perform crop inspection type activities. For use on winter oilseed rape, a single application of 0.8 L product/ha (corresponding to 0.2 kg a.s./ha) is assessed. Re-entry could potentially occur once spray deposits have dried.

B.6.14.3.1 Estimation of worker exposure assuming personal protective equipment is not used

Estimates of worker exposure are based on the exposure model proposed in the EUROPOEM II Re-entry Working Group report (van Hemmen et al. (2002). Post-application exposure of workers to pesticides in agriculture. Report of the re-entry working group, EUROPOEM II project: FAIR3-CT96-. Policy paper on agricultural transfer coefficients.).

In the absence of a specific transfer coefficient (TC) for workers performing crop inspection activities in winter oilseed rape (BBCH 63-67), the EUROPOEM TC value for workers harvesting ornamental flowers has been assumed. This TC value, 5000 cm²/hour, is the highest of the EUROPOEM recommended TC values and was derived from activities where workers cut, sorted and carried ornamental flowers grown in a glasshouse. These activities involved frequent contact with the cut flowers to the hand, arm and chest regions of the workers body. Use of this TC value is therefore expected to provide a precautionary estimate of exposure for workers performing crop inspections in winter oilseed rape.

The EUROPOEM re-entry model assumes a default dislodgeable foliar residues value of 3 µg a.s./cm² per kg a.s./ha. Systemic exposure is predicted assuming a dermal absorption of 8%, an exposure duration of 2 hours for crop inspection, and a 60 kg worker body weight. Detailed calculations are presented in Appendix 1.

Table B.6.14.3.1-1: Estimation of worker exposures – no PPE

Crop scenario	Application rate (kg a.s./ha)	Number of applications	DFR (µg/cm ²)	TC (cm ² /h)	Worker exposure	
					mg/person/ day	% AOEL
No PPE						
Oilseed rape	0.2	1	3	5000	0.48	0.9

No risk for workers/crop inspectors is identified.

B.6.14.3.2 Estimation of worker exposure assuming personal protective equipment is used

As predicted levels of exposure using the EUROPOEM re-entry exposure model are below the AOEL for the unprotected worker, estimates of exposure for workers using PPE are not required.

B.6.14.3.3 Estimation of operator exposure assuming personal protective equipment is used and using data generated on dislodgeable residues under the proposed conditions of use

Since the risk assessment performed indicates that the health-based limit value (AOEL) will not be exceeded under practical conditions of use, a refinement of the exposure estimate by using data on dislodgeable residues instead of the default assumption was not considered necessary.

B.6.14.3.4 Measurement of worker exposure

A study to measure worker exposure to S-2200 25SC under practical conditions of use was not conducted since the estimates of exposure under worst case assumptions demonstrated that there is an acceptable margin of safety between the estimated exposure levels and the systemic AOEL for the active substance mandestrobin.

B.6.14.3.5 Summary on worker exposure

For the intended use there is a safe use given for worker even without use of PPE.

B.6.15 Epidemiology (Annex IIIA 7.8)

S-2200 is a new chemical currently in development. There has been no exposure of the general population or epidemiology study. No indication of S-2200-related ill-health have been detected by, or reported to, medical staff at the manufacturing site.

WARNING: This document forms part of an EC evaluation data package and should not be read in isolation. Read the full document for more information. This document must be graded on the basis of this document.

Appendix 1: Exposure models

THE GERMAN MODEL (GEOMETRIC MEAN VALUES) – with and without PPE

Estimation of operator exposure (acc. to the German model)				
Active substance (a.s.)	Mandestrobin			
Product	S-2200 25SC			
Intended use(s)	winter oilseed rape			
Type of preparation	Field Crops, Tractor Mounted (FCTM)			
Type of preparation	Liquid			
Application rate (AR)	0,2	kg a.s./ha		
Treated area per day (A)	20	ha/d		
Systemic AOEL	0,91	mg/kg bw/d		
Dermal absorption (DA)	0,1	% for mixing/loading (m/l)		
	8	% for application (appl.)		
Inhalation absorption (IA)	100	%		
Body weight (BW)	70	kg		

Personal protective equipment:	BVL code	Reduction factor	to lower:	
Particle filtering half mask (m/l) ¹⁾	ST1102	0,08	I _M	<input type="checkbox"/>
Half mask with combined filter (m/l) ¹⁾	ST2102	0,02	I _M	<input type="checkbox"/>
Particle filtering half mask (appl.) ¹⁾	ST1203	0,08	I _A	<input type="checkbox"/>
		0,8	D _{A(C)}	
Half mask with combined filter (appl.) ¹⁾	ST2202	0,02	I _A	<input type="checkbox"/>
		0,8	D _{A(C)}	
Protective gloves (m/l) ²⁾	SS110	0,01	D _{M(H)}	<input checked="" type="checkbox"/>
Protective gloves (appl.) ²⁾	SS120	0,01	D _{A(H)}	<input checked="" type="checkbox"/>
Protective garment + sturdy footwear (appl.) ²⁾	SS2202	0,05	D _{A(B)}	<input type="checkbox"/>
Broad-brimmed headgear (appl.) ²⁾	SS420	0,5	D _{A(C)}	<input type="checkbox"/>
Hood and visor (appl.) ²⁾	SS520	0,05	D _{A(C)}	<input type="checkbox"/>

¹⁾ DIN EN 149 (2001), ²⁾ BVL (2006) Guidelines for requirements concerning personal protective equipment in plant protection

Estimated inhalation exposure:	Personal protective equipment (PPE)	Factor
I _M	no PPE	1
I _A	no PPE	1

Estimated dermal exposure:	Personal protective equipment (PPE)	Factor
D _{M(H)}	SS110	0,01
D _{A(H)}	SS120	0,01
D _{A(C)}	no PPE	1
D _{A(B)}	no PPE	1

Estimation of operator exposure: German model

Input parameters considered for the estimation of operator exposure:

Formulation type:	Liquid	Application technique:	Field Crops, Tractor Mounted (FCTM)
Application rate (AR):	0,2 kg	Dermal hands m/l (D_{M(H)}):	2,4 mg/person/kg a.s.
Area treated per day (A):	20 ha	Dermal hands appl. (D_{A(H)}):	0,38 mg/person/kg a.s.
Dermal absorption (DA):	0,1 % (concentr.)	Dermal body appl. (D_{A(B)}):	1,6 mg/person/kg a.s.
	8 % (dilution)	Dermal head appl. (D_{A(C)}):	0,06 mg/person/kg a.s.
Inhalation absorption (IA):	100 %	Inhalation m/l (I_M):	0,0006 mg/person/kg a.s.
Body weight (BW):	70 kg/person	Inhalation appl. (I_A):	0,001 mg/person/kg a.s.
AOEL	0,91 mg/kg bw/d		

Operator exposure towards Madestrobin				
Without PPE			With PPE	
Operators: Systemic dermal exposure after application in winter oilseed rape				
Dermal exposure during mixing/loading				
Hands			Hands	
$SDE_{OM(H)} = (D_{M(H)} \times AR \times A \times DA) / BW$			$SDE_{OM(H)} = (D_{M(H)} \times AR \times A \times PPE^1 \times DA) / BW$	
$(2,4 \times 0,2 \times 20 \times 0,1\%) / 70$			$(2,4 \times 0,2 \times 20 \times 0,01 \times 0,1\%) / 70$	
External dermal exposure	9,6	mg/person	External dermal exposure	0,096 mg/person
External dermal exposure	0,1371429	mg/kg bw/d	External dermal exposure	0,0013714 mg/kg bw/d
Systemic dermal exposure	0,000137	mg/kg bw/d	Systemic dermal exposure	0,000001 mg/kg bw/d
Dermal exposure during application				
Hands			Hands	
$SDE_{OA(H)} = (D_{A(H)} \times AR \times A \times DA) / BW$			$SDE_{OA(H)} = (D_{A(H)} \times AR \times A \times PPE^1 \times DA) / BW$	
$(0,38 \times 0,2 \times 20 \times 8\%) / 70$			$(0,38 \times 0,2 \times 20 \times 0,01 \times 8\%) / 70$	
External dermal exposure	1,52	mg/person	External dermal exposure	0,0152 mg/person
External dermal exposure	0,0217143	mg/kg bw/d	External dermal exposure	0,0002171 mg/kg bw/d
Systemic dermal exposure	0,001737	mg/kg bw/d	Systemic dermal exposure	0,000017 mg/kg bw/d
Body			Body	
$SDE_{OA(B)} = (D_{A(B)} \times AR \times A \times DA) / BW$			$SDE_{OA(B)} = (D_{A(B)} \times AR \times A \times PPE^2 \times DA) / BW$	
$(1,6 \times 0,2 \times 20 \times 8\%) / 70$			$(1,6 \times 0,2 \times 20 \times 1 \times 8\%) / 70$	
External dermal exposure	6,4	mg/person	External dermal exposure	6,4 mg/person
External dermal exposure	0,0914286	mg/kg bw/d	External dermal exposure	0,0914286 mg/kg bw/d
Systemic dermal exposure	0,007314	mg/kg bw/d	Systemic dermal exposure	0,007314 mg/kg bw/d
Head			Head	
$SDE_{OA(C)} = (D_{A(C)} \times AR \times A \times DA) / BW$			$SDE_{OA(C)} = (D_{A(C)} \times AR \times A \times PPE^3 \times DA) / BW$	
$(0,06 \times 0,2 \times 20 \times 8\%) / 70$			$(0,06 \times 0,2 \times 20 \times 1 \times 8\%) / 70$	
External dermal exposure	0,24	mg/person	External dermal exposure	0,24 mg/person
External dermal exposure	0,0034286	mg/kg bw/d	External dermal exposure	0,0034286 mg/kg bw/d
Systemic dermal exposure	0,000274	mg/kg bw/d	Systemic dermal exposure	0,000274 mg/kg bw/d
Total systemic dermal exposure: $SDE_O = SDE_{OM(H)} + SDE_{OA(H)} + SDE_{OA(B)} + SDE_{OA(C)}$			Total systemic dermal exposure: $SDE_O = SDE_{OM(H)} + SDE_{OA(H)} + SDE_{OA(B)} + SDE_{OA(C)}$	
Total external dermal exposure	17,76	mg/person	Total external dermal exposure	6,7512 mg/person
Total external dermal exposure	0,2537143	mg/kg bw/d	Total external dermal exposure	0,0964457 mg/kg bw/d
Total systemic dermal exposure	0,00946	mg/kg bw/d	Total systemic dermal exposure	0,00761 mg/kg bw/d

– 295 –

Mandestrobin – Volume 3, Annex B.6 Toxicology and metabolism

January 2014

Operators: Systemic inhalation exposure after application in winter oilseed rape					
Inhalation exposure during mixing/loading					
$SIE_{OM} = (I_M \times AR \times A \times IA) / BW$ $(0,0006 \times 0,2 \times 20 \times 100\%) / 70$			$SIE_{OM} = (I_M \times AR \times A \times PPE^4 \times IA) / BW$ $(0,0006 \times 0,2 \times 20 \times 1 \times 100\%) / 70$		
External inhalation exposure	0,0024	mg/person	External inhalation exposure	0,0024	mg/person
External inhalation exposure	3,429E-05	mg/kg bw/d	External inhalation exposure	3,429E-05	mg/kg bw/d
Systemic inhalation exposure	0,000034	mg/kg bw/d	Systemic inhalation exposure	0,000034	mg/kg bw/d
Inhalation exposure during application					
$SIE_{OA} = (I_A \times AR \times A \times IA) / BW$ $(0,001 \times 0,2 \times 20 \times 100\%) / 70$			$SIE_{OA} = (I_A \times AR \times A \times PPE^4 \times IA) / BW$ $(0,001 \times 0,2 \times 20 \times 1 \times 100\%) / 70$		
External inhalation exposure	0,004	mg/person	External inhalation exposure	0,004	mg/person
External inhalation exposure	5,714E-05	mg/kg bw/d	External inhalation exposure	5,714E-05	mg/kg bw/d
Systemic inhalation exposure	0,000057	mg/kg bw/d	Systemic inhalation exposure	0,000057	mg/kg bw/d
Total systemic inhalation exposure: $SIE_O = SIE_{OM} + SIE_{OA}$			Total systemic inhalation exposure: $SIE_O = SIE_{OM} + SIE_{OA}$		
Total external inhalation exposure	0,006400	mg/person	Total external inhalation exposure	0,006400	mg/person
Total external inhalation exposure	0,000091	mg/kg bw/d	Total external inhalation exposure	0,000091	mg/kg bw/d
Total systemic inhalation exposure	0,000091	mg/kg bw/d	Total systemic inhalation exposure	0,000091	mg/kg bw/d
Total systemic exposure: $SE_O = SDE_O + SIE_O$			Total systemic exposure: $SE_O = SDE_O + SIE_O$		
Total systemic exposure	0,66880	mg/person	Total systemic exposure	0,53891	mg/person
Total systemic exposure	0,009554	mg/kg bw/d	Total systemic exposure	0,007699	mg/kg bw/d
% of AOEL	1,0	%	% of AOEL	0,8	%
¹⁾ reduction factor for gloves is 0.01 (professional applications) and 0.5 (home/allotment garden applications), resp. ²⁾ reduction factor for protective garment is 0.05 (prof. appl.) and 0.5 (workwear, home/allotment garden appl.), resp. ³⁾ reduction factor for broad brimmed headgear and hood and visor is 0.5 and 0.05, respectively (professional appl.) ⁴⁾ reduction factor for RPE is 0.08 (particle filter) and 0.02 (combined vapour and particle filter), resp. (prof. appl.)					

WARNING: This document forms part of an EC evaluation data package and should not be isolated or used on the basis of this document.

THE UK PREDICTIVE OPERATOR EXPOSURE MODEL (POEM) - NO PPE USED

THE UK PREDICTIVE OPERATOR EXPOSURE MODEL (POEM)

Application method	Tractor-mounted/trailed boom sprayer: hydraulic nozzles		
Product	S-2200 25SC	Active substance	Mandestrobin
Formulation type	water-based	a.s. concentration	250 mg/ml
Dermal absorption from product	0,1 %	Dermal absorption from spray	8,00 %
Container	1 litre any closure		
PPE during mix/loading	None	PPE during application	None
Dose	0,8 l/ha	Work rate/day	50 ha
Application volume	100 l/ha	Duration of spraying	6 h

EXPOSURE DURING MIXING AND LOADING

Container size	1 litres
Hand contamination/operation	0,01 ml
Application dose	0,8 litres product/ha
Work rate	50 ha/day
Number of operations	40 /day
Hand contamination	0,4 ml/day
Protective clothing	None
Transmission to skin	100 %
Dermal exposure to formulation	0,4 ml/day

DERMAL EXPOSURE DURING SPRAY APPLICATION

Application technique	Tractor-mounted/trailed boom sprayer: hydraulic nozzles		
Application volume	100 spray/ha		
Volume of surface contamination	10 ml/h		
Distribution	Hands	Trunk	Legs
	65%	10%	25%
Clothing	None	Permeable	Permeable
Penetration	100%	5%	15%
Dermal exposure	6,5	0,05	0,375 ml/h
Duration of exposure	6 h		
Total dermal exposure to spray	41,55 ml/day		

ABSORBED DERMAL DOSE

	Mix/load	Application
Dermal exposure	0,4 ml/day	41,55 ml/day
Concen. of a.s. product or spray	250 mg/ml	2 mg/ml
Dermal exposure to a.s.	100 mg/day	83,1 mg/day
Percent absorbed	0,1 %	8 %
Absorbed dose	0,1 mg/day	6,648 mg/day

INHALATION EXPOSURE DURING SPRAYING

Inhalation exposure	0,01 ml/h
Duration of exposure	6 h
Concentration of a.s. in spray	2 mg/ml
Inhalation exposure to a.s.	0,12 mg/day
Percent absorbed	100 %
Absorbed dose	0,12 mg/day

PREDICTED EXPOSURE

Total absorbed dose	6,868 mg/day
Operator body weight	60 kg
Operator exposure	0,114466667 mg/kg bw/day
AOEL	0,91 mg/kg bw/day
% AOEL	12,57875458

WARNING: This document forms part of an EC evaluation data package and should not be released on the basis of this document.

THE UK PREDICTIVE OPERATOR EXPOSURE MODEL (POEM) - PPE USED DURING MIXING/LOADING AND APPLICATION

THE UK PREDICTIVE OPERATOR EXPOSURE MODEL (POEM)

Application method	Tractor-mounted/trailed boom sprayer: hydraulic nozzles		
Product	S-2200 25SC	Active substance	Mandestrobin
Formulation type	water-based	a.s. concentration	250 mg/ml
Dermal absorption from product	0,1 %	Dermal absorption from spray	8,00 %
Container	1 litre any closure		
PPE during mix/loading	Gloves	PPE during application	Gloves
Dose	0,8 l/ha	Work rate/day	50 ha
Application volume	100 l/ha	Duration of spraying	6 h

EXPOSURE DURING MIXING AND LOADING

Container size	1 litres
Hand contamination/operation	0,01 ml
Application dose	0,8 litres product/ha
Work rate	50 ha/day
Number of operations	40 /day
Hand contamination	0,4 ml/day
Protective clothing	Gloves
Transmission to skin	5 %
Dermal exposure to formulation	0,02 ml/day

DERMAL EXPOSURE DURING SPRAY APPLICATION

Application technique	Tractor-mounted/trailed boom sprayer: hydraulic nozzles		
Application volume	100 spray/ha		
Volume of surface contamination	10 ml/h		
Distribution	Hands	Trunk	Legs
	65%	10%	25%
Clothing	Gloves	Permeable	Permeable
Penetration	10%	5%	15%
Dermal exposure	0,65	0,05	0,375 ml/h
Duration of exposure	6 h		
Total dermal exposure to spray	6,45 ml/day		

ABSORBED DERMAL DOSE

	Mix/load	Application
Dermal exposure	0,02 ml/day	6,45 ml/day
Concen. of a.s. product or spray	250 mg/ml	2 mg/ml
Dermal exposure to a.s.	5 mg/day	12,9 mg/day
Percent absorbed	0,1 %	8 %
Absorbed dose	0,005 mg/day	1,032 mg/day

INHALATION EXPOSURE DURING SPRAYING

Inhalation exposure	0,01 ml/h
Duration of exposure	6 h
Concentration of a.s. in spray	2 mg/ml
Inhalation exposure to a.s.	0,12 mg/day
Percent absorbed	100 %
Absorbed dose	0,12 mg/day

PREDICTED EXPOSURE

Total absorbed dose	1,157 mg/day
Operator body weight	60 kg
Operator exposure	0,019283333 mg/kg bw/day
AOEL	0,91 mg/kg bw/day
% AOEL	2,119047619

WARNING: This document forms part of an EC evaluation data package and should not be released without the approval of the competent authority. The information contained herein is confidential and should not be used for any other purpose than the one for which it was provided. The use of this information is subject to the terms and conditions of the license agreement. The information contained herein is confidential and should not be used for any other purpose than the one for which it was provided. The use of this information is subject to the terms and conditions of the license agreement.

BYSTANDER AND RESIDENT EXPOSURE AFTER MARTIN ET AL

Estimation of bystander and resident exposure (adults and children)			
Active substance (a.s.)	Mandestrobin		
Product	S-2200 25SC		
Intended uses	Field Crops, Tractor Mounted (FCTM)		
Treated area per day (A)	20	ha/d	
Application rate (AR)	0,2	kg a.s./ha	
Number of applications (NA)	1		¹⁾
¹⁾ Consideration of more than two applications are not necessary if degradation of the active substance on foliage of at least 50 % can be assumed between two applications (otherwise use multiple application factor).			
Dermal absorption (DA)	8	% (worst case, e.g. during application)	
Inhalation absorption (IA)	100	%	
Oral absorption (OA)	100	%	
Systemic AOEL	0,91	mg/kg bw/d	
Body weight (BW)	60	kg/person (adults)	
	16,15	kg/person (children)	
Distance between application and bystander or resident:			
FCTM:	10	m	
High crops not selected			
	0	m	
Home & garden not selected			
		m	
Drift deposit (D) for 1 appl. based on appl. technique and distance:			0,29 % (FCTM, 10 m)
Airborne vapour concentration (ACv)	0,001	mg/m ³ ²⁾	
²⁾ 1 µg/m ³ for semivolatile substances, i.e. vapour pressure (20 °C): ≥ 1x10 ⁻⁵ - < 5x10 ⁻³ Pa; 15 µg/m ³ for volatile substances, i.e. vapour pressure (20 °C): ≥ 5x10 ⁻³ Pa			

Estimation of bystander exposure during/after application in Field Crops, Tractor Mounted

Input parameters considered for the estimation of bystander exposure:

Intended use(s):		Drift (D):	0,29	% (FCTM, 10 m)
Application rate (AR):	0,2 kg a.s./ha	Exposed Body Surface Area (BSA):	1	m ² (adults)
			0,21	m ² (children)
Body weight (BW):	60 kg/person (adults)	Specific Inhalation Exposure (I* _A):	0,001	mg/kg a.s. (6 hours, adults)
	16,15 kg/person (children)		0,00057	mg/kg a.s. (6 hours, children)
Dermal absorption (DA):	8,00 % ('worst case')	Area Treated (A):	20	ha/d (based on Field Crops, Tractor Mounted)
Inhalation absorption (IA):	100 %	Exposure duration (T):	5	min
AOEL:	0,91 mg/kg bw/d			

Bystander exposure towards Mandestrobin					
Adults			Children		
Bystander: Dermal exposure after application in (via spray drift)					
$SDE_B = (AR \times D \times BSA \times DA) / BW$			$SDE_B = (AR \times D \times BSA \times DA) / BW$		
$(20 \times 0,29\% \times 1 \times 8\%) / 60$			$(20 \times 0,29\% \times 0,21 \times 8\%) / 16,15$		
External exposure	0,058	mg/person	External exposure	0,01218	mg/person
External exposure	0,00096667	mg/kg bw/d	External exposure	0,00075418	mg/kg bw/d
Absorbed dose:	0,0000773	mg/kg bw/d	Absorbed dose:	0,0000603	mg/kg bw/d
Bystander: Inhalation exposure after application in					
$SIE_B = (I^*_A \times AR \times A \times T \times IA) / BW$			$SIE_B = (I^*_A \times AR \times A \times T \times IA) / BW$		
$(0,001 / 360 \times 0,2 \times 20 \times 5 \times 100\%) / 60$			$(0,001 / 360 \times 0,2 \times 20 \times 5 \times 100\%) / 16,15$		
External exposure	5,5556E-05	mg/person	External exposure	3,1928E-05	mg/person
External exposure	9,2593E-07	mg/kg bw/d	External exposure	1,977E-06	mg/kg bw/d
Absorbed dose:	0,0000009	mg/kg bw/d	Absorbed dose:	0,0000020	mg/kg bw/d
Total systemic exposure: $SE_B = SDE_B + SIE_B$			Total systemic exposure: $SE_B = SDE_B + SIE_B$		
Total systemic exposure (absorbed dose)	0,00469556	mg/person	Total systemic exposure (absorbed dose)	0,00100633	mg/person
Total systemic exposure (absorbed dose)	0,0000783	mg/kg bw/d	Total systemic exposure (absorbed dose)	0,0000623	mg/kg bw/d
% of AOEL:	0.01	%	% of AOEL:	0.01	%

Estimation of resident exposure after application in Field Crops, Tractor Mounted (FCTM)

Input parameters considered for the estimation of resident exposure:

Input parameters considered for the estimation of Residue exposure:				
Intended use(s):			Drift (D):	0,29 % (FCTM, 10 m)
Application rate (AR):	0,2	kg a.s./ha	Transfer coefficient (TC):	7300 cm ² /h (adults)
				2600 cm ² /h (children)
Number of applications (NA):	1		Turf Transferable Residues (TTR):	5 %
Body weight (BW):	60	kg/person (adults)	Exposure Duration (H):	2 h
	16,15	kg/person (children)	Airborne Concentration of Vapour (ACV):	0,001 mg/m3
Dermal absorption (DA):	8,00	% ('worst case')	Inhalation Rate (IR):	16,57 m ³ /d (adults),
Inhalation absorption (IA):	100	%		8,31 m ³ /d (children)
Oral absorption (OA)	100	%	Saliva Extraction Factor (SE):	50 %
AOEL	0,91	mg/kg bw/d	Surface Area of Hands (SA):	20 cm ²
			Frequency of Hand to Mouth (Freq):	20 events/h
			Dislodgeable foliar residues (DFR):	20 %
			Ingestion Rate for Mouthing of Grass/Day (IgR):	25 cm ² /d

Resident exposure towards Mandestrobin					
Adults			Children		
Residents: Dermal exposure after application in (via deposits caused by spray drift)					
$SDE_R = (AR \times NA \times D \times TTR \times TC \times H \times DA) / BW$			$SDE_R = (AR \times NA \times D \times TTR \times TC \times H \times DA) / BW$		
$(0,002 \times 1 \times 0,29\% \times 5\% \times 7300 \times 2 \times 8\%) / 60$			$(0,002 \times 1 \times 0,29\% \times 5\% \times 2600 \times 2 \times 8\%) / 16,15$		
External exposure	0,004234	mg/person	External exposure	0,001508	mg/person
External exposure	7,0567E-05	mg/kg bw/d	External exposure	9,3375E-05	mg/kg bw/d
Absorbed dose:	0,0000056	mg/kg bw/d	Absorbed dose:	0,0000075	mg/kg bw/d
Residents: Inhalation exposure to vapour					
$SIE_R = (AC_V \times IR \times IA) / BW$			$SIE_R = (AC_V \times IR \times IA) / BW$		
$(0,001 \times 16,57 \times 100\%) / 60$			$(0,001 \times 8,31 \times 100\%) / 16,15$		
External exposure	0,01657	mg/person	External exposure	0,00831	mg/person
External exposure	0,00027617	mg/kg bw/d	External exposure	0,00051455	mg/kg bw/d
Absorbed dose:	0,0002762	mg/kg bw/d	Absorbed dose:	0,0005146	mg/kg bw/d
			Residents: Oral exposure (hand-to-mouth transfer)		
			$SOE_H = (AR \times NA \times D \times TTR \times SE \times SA \times Freq \times H \times OA) /$		
			$(0,002 \times 1 \times 0,29\% \times 5\% \times 50\% \times 20 \times 20 \times 2 \times 100\%) / 16,15$		
			External exposure	0,000116	mg/person
			External exposure	7,1827E-06	mg/kg bw/d
			Absorbed dose	0,0000072	mg/kg bw/d
			Residents: Oral exposure (object-to-mouth transfer)		
			$SOE_O = (AR \times NA \times D \times BFR \times IgR \times OA) / BW$		
			$(0,002 \times 1 \times 0,29\% \times 20\% \times 25 \times 100\%) / 16,15$		
			External exposure	0,000029	mg/person
			External exposure	1,7957E-06	mg/kg bw/d
			Absorbed dose	0,0000018	mg/kg bw/d
Total systemic exposure: $SE_R = SDE_R + SIE_R$			Total systemic exposure: $SE_R = SDE_R + SIE_R + SOE_H + SOE_O$		
Total systemic exposure (absorbed dose)	0,01690872	mg/person	Total systemic exposure (absorbed dose)	0,00857564	mg/person
Total systemic exposure (absorbed dose)	0,0002818	mg/kg bw/d	Total systemic exposure (absorbed dose)	0,0005310	mg/kg bw/d
% of AOEL:	0.03	%	% of AOEL:	0.06	%

WORKER EXPOSURE AFTER EUROPOEM II

WORKER EXPOSURE		EUROPOEM II MODEL	
form	S-2200 25SC	Re-entry in the field	
a.s.	Mandestrobin		
Parameter	Value	Unit	References, comments
Re-entry activities in the field			
AR Application rate	0,2	kg a.s./ha	summary of intended uses
Worker			
Duration			
T	2	hours / day	default: 6 h (Europoem II)
Inhalation Exposure			
no model available	-		w without PPE
Dermal Exposure			
DFR Dislodgeable foliar residue	30	mg a.s./m2/kg a.s./ha	default (Europoem II)
TC Transfer coefficient	0,5	m2/ hour	vegetable (field): 0.25; ornamentals: 0.5; small fruit: 0.3; large fruit: 0.45 (Europoem II)
Dermal Exposure	6	mg a.s./ day	DE = DFR x AR x TC x T
Internal exposure			
DA Dermal Absorption	8	%	
PPE-factor dermal	5		gloves*
AOEL	54,6	mg a.s./ day	based on 60 kg bw
	Without PPE	With PPE	
Internal exposure	[mg a.s./ day]	[mg a.s./ day]	
Inhalation	-	-	no model available
Dermal	0,480	0,096	DE(int) = DE x (DA/100)
Total	0,480	0,096	sum
% AOEL			
Inhalation	-	-	no model available
Dermal	1	0	%AOEL = 100 x DE(int) / AOEL
Total	1	0	sum

* It is assumed in the used TC values, that body exposure is already reduced by (protective) clothing. The use of gloves will result in an extra reduction factor of 5.

Appendix 2: Position papers

Two position papers were submitted by the notifier and are included in full in this Appendix.

- 1.) The toxicological relevance of the liver and thyroid alterations observed in rats treated with S-2200TG based on mode of action (██████████ 2012a)
- 2.) Up dated interpretation of higher incidence of ovarian sex-cord stromal tumour in female rats treated with S-2200TG in a 2-year carcinogenicity study (██████████ 2013)

WARNING: This document forms part of an EC evaluation data package and should not be read in isolation. Reactions must be graded on the basis of this document.

Document Title

Author

Date

October 10, 2012

Title: **The toxicological relevance of the liver and thyroid alterations observed in rats treated with S-2200TG based on mode of action**

Facility: [REDACTED]
[REDACTED]
[REDACTED]
[REDACTED]

Personnel

Author:

Date:

[REDACTED]

Approved by:

Date:

[REDACTED]

Group Manager, Toxicology group

WARNING: This document forms part of an EC evaluation data package and should not be read in isolation. Ration must be granted on the basis of this document.

Page

I. Summary.....	4
II. Introduction	5
III. General information on liver hypertrophy.....	5
IV. General information on thyroid hypertrophy	6
V. Assessment of toxicological relevance of S-2200TG-induced liver and thyroid alterations	6
V-1. Genotoxicity and carcinogenicity of S-2200TG	6
V-2. Evidence for enzyme induction by S-2200TG.....	7
A. Rat.....	7
B. Mouse	12
V-3. Assessment for toxicological relevance of liver hypertrophy observed in general toxicity and/or carcinogenicity studies with S-2200TG	13
A. Rat.....	13
B. Mouse	16
C. Dog	17
V-4. Assessment for toxicological relevance of thyroid hypertrophy observed in general toxicity and/or carcinogenicity studies with S-2200TG	20
A. Postulated mode of action for the induction of thyroid follicular-cell hypertrophy in rat	21
B. Key events in experimental animals	21
C. Dose-response relationship	22
D. Temporal association	25
E. Strength, consistency, and specificity of association of the response with key event.....	26
F. Biological plausibility and coherence	26
G. Other modes of action	27
H. Uncertainties, inconsistencies, and data gaps.....	27
I. Assessment of postulated mode of action	27
J. Human applicability of the proposed mode of action	28
K. Conclusion: statement of confidence, analysis, and implication	31
VI. Conclusions	31
VII. References	32

I. Summary

S-2200 Technical Grade (abbreviated as S-2200TG in this document) is a candidate novel strobilurin fungicide developed by Sumitomo Chemical Co., Ltd. The general toxicity of S-2200TG has been studied in experimental animals including rats, mice, and dogs in standard bioassays. These studies have revealed that the main toxicologic target organ of S-2200TG is the liver in all species examined. In addition, thyroid was also a target organ in the rat but not in the mouse and dog. The liver finding was mainly liver hypertrophy (increased liver weight and/or hepatocellular hypertrophy), and the thyroid finding was thyroid follicular-cell hypertrophy. However, no tumourigenic findings were observed in rat or mouse carcinogenicity studies. In this document, the toxicological significance of the liver and thyroid hypertrophy caused by S-2200TG is discussed based on experimental data of S-2200TG and published information.

In this assessment, we firstly conclude that S-2200TG is a hepatic enzyme inducer via at least constitutive androstane receptor (CAR) activation in rat, similar to phenobarbital (PB), evidenced by induction of CYP2B activity and UDP-glucuronosyltransferase activity toward thyroxine (T4) (T4-UGT), and proliferation of smooth endoplasmic reticulum (SER). Therefore, the liver hypertrophy (i.e., increased liver weight and/or hepatocellular hypertrophy) caused by S-2200TG is judged to be an adaptive response by enzyme induction at least via CAR and not adverse. This activity also appears to be plausible in mouse and dog. Furthermore, this activity would theoretically operate in humans, as demonstrated by CYP2B inducers. S-2200TG at a much higher dose level induced adverse effects on liver as some additional functional changes or additional pathological findings to the hypertrophy were observed. However, the adverse effects occurred in a dose related manner and there was a threshold at relatively high exposure level; and most importantly, S-2200TG did not induce liver tumours in rat and mouse, reducing concern in human risk assessment.

Secondly, we obtained data indicating that S-2200TG increased T4-UGT and indirectly perturbed the hypothalamus-pituitary-thyroid hormone axis, and then increased thyroid follicular-cell hypertrophy in rats, which is also similar to PB, a CAR activator. The relevance of the rat thyroid abnormality to human health was assessed by using the 2006 IPCS Human Relevance Framework. The postulated mode of action (MOA) for possible induction of thyroid follicular-cell hypertrophy in rats was tested against the Bradford Hill criteria, and was found to satisfy the conditions of dose and temporal concordance, biological plausibility, coherence, strength, consistency, and specificity that fit with a well-established MOA for thyroid follicular-cell hypertrophy. Although the postulated MOA could theoretically operate in humans, marked quantitative differences in the inherent susceptibility for thyroid abnormality, especially tumour induction, to thyroid hormone imbalance in rats is not relevant to humans.

Therefore, even though the liver and thyroid hypertrophy were induced by S-2200TG treatment in experimental animals, the findings from a MOA analysis allow for the conclusion that S-2200TG does not pose a hazard to humans.

II. Introduction

S-2200TG is a candidate novel strobilurin fungicide developed by Sumitomo Chemical Co., Ltd. The general toxicity of S-2200TG has been studied in experimental animals including rats, mice, and dogs in standard bioassays under the guidelines of Good Laboratory Practice and the test protocols designated by the European Community (EC), Organisation for Economic Co-operation and Development (OECD), US. Environmental Protection Agency (US.EPA), and Ministry of Agriculture, Forestry and Fisheries of Japan (Japan MAFF). These studies have revealed that the main toxicologic target organ of S-2200TG is liver in all species examined. In addition, thyroid was also a target organ in the rat but not in mouse and dog. The liver finding was mainly liver hypertrophy (increased liver weight and/or hepatocellular hypertrophy), and the thyroid finding was thyroid follicular-cell hypertrophy. However, no tumourigenic findings were observed in rat or mouse carcinogenicity studies.

In this document, the toxicological relevance of the liver and thyroid hypertrophy caused by S-2200TG is discussed based on experimental data of S-2200TG and published information.

III. General information on liver hypertrophy

“Hepatocellular hypertrophy” is reviewed as below in “JMPR Summary Report, 5. Guidance on the interpretation of hepatocellular hypertrophy” (JMPR, 2006).

Hepatocellular hypertrophy is a general increase in the size of the liver because of cell enlargement and accumulation of fluids. It is not attributable to tumour formation or to an increase in the number of cells (hyperplasia). An indication that hypertrophy is occurring in hepatocytes is usually an increase in the size and weight of the liver. At the cellular level, the response is a [involves] proliferation of the smooth endoplasmic reticulum (SER) that would be evident microscopically at an early stage at the tissue level as an increase in acidophilia (e.g. eosinophilia). Proliferation of SER would be confirmed by electron microscopy. Hepatocellular hypertrophy is typically related to increased functional capacity. To maintain homeostasis in the whole organism, the hepatocyte frequently responds to xenobiotic exposure by increasing its metabolic capacity via induction of xenobiotic metabolizing enzymes. Such hepatic adaptive responses usually result from chemical interaction with cellular regulatory pathways (often receptor-mediated), leading to changes in gene expression and protein synthesis, and eventually cell growth and alteration of microsomal enzyme activities. Adaptive responses are potentially beneficial in that they enhance the capacity of the organism to respond to chemical-induced stress, and are reversible. However, there are limits to these homeostatic responses and it is important to recognize when these limits have been exceeded. Because toxicity is an exposure-related phenomenon, there are lower exposures that produce effects within the control of homeostatic mechanisms and higher exposures that result in effects that exceed the capacity of these mechanisms to return the organism to its previous condition once exposure has ceased.

No single effect is generally sufficient to support a determination that liver hypertrophy is adaptive or adverse. Determination of hepatotoxicity involves a detailed consideration of clinical chemistry and histopathology (or other relevant information such as histochemistry, morphometry and electron microscopy). The type, severity or magnitude, and dose-response relationship of observed effects, as well as the progression of observed lesions with duration of dosing, should be considered. It is important to evaluate whether the observed effects present a biologically plausible and consistent pattern of changes in clinical chemistry and histopathology indicative of hepatotoxicity. Sustained effects should be given more weight than transient effects.

Furthermore, we should recognize that sustained treatment with enzyme inducers in rodents for a long time often (but not always) induce liver tumours; e.g., activators via constitutive androstane receptor (CAR) and peroxisome proliferators activated receptor alpha (PPAR α) have been well studied (Cohen and Arnold, 2011; Klaunig et al., 2003; Osimitz and Lake, 2009; Yamada et al., 2009). The nuclear receptor CAR mediates the response evoked by a class of xenobiotics known as the 'phenobarbital-like inducers' (Wei et al., 2000).

IV. General information on thyroid hypertrophy

There is an excellent review of thyroid biology (Dellarco, et al., 2006). Current understanding about thyroid related-alterations is summarized below:

There are considerable data from studies in laboratory rodents demonstrating the relationship between sustained perturbation of the hypothalamic–pituitary–thyroid axis, prolonged stimulation of the thyroid gland by thyroid stimulating hormone (TSH), and the progression of thyroid follicular-cells to hypertrophy, hyperplasia, and eventually neoplasia (Hard, 1998; Hurley et al., 1998; IARC, 2001; McClain, 1995). Increased secretion of TSH may result via several mechanisms, including increased hepatic clearance of thyroxine (T4) by UDP-glucuronosyltransferase activity toward T4 (T4-UGT).

Circulating levels of T4 are monitored by the thyrotropic cells of the pituitary gland that are responsible for the synthesis of TSH. In the pituitary gland T4 is metabolized by 5-deiodinase type II to T3, which then binds to specific receptors in the [pituitary] cell nucleus [(Capen, 1997)]. A decrease in T3 receptor occupancy results in stimulation of TSH synthesis and secretion. Studies *in vivo* have shown that injection of rats with TSH leads to reductions in thyroid follicular-cell nuclear stain, a non-proliferation specific nuclear antigen, indicating that these cells were leaving the nondividing state to resume the cell cycle (Bayer et al., 1992). This study showed that low, repeated doses of TSH (0.25 IU/rat twice daily) produced a cumulative response in nuclear stain levels over 10 days, and the response returned to normal resting levels within 5 days of cessation of TSH injections. Reduction in nuclear stain is also an early event that parallels the earliest known pinocytotic response to TSH. These data are consistent with increased TSH concentrations alone causing thyroid follicular-cells of rats to enter a state of preproliferation. [Hepatic UGT activity is regulated by the nuclear receptor CAR (Sugatani et al., 2001), and its stimulation leads to metabolism of T4 and consequent increased TSH and thyroid hypertrophy and proliferation.]

V. Assessment of toxicological relevance of S-2200TG-induced liver and thyroid alterations

V-1. Genotoxicity and carcinogenicity of S-2200TG

S-2200TG was not genotoxic in a battery of *in vitro* and *in vivo* assays: reverse mutation test in a bacterial system, gene mutation test in Chinese Hamster V79 cells, chromosomal abbreviation test in Chinese Hamster lung cells (CHL/IU), and micronucleus test in CD-1 mice.

The carcinogenicity of S-2200TG has been studied in male and female rats and mice in standard bioassays under the guidelines of Good Laboratory Practice.

Male and female Crl:CD1(ICR) mice were fed 0 (control), 700, 2000, or 7000 ppm S-2200TG (purity, 93.4%) in the diet for 78 weeks (average chemical intakes: 83, 239, and 824 mg/kg/day for males; 99, 280, and 994 mg/kg/day for females, respectively). The No Observed Adverse Effect Level (NOAEL) for this study was considered to be 7000 ppm (824 mg/kg/day for males and 994 mg/kg/day for females) following 78 weeks of treatment since no increased tumourigenicity occurred. Male and female Crl:WI(Han) rats were fed 0 (control), 400, 2000, 7000, or 15000 ppm S-2200TG (purity, 93.4%) in the diet for 104 weeks (average chemical intakes: 21, 105, 376, and 804 mg/kg/day for males; 27, 135, 475 and 1016 mg/kg/day for females, respectively). Body weight suppressions were observed at 15000 ppm in males and 2000 ppm and higher in females. The NOAEL for this study was considered to be 2000 ppm (105 mg/kg/day) for males and 400 ppm (26.7 mg/kg/day) for females following 104 weeks of treatment.

No statistically-significant increase of neoplastic findings was observed in any organs of treated animals in both studies. Therefore, S-2200TG is concluded not to be carcinogenic.

V-2. Evidence for enzyme induction by S-2200TG

Based on experimental data described below, S-2200TG is a hepatic enzyme inducer via at least CAR activation in rat and mouse, similar to phenobarbital (PB).

A. Rat

As an analysis for mode of action (MOA) for S-2200TG-induced liver and thyroid alterations

in rats, a study was conducted to evaluate the dose response, time course, and reversibility of alterations at an early phase of treatment with S-2200TG, mainly focusing on liver enzyme induction, hepatocellular replicative DNA synthesis, and thyroid hormone levels and morphology. Details are presented in the Technical Report (Asano, 2012). Male and female Crlj:WI rats were fed diets containing 0 (control), 400, 2000, 7000 and 15000 ppm S-2200TG for 7 days. These dose levels are identical to those in the 2-year bioassay. To evaluate time course of alterations at 15000 ppm, male and female Crlj:WI rats were also fed diets containing 0 and 15000 ppm for 14 days, and data from both 7 and 14-day treatment groups were compared to determine whether enhancement or attenuation of alterations due to longer treatment was observed. Furthermore, to evaluate reversibility, male and female Crlj:WI rats were fed diets containing 0 and 15000 ppm S-2200TG for 7 days followed by 7-day cessation of the treatment. As a positive control for a CAR activator, 1000 ppm PB groups were included in each phase of the study.

Under conditions of the present study, treatment with S-2200TG caused no deaths, did not show severe toxicity or marked abnormalities in clinical signs, body weight or food consumption that could confound evaluation of the main target endpoints.

In rats treated with S-2200TG for 7 days, hepatic alterations were observed in a dose-related manner; they included increases of liver weight, enlarged liver, diffuse hepatocellular hypertrophy, CYP2B and T4-UGT activities, and replicative DNA synthesis of hepatocytes (determined by 5-bromo-2'-deoxyuridine (BrdU) labeling index). In general, clear effects were observed at 7000 ppm and higher in both sexes. However, CYP4A activity revealed no remarkable increase. Electron microscopic examination revealed SER was increased, but peroxisome was not changed in the hepatocytes from the animals administered S-2200TG at 15000 ppm. These findings are summarized in Tables 1 and 2.

Table 1. Summary findings from 7-day treatment study in rats (Male)

Endpoints	7-day Treatment group					
	Control	S-2200TG				PB
		400ppm	2000ppm	7000ppm	15000ppm	1000ppm
Test Item Intake (mg/kg/day)	0	23.3	115.7	378.9	744.4	57
Death #	0/10	0/10	0/10	0/10	0/10	0/10
Final Body Weight (g)	407±23.3	394±17.4	394±10.1	388±14.8*	386±31.9	398±29.7
Body Weight Gain (g)	38±5.0	34±6.5	34±4.9	28±5.6**	27±12	35±12
Food Consumption (g/animal/day)	23±0.7	22±0.5	22±0.8	21±0.8*	21±1.8*	21±1.1*
Liver Weight						
Absolute (g)	14.86±1.381	14.26±1.07	14.83±1.005	15.6±0.835	17.52±2.173*	17.95±1.643**
(vs. control, fold)	1	0.96	1.00	1.05	1.18	1.21
Relative (g/body weight)	3.65±0.22	3.61±0.177	3.77±0.214	4.03±0.215**	4.53±0.342**	4.51±0.278**
(vs. control, fold)	1	0.99	1.03	1.10	1.24	1.24
Thyroid Weight						
Absolute (g)	21±3	22±3.4	23±6.3	21±3.8	24±3.3	25±5.7
(vs. control, fold)	1	1.05	1.10	1.00	1.14	1.19
Relative (g/body weight)	5.2±0.74	5.6±0.98	5.8±1.58	5.4±0.88	6.2±0.92	6.2±1.43
(vs. control, fold)	1	1.08	1.12	1.04	1.19	1.19
BrdU labeling index of hepatocytes (%)	0.94±0.47	0.92±0.52	1.71±1.01	2.19±0.92**	3.66±2.47**	3.65±1.87**
(vs. control, fold)	1	0.98	1.82	2.33	3.89	3.88
Hepatic CYP2B activity (pmol/min/mg S9 protein)	32±15.3	47±10.1	92±9.7*	278±94.3*	433±119.6*	800±263.7*
(vs. control, fold)	1	1.47	2.88	8.69	13.53	25.00
Hepatic CYP4A activity (pmol/min/mg S9 protein)	125±19.1	122±24.4	124±16.5	151±13.7	155±24.6	209±24.5**
(vs. control, fold)	1	0.98	0.99	1.21	1.24	1.67
Hepatic T4-UGT (pmol/min/mg S9 protein)	0.41±0.054	0.51±0.024*	0.54±0.075	0.62±0.051*	0.61±0.046*	0.79±0.122*
(vs. control, fold)	1	1.24	1.32	1.51	1.49	1.93
Serum TSH (ng/mL)	8.6±2.76	7.3±2.33	7.5±2.5	6.7±2.12	7.5±2.96	10.6±3.42
(vs. control, fold)	1	0.85	0.87	0.78	0.87	1.23
Serum T3 (µg/dL)	0.5±0.07	0.5±0.1	0.5±0.08	0.4±0.08	0.5±0.1	0.5±0.11
(vs. control, fold)	1	1.00	1.00	0.80	1.00	1.00
Serum T4 (µg/dL)	5.04±0.722	5.35±0.848	5.36±0.71	4.56±0.897	4.09±0.869*	4.45±1.305
(vs. control, fold)	1	1.06	1.06	0.90	0.81	0.88
Gross pathology #						
Liver						
Enlarged	0/10	0/10	0/10	0/10	3/10	0/10
Histopathology #						
Liver						
Hypertrophy, hepatocyte, centrilobular	0/10	0/10	0/10	0/10	0/10	10/10**
Hypertrophy, hepatocyte, diffuse	0/10	0/10	1/10	8/10**	9/10**	0/10
Thyroid						
Hypertrophy, follicular, diffuse	1/10	1/10	2/10	2/10	3/10	5/10
Histopathology #						
Liver						
electron microscopy						
Proliferation, SER	0/2	ND	ND	ND	1/2	ND

Data presents mean±SD, n=6-10. #: Data is scored as number of animals exhibiting findings out of total number of animals examined.

*: p<0.05, **: p<0.01. Red shadow presents biologically significant change. ND: Not Determined

Table 2. Summary findings from 7-day treatment study in rats (Female)

Endpoints		7-day treatment group						
			S-2200TG				PB	
		Control	400ppm	2000ppm	7000ppm	15000ppm	1000ppm	
Test Item Intake (mg/kg/day)		0	25.7	131.2	420.2	811.8	66.2	
Death #		0/10	0/10	0/10	0/10	0/10	0/10	
Final Body Weight (g)		271±17.5	266±10.7	266±12.2	263±8.8	255±17.6	269±16.4	
Body Weight Gain (g)		24±11.6	23±6.9	23±8.9	21±9.2	12±5.1**	19±12.7	
Food Consumption (g/animal/day)		17±0.5	17±1.1	17±1.1	16±0.5	15±1.1	16±0.9	
Liver Weight		Absolute (g)	9.3±0.797	9.08±0.761	9.99±0.544	10.67±0.943**	10.96±1.197**	
	(vs. control, fold)	1	0.98	1.04	1.07	1.15	1.18	
	Relative (g/body weight)	3.44±0.125	3.41±0.241	3.63±0.12*	3.79±0.095**	4.17±0.118**	4.07±0.232**	
	(vs. control, fold)	1	0.99	1.06	1.10	1.21	1.18	
Thyroid Weight		Absolute (g)	18±4	18±2.8	18±3.1	23±3.1**	22±3.5*	21±3.4
	(vs. control, fold)	1	1.00	1.00	1.28	1.22	1.17	
	Relative (g/body weight)	6.5±1.38	6.9±1.09	6.7±1.17	8.6±1.12**	8.5±1.38**	7.9±1.04*	
	(vs. control, fold)	1	1.06	1.03	1.32	1.31	1.22	
BrdU labeling index of hepatocytes (%)		0.99±0.63	1.90±0.71	1.95±1.06	2.23±0.96*	2.39±1.55**	3.05±1.10**	
	(vs. control, fold)	1	1.92	1.97	2.25	2.41	3.08	
Hepatic CYP2B activity (pmol/min/mg S9 protein)		1.5±1.05	1.7±0.77	4.5±2.15*	38.1±15.39*	117.9±70.55*	397.8±79.35*	
	(vs. control, fold)	1	1.13	3	25.4	78.6	265.2	
Hepatic CYP4A activity (pmol/min/mg S9 protein)		177±29.2	167±41.3	152±35.5	158±31.3	150±36.1	229±16.9*	
	(vs. control, fold)	1	0.94	0.86	0.89	0.85	1.29	
Hepatic T4-UGT (pmol/min/mg S9 protein)		0.46±0.069	0.43±0.033	0.46±0.071	0.53±0.057	0.62±0.074**	0.56±0.06*	
	(vs. control, fold)	1	0.93	1	1.15	1.35	1.22	
Serum TSH (ng/mL)		6.1±1.71	6.3±2.13	6.7±2.14	9.1±4.99	15.1±6.22**	10.2±3.8**	
	(vs. control, fold)	1	1.03	1.10	1.49	2.48	1.67	
Serum T3 (µg/dL)		0.6±0.19	0.6±0.12	0.6±0.21	0.6±0.15	0.6±0.08	0.6±0.14	
	(vs. control, fold)	1	1.00	1.00	1.00	1.00	1.00	
Serum T4 (µg/dL)		4.7±1.412	4.7±1.124	4.52±1.331	4.34±1.24	3.95±1.311	3.17±0.641**	
	(vs. control, fold)	1	1.00	0.96	0.92	0.84	0.67	
Gross pathology # Liver								
	Enlarged	0/10	0/10	0/10	0/10	0/10	5/10*	
Histopathology # Liver								
	Hypertrophy, hepatocyte, centrilobular	0/10	0/10	0/10	0/10	0/10	10/10**	
	Hypertrophy, hepatocyte, diffuse	1/10	1/10	5/10	6/10*	8/10**	0/10	
Thyroid								
	Hypertrophy, follicular, diffuse	0/10	1/10	1/10	4/10*	6/10**	3/10	
Histopathology # Liver								
	electron microscopy							
	Proliferation, SER	0/2	ND	ND	ND	1/2	ND	
	Enlargement, lipid droplets	0/2	ND	ND	ND	1/2	ND	

Data present mean±SD, n=6-10. #: Data is scored as number of animals exhibiting findings out of total number of animals examined.

*: p<0.05, **: p<0.01. Red shadow presents biologically significant change. ND: Not Determined

Figure 1 shows that CYP2B and T4-UGT activities were increased in both sexes. These increases were less than that induced by PB 1000 ppm except for T4-UGT in females.

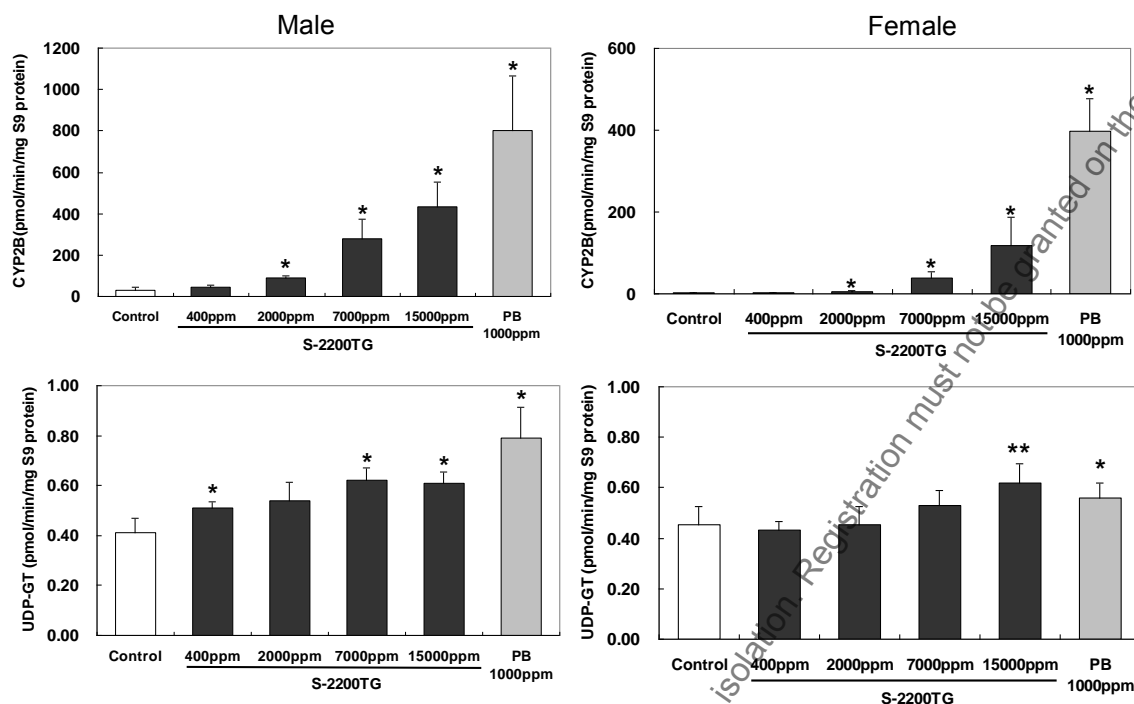


Figure 1. Hepatic CYP2B and T4-UGT induction in rats treated with S-2200TG or phenobarbital (PB) for 7 days. Data represent mean \pm SD, N=6. * $p<0.05$, ** $p<0.01$.

Compared to those observed after 7-day treatment, 14-day treatment revealed equivalent or slightly enhanced alterations of liver weight and morphology. For replicative DNA synthesis, continuous treatment with S-2200TG 15000 ppm for 14 days attenuated the increase of BrdU labeling indices, indicating a transient increase of rate of replicative DNA synthesis (Figure 2). These findings were consistent with those caused by PB.

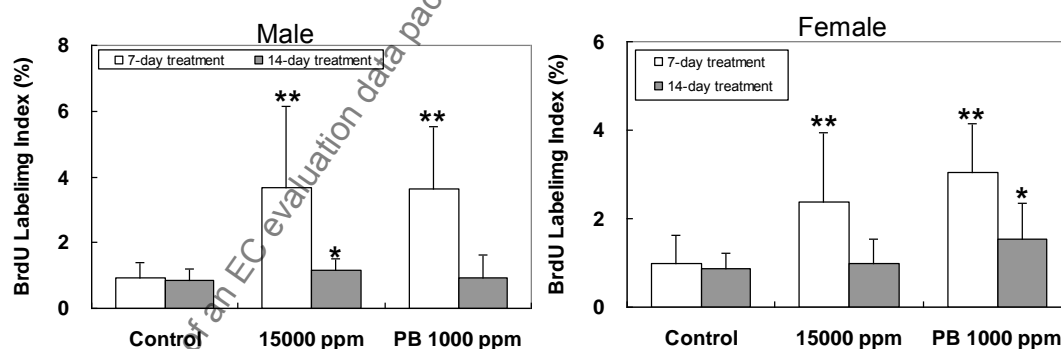


Figure 2. Hepatic replicative DNA synthesis determined by BrdU labeling indices in rats treated with S-2200TG or phenobarbital (PB) for 7 and 14 days. Data represent mean \pm SD, N=10. * $p<0.05$, ** $p<0.01$.

In rats treated with S-2200TG for 7 days, slight decrease of serum T4 levels, slight increase of TSH, and slight increase of thyroid weight with diffuse follicular-cell hypertrophy were observed in females in a dose-related manner irrespective of statistical significance (Tables 1 and 2). These effects were not recognized in males although serum T4 was significantly decreased (Tables 1 and 2). However, after 14-day treatment with S-2200TG, these findings were more clearly observed in both sexes (Figure 3). Taken together with induction of T4-UGT, these findings suggest that the decrease of T4 occurred prior to the increase of TSH and thyroid hypertrophy.

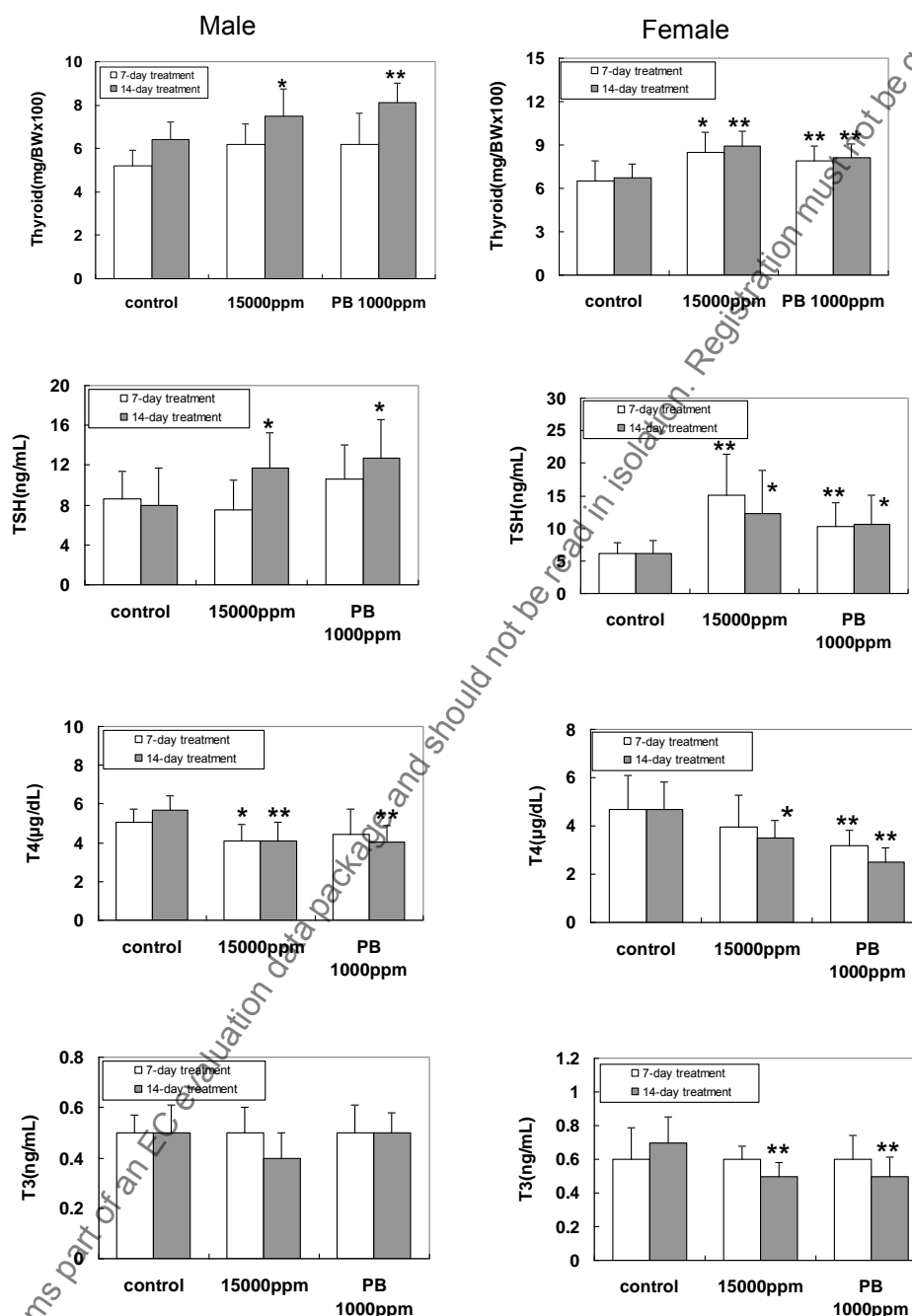


Figure 3. Comparison of alterations on relative thyroid weight and serum hormone levels in rats treated with S-2200TG or phenobarbital (PB) for 7 or 14 days. Data represent mean \pm SD, N=10. * p<0.05, ** p<0.01.

Regarding reversibility, all events including increased liver weights, increased enzyme induction, hepatocellular hypertrophy, decreased T4, increased TSH, increased thyroid weight, and thyroid follicular-cell hypertrophy were attenuated after 7-day cessation of S-2200TG treatment,

suggesting that the alterations of rat liver and thyroid by S-2200TG are reversible similar to what occurs with PB.

In conclusion, the present study demonstrated that treatment with S-2200TG in the rat revealed increased liver weight with diffuse hepatocellular hypertrophy and proliferation of SER, remarkable increase of CYP2B activity, and transient increase of rate of replicative DNA synthesis, with a dose response and reversibility. Furthermore, S-2200TG increased T4-UGT activity and secondarily perturbed the hypothalamus-pituitary-thyroid hormone axis. These effects are similar to PB, a CAR activator. Therefore, it is reasonable to conclude that S-2200TG is a hepatic enzyme inducer via at least CAR activation in rat, similar to PB.

B. Mouse

The study was conducted to evaluate whether S-2200TG induces hepatic metabolic enzymes via CAR activation in mouse liver (Yamada, 2012). Male Crlj:CD1(ICR) mice were fed diets containing 0 (control) or 7000 ppm S-2200TG for 7 days, and then hepatic CYP2B activity (determined by 7-pentoxoresorufin O-depentyldase activity) was examined. 7000 ppm is the highest dose in the mouse 1.5-year bioassay. In addition, replicative DNA synthesis of hepatocytes (determined by BrdU labeling index) was also examined because CAR activators often increase BrdU labeling index of hepatocytes at an early phase of treatment (Jones et al., 2009). Under conditions of the present study, treatment with S-2200TG caused no deaths, did not show severe toxicity, that might confound evaluation of hepatic CYP2B induction or BrdU labelling index, and no major clinical signs, body weight or food consumption changes occurred. The findings are summarized in Table 3.

Absolute liver weight tended to increase but not statistically significantly (1.06-fold of control value), whereas relative liver weight was significantly increased by S-2200TG treatment (1.07-fold of control value). No clear alterations were observed in liver gross pathology. By histopathology, slight eosinophilic change/hypertrophy of hepatocytes was observed in three of ten animals treated with S-2200TG 7000 ppm, none in controls. No necrosis was observed. Hepatic CYP2B activity was also significantly increased by S-2200TG treatment (1.71-fold of control value). No clear alterations were observed in replicative DNA synthesis of hepatocytes.

In conclusion, the present study demonstrated that treatment with S-2200TG in mouse revealed an increase of liver weight with slight eosinophilic change/hypertrophy of hepatocyte and increase of CYP2B activity, but much less than with PB (unpublished data). Therefore, it is reasonable to conclude that S-2200TG is a weak hepatic enzyme inducer via at least CAR activation in mouse. However, in contrast to PB (Jones, et. al, 2009), S-2200TG at this dose level tested did not significantly enhance replicative DNA synthesis of hepatocytes at this early phase of treatment.

Table 3. Summary findings from 7-day treatment study in mice

Endpoints	Groups	
	Control	S-2200TG 7000 ppm
Test Item Intake (mg/kg/day)	0	814
Death #	0/10	0/10
Final Body Weight (g)	43.8 ± 1.89	43.3 ± 2.26
Total Body Weight Gain (g)	2.8 ± 0.88	2.3 ± 0.84
Food Consumption at Day 6 (g/animal/day)	5.2 ± 0.48	5.2 ± 0.36
Liver Weight	Absolute (g)	2.50 ± 0.193
	(Fold change compared to control)	1
	Relative (g/body weight)	5.72 ± 0.356
	(Fold change compared to control)	1
S9 protein (mg/g liver)		167 ± 19.2
	(Fold change compared to control)	1
Hepatic CYP2B activity (pmol/min/mg S9 protein)		35 ± 4.2
	(Fold change compared to control)	1
BrdU labeling index of hepatocytes (%)		3.59 ± 1.93
	(Fold change compared to control)	1
Gross pathology #	Liver	
	No remarkable findings	10/10
Histopathology #	Liver	
	No remarkable findings	10/10
	Eosinophilic change/hypertrophy, hepatocyte	0/10
	Brownish pigment, hepatocyte, focal	0/10
	Cell infiltration, mononuclear cell, focal	2/10
	Necrosis, focal	2/10

Data presents mean±SD, N=6-10. #: Data is scored as number of animals exhibiting findings out of total number of animals examined. *p<0.05, **p<0.01. Red shadow presents biologically significant change.

V-3. Assessment for toxicological relevance of liver hypertrophy observed in general toxicity and/or carcinogenicity studies with S-2200TG

As mentioned above, several toxicology studies have revealed that the main toxicologic target organ of S-2200TG is liver in all species examined. The liver finding was mainly liver hypertrophy (increased liver weight and/or hepatocellular hypertrophy). In addition, thyroid was also a target organ in the rat but not in mouse and dog. The thyroid finding was thyroid follicular-cell hypertrophy. Toxicological relevance of the liver findings were first assessed in line mainly with the guidance in the JMPR Summary Report (JMPR, 2006), and then the toxicological relevance of the thyroid findings were assessed.

A. Rat

The findings in rat studies are summarized in Tables 4 and 5. The general toxicity and carcinogenicity studies of S-2200TG in rats revealed liver hypertrophy (i.e., increased liver weight and/or hepatocellular hypertrophy) and thyroid hyperplasia (weight change of thyroid was not examined but thyroid follicular-cell hypertrophy was observed by histopathological examination). Apart from the increased liver weight and hepatocellular hypertrophy, γ -glutamyltranspeptidase and total cholesterol in peripheral blood were increased at some dose levels. However, no changes were observed in alanine aminotransferase and aspartate aminotransferase at any doses examined. Histopathologically, other findings, including cytotoxicity and necrosis, were not observed in all phases examined. Only hepatocellular vacuolation was increased at 7000 and 15000 ppm after 2-year treatment in both sexes. Furthermore, neoplastic and pre-neoplastic findings were not observed.

Regarding the liver hypertrophy caused by S-2200TG, since induction of microsome enzyme by S-2200TG is already evident as mentioned above (Asano, 2012; Yamada, 2012), it could be an adaptive response when neither concomitant abnormality of several endpoints in blood biochemistry or other pathological findings were observed. Therefore, in the 90-day study, the Lowest Adverse Effect Level (LOAEL) on the liver was concluded to be at 20000 ppm of both

sexes because significant changes of two parameters such as γ -glutamyltranspeptidase and total cholesterol accompanied the liver hypertrophy. However, at 10000 ppm of both sexes, only serum total cholesterol was slightly increased. Therefore, the findings at 10000 ppm are considered an adaptive response but not adverse.

After 1-year treatment (satellite group in the combined chronic and carcinogenicity study), similar changes to 20000 ppm in the 90-day study were observed at 15000 ppm but not at 7000 ppm. Therefore, the LOAEL in the liver after 1-year treatment is 7000 ppm in both sexes. After 2-year treatment, the liver hypertrophy was observed at 7000 and 15000 ppm in males and 2000, 7000, and 15000 ppm in females. Although blood biochemistry was not examined in this carcinogenicity study, increased hepatocellular vacuolation was also observed in addition to hypertrophy at 7000 and 15000 ppm. Thus, the LOAEL for the liver after 2-year treatment is considered to be 7000 ppm in both sexes.

Table 4. Summary of liver and thyroid findings in male rats

Endpoints	Dose levels (ppm)								
	0	400	800	2000	4000	7000	10000	15000	20000
90-Day Study									
Chemical intake (mg/kg/day)	0	na	54	na	282.6	na	742.7	na	1544.6
Final body weight	100	na	103	na	99	na	96	na	92
Absolute liver weight	100	na	108	na	114**	na	122**	na	144**
Relative liver weight	100	na	105	na	115**	na	128**	na	157**
Hepatocellular hypertrophy #	0/12	na	0/12	na	12/12	na	12/12	na	12/12
γ -Glutamyltranspeptidase	2	na	2	na	2	na	2	na	5**
Total cholesterol	100	na	110	na	115	na	130**	na	140**
Thyroid follicular-cell hypertrophy #	2/12	na	2/12	na	6/12	na	9/12*	na	7/12
1-Year Study									
Chemical intake (mg/kg/day)	0.0	25.5	na	130.3	na	448.8	na	991.8	na
Final body weight	100	109*	na	99	na	96	na	92	na
Absolute liver weight	100	117**	na	109	na	112*	na	127**	na
Relative liver weight	100	107	na	110**	na	117**	na	140**	na
Hepatocellular eosinophilia / hypertrophy #	0/19	0/19	na	0/19	na	15/20**	na	20/20**	na
γ -Glutamyltranspeptidase	2	2	na	2	na	2	na	8**	na
Total cholesterol	100	100	na	104	na	117	na	126*	na
Thyroid follicular-cell hypertrophy #	1/19	0/19	na	1/19	na	9/20**	na	18/20**	na
2-Year Study									
Chemical intake (mg/kg/day)	0.0	21.0	na	105.1	na	375.6	na	804.3	na
Final body weight	100	101	na	94	na	96	na	89**	na
Absolute liver weight	100	103	na	97	na	114	na	103	na
Relative liver weight	100	99	na	100	na	108	na	114*	na
Hepatocellular eosinophilia / hypertrophy #	7/50	11/50	na	13/50	na	30/50**	na	37/50**	na
Hepatocellular vacuolation #	28/50	39/50*	na	37/50	na	38/50*	na	45/50**	na
Eosinophilic focus #	0/50	0/50	na	1/50	na	1/50	na	0/50	na
Hepatocellular adenoma #	1/50	0/50	na	0/50	na	0/50	na	2/50	na
Thyroid follicular-cell hypertrophy #	1/50	2/50	na	0/50	na	0/50	na	11/50**	na
Thyroid follicular-cell adenoma #	7/50	4/50	na	7/50	na	7/50	na	7/50	na
Thyroid follicular-cell carcinoma #	0/50	0/50	na	2/50	na	1/50	na	0/50	na

Data presents relative values when control value is shown as 100. #: Data is scored as number of animals exhibiting findings out of total number of animals examined. *: $p < 0.05$, **: $p < 0.01$. Shadow presents biologically significant change. †: The finding in males of 7000 ppm is considered toxicologically significant because data from terminal-kill animals indicates statistically significant increase. "na" means data not available.

Table 5. Summary of liver and thyroid findings in female rats

	Dose levels (ppm)								
	0	400	800	2000	4000	7000	10000	15000	20000
90 Day Study									
Chemical intake (mg/kg/day)	0	na	61.6	na	320.1	na	788.5	na	1886.5
Final body weight	100	na	97	na	97	na	98	na	96
Absolute liver weight	100	na	95	na	105	na	117**	na	144**
Relative liver weight	100	na	98	na	108	na	119**	na	150**
Hepatocellular hypertrophy #	0/12	na	0/12	na	4/11*	na	9/12**	na	12/12**
γ -Glutamyltranspeptidase	2	na	2	na	2	na	3	na	4**
Total cholesterol	100	na	113	na	120	na	167**	na	173**
Thyroid follicular-cell hypertrophy #	2/12	na	1/12	na	4/11	na	5/12	na	6/12
1-Year Study									
Chemical intake (mg/kg/day)	0.0	31.3	na	151.4	na	535.3	na	1138.9	na
Final body weight	100	96	na	98	na	97	na	91*	na
Absolute liver weight	100	96	na	103	na	117**	na	124**	na
Relative liver weight	100	100	na	105	na	121**	na	135**	na
Hepatocellular eosinophilia / hypertrophy #	0/20	1/20	na	1/19	na	17/19**	na	15/20**	na
γ -Glutamyltranspeptidase	2	2	na	2	na	2	na	6**	na
Total cholesterol	100	90	na	115	na	155**	na	150**	na
Thyroid follicular-cell hypertrophy #	0/20	0/20	na	0/19	na	9/19**	na	15/20**	na
2-Year Study									
Chemical intake (mg/kg/day)	0.0	26.7	na	135.2	na	475.0	na	1016.2	na
Final body weight	100	97	na	89**	na	89**	na	80**	na
Absolute liver weight	100	104	na	105	na	103	na	111	na
Relative liver weight	100	95	na	115*	na	114*	na	128**	na
Hepatocellular eosinophilia / hypertrophy #	16/50	22/50	na	32/50**	na	43/50**	na	42/50**	na
Hepatocellular vacuolation #	20/50	13/50	na	19/50	na	29/50 ^s	na	38/50**	na
Eosinophilic focus	0/50	0/50	na	0/50	na	1/50	na	1/50	na
Hepatocellular adenoma	0/50	0/50	na	0/50	na	0/50	na	1/50	na
Thyroid follicular-cell hypertrophy #	0/50	1/50	na	2/50	na	0/50	na	4/50	na
Thyroid follicular-cell adenoma #	2/50	2/50	na	2/50	na	5/50	na	1/50	na
Thyroid follicular-cell carcinoma #	0/50	0/50	na	0/50	na	1/50	na	0/50	na

Data presents relative values when control value is shown as 100. #: Data is scored as number of animals exhibiting findings out of total number of animals examined. *: $p < 0.05$, **: $p < 0.01$. Shadow presents biologically significant change. ^s: The finding in females at 7000 ppm is considered toxicologically significant because data from terminal-kill animals indicates statistically significant increase. "na" means data not available.

To determine whether progression of liver hypertrophy with duration of treatment is observed, further analysis was conducted. As shown in Figure 4, the relative liver weight was increased by S-2200TG treatment in a dose related manner. Generally, dose response of the liver weight change adjusted by chemical intake was similar among the three studies, i.e., potency of liver hypertrophy is similar irrespective of the treatment period. These findings suggest that the stress caused by S-2200TG at dose levels tested was within the control of homeostatic mechanisms. In fact, as mentioned above, no cytotoxic finding was observed.

Regarding thyroid, details are discussed later in this document.

No increase of tumours in any organs, including liver and thyroid, was observed in rats treated with S-2200TG at 15000 ppm which is the limiting dose level in the 2 year study.

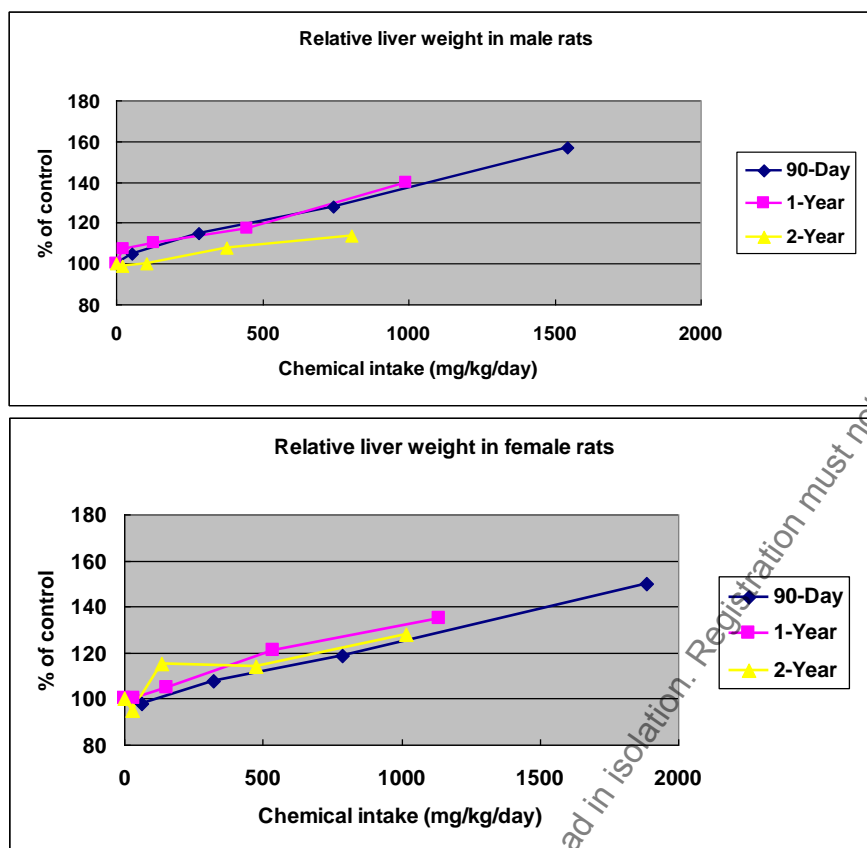


Figure 4. Relative liver weight changes in rats treated with S-2200TG for 90 days, 1 year, and 2 years. Data represent % of control values at each dose levels; 0, 800, 4000, 10000, and 20000 ppm for the 90-Day study; 0, 400, 2000, 7000, and 15000 ppm for the 1- and 2-Year studies.

B. Mouse

As shown in Table 6, S-2200TG also increased mouse liver weight in the 90-day, 1-year (satellite group in the carcinogenicity study), and 1.5-year studies, but without any associated liver pathological findings including hepatocellular hypertrophy and changes of blood biochemistry. S-2200TG is also a CYP2B inducer in mouse liver (Yamada, 2012). Therefore, these findings suggest that the increased liver weight by S-2200TG in mice is an adaptive response, but not adverse effect.

No apparent abnormality compared to control was observed in mouse thyroid.

No increase of tumours in any organs, including liver and thyroid, was observed in mice treated with S-2200TG at the limited dose level for 1.5 year.

Table 6. Summary of liver findings in mice

	Dose levels (ppm)											
	Male						Female					
	0	700	1750	2000	3500	7000	0	700	1750	2000	3500	7000
90-Day Study												
Chemical Intake (mg/kg/day)	0	na	204	na	405	807	0	na	252	na	529	1110
Final body weight	100	na	101	na	98	102	100	na	98	na	96	94
Absolute liver weight	100	na	105	na	107	117**	100	na	110	na	104	114
Relative liver weight	100	na	104	na	109**	115**	100	na	112*	na	108	122**
1-Year Study												
Chemical Intake (mg/kg/day)	0	88.4	na	255	na	883	0	104	na	325	na	1050
Final body weight	100	99	na	95	na	95	100	100	na	102	na	89
Absolute liver weight	100	105	na	103	na	111	100	103	na	110	na	113
Relative liver weight	100	106	na	108	na	115**	100	103	na	108	na	125**
1.5-Year Study												
Chemical Intake (mg/kg/day)	na	82.5	na	239	na	824	na	99.2	na	280	na	994
Final body weight	100	99	na	102	na	98	100	102	na	105	na	97
Absolute liver weight	100	106	na	106	na	115	100	114	na	120	na	109
Relative liver weight	100	106	na	105	na	115**	100	105	na	112	na	111
Hepatocellular adenoma #	7/51	4/51	na	10/51	na	6/51	0/51	0/51	na	0/51	na	0/51
Hepatocellular carcinoma #	0/51	2/51	na	1/51	na	0/51	0/51	0/51	na	0/51	na	0/51

Data presents relative values when control value is shown as 100. #: Data is scored as number of animals exhibiting findings out of total number of animals examined. *: p<0.05, **: p<0.01. Shadow presents statistically significant change but toxicologically not significant because no concomitant other histopathology and blood biochemistry. "na" means data not available.

C. Dog

In the dog 90-day study, S-2200TG also increased liver weight (in males, 12000 and 40000 ppm; and in females, 4000 ppm and above) (Table 7). Histopathologically, centrilobular hepatocellular swelling was present in males administered 12000 and 40000 ppm and in females administered 4000 and 12000 ppm. Females at 40000 ppm did not show this finding. Reason for lack of this finding is unknown, but severe suppression of body weight may be related. [Notifier considers that "centrilobular hepatocellular swelling" means "centrilobular hepatocellular hypertrophy" because "centrilobular hepatocellular hypertrophy" was recorded in the 1-year dog study.] We do not have direct evidence for hepatic enzyme induction by S-2200TG in dog liver. However, we know that S-2200TG induces at least CYP2B accompanied with increased liver weight and hepatocellular hypertrophy in rat and mouse liver. These finding suggests that S-2200TG is also likely to induce hepatic enzyme in dogs.

Some additional associated changes in blood biochemistry and other pathological findings were observed at 12000 ppm and higher in both sexes. The alterations of blood biochemistry were increases in aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma glutamyltransferase, triglyceride, and globulin; and decreases in total cholesterol, albumin, albumin/globulin ratios and glucose. In addition, other abnormalities were also observed; pigment and centrilobular degeneration, periportal/centrilobular fibrosis, etc. However, no additional findings were observed in females at 4000 ppm. Therefore, the increased liver weight and hepatocellular swelling (hypertrophy) at 4000 ppm in females are not adverse but adaptive responses. Based on these findings, the LOAEL in the liver in the 90-day dog study was determined to be 12000 ppm in both sexes.

As shown in Table 8, in the dog 1-year study, S-2200TG also increased liver weight (males, 4000 and 8000 ppm; and females, 8000 ppm). S-2200TG-related disturbances in blood biochemistry parameters includes increases in aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and gamma glutamyltransferase (males, 4000 and/or 8000 ppm; females, 8000 ppm), and decreases in albumin and total cholesterol (females, 8000 ppm). Histopathologically, liver hepatocyte hypertrophy was recorded for males and females administered

4000 and 8000 ppm. Increased levels of hepatocyte pigment (males, 4000 and 8000 ppm; and females, 8000 ppm) together with a marginal increase in pigmented macrophages (both sexes, 8000 ppm), centrilobular degeneration (one male and one female administered 8000 ppm), and portal fibrosis/bile duct proliferation (one male administered 8000 ppm) were observed. Overall, since S-2200TG induced liver hypertrophy with concomitant biochemical changes and other histopathological findings, the LOAEL in the liver in the dog 1-year study was determined to be 4000 ppm in males and 8000 ppm in females.

No apparent abnormality was observed in dog thyroid.

WARNING: This document forms part of an EC evaluation data package and should not be read in isolation. Registration must not be granted on the basis of this document.

Table 7. Summary of liver findings in the dog 90-day study

Endpoints	Examination (Week)	Dose levels (ppm)							
		Male				Female			
		0	4000	12000	40000	0	4000	12000	40000
Chemical intake (mg/kg/day)		0	90.9	267.8	933.1	0	102.7	304.4	820.4
Final body weight		100	96	97	79*	100	96	96	73**
Absolute liver weight		100	102	115	114	100	124	124	106
Relative liver weight		100	106	120	146**	100	131**	131**	148**
Histopathology, Liver:									
Centrilobular hepatocellular swelling #, \$		0/4	0/4	3/4	1/4	0/4	3/4	3/4	0
Pigment #		0/4	0/4	3/4	4/4*	0/4	0/4	3/4	4*
Periportal / centrilobular fibrosis #		0/4	0/4	0/4	4/4*	0/4	0/4	0	1/4
Centrilobular degeneration #		0/4	0/4	2/4	3/4	0/4	0/4	4/4*	4/4*
Aspartate aminotransferase	4	100	52	63	130	100	97	131	207*
	8	100	119	135	208**	100	100	128	207**
	13	100	116	113	156**	100	103	130	157*
Alanine aminotransferase	4	100	74	271	743	100	97	354	917*
	8	100	82	306	679**	100	95	341	722*
	13	100	81	278	581**	100	119	327	535*
Alkaline phosphatase	4	100	93	144	328**	100	94	150	243*
	8	100	102	163*	332**	100	109	189*	308**
	13	100	107	174*	294**	100	134	229**	349**
γ-Glutamyltranspeptidase	4	2	2	2	6*	100	100	100	200
	8	2	2	2	6	100	100	150	350
	13	4	3	3	10*	100	133	167	267*
Triglyceride	4	100	108	140	168	100	91	98	221*
	8	100	103	110	156**	100	100	110	151*
	13	100	126	124	182**	100	103	111	176*
Albumin	4	100	100	100	85**	100	97	92	92
	8	100	92	94	78**	100	100	97	86*
	13	100	89	94	75**	100	100	95	78**
Globulin	13	100	110	100	145**	100	113	113	138
Albumin/globulin ratios	4	100	93	93	71**	100	88	88	88*
	8	100	94	100	61**	100	86	91	68*
	13	100	89	94	56**	100	83	83	58**
Total cholesterol	4	100	97	97	63**	100	93	83	97
	8	100	88	86	49**	100	95	80	80
	13	100	82	84	48**	100	91	78	73*
Glucose	4	100	96	96	86**	100	96	92	85**
	8	100	106	102	89**	100	98	102	83**
	13	100	100	102	79**	100	104	104	88*

Data presents relative values when control value is shown as 100. #: Data is scored as number of animals exhibiting findings out of total number of animals examined. *: p<0.05, **: p<0.01. Shadow presents biologically significant change. "na" means data not available. \$: Notifier considers that "centrilobular hepatocellular swelling" means "centrilobular hepatocellular hypertrophy" because "centrilobular hepatocellular hypertrophy" was recorded in the 1-year dog study.

Table 8. Summary of liver findings in the dog 1-year study

Endpoints	Examination (Week)	Dose levels (ppm)									
		Male					Female				
		0	200	800	4000	8000	0	200	800	4000	8000
Chemical intake (mg/kg/day)		0	4.3	19.2	92.0	180.7	0	4.5	20.4	92.0	225.7
Final body weight		100	104	91	96	94	100	97	96	102	84
Absolute liver weight		100	97	94	111	112	100	102	102	104	106
Relative liver weight		100	93	103	113	120	100	103	106	103	127*
Histopathology, Liver:											
Centrilobular hepatocellular hypertrophy #		0/4	0/4	0/4	2/4	3/4*	0/4	0/4	0/4	1/4	4/4*
Pigment #		1/4	0/4	1	3/4	4/4*	1/4	1/4	0/4	1/4	4/4*
Pigmented macrophages #		1/4	0/4	0/4	0/4	2/4	1/4	0/4	0/4	1/4	2/4
Portal fibrosis/bile duct proliferation #		0/4	0/4	0/4	0/4	1/4	0/4	0/4	0/4	0/4	0/4
Centrilobular degeneration #		0/4	0/4	0/4	0/4	1/4	0/4	0/4	0/4	0/4	1/4
Aspartate aminotransferase	26	100	100	97	97	203*	100	115	108	88	108
Alanine aminotransferase	13	100	111	116	121	358	100	124	131	135	272
	26	100	114	114	102	269	100	116	148	100	184
	52	100	133	128	118	320	100	121	172	114	203
Alkaline phosphatase	13	100	108	163	190	283*	100	84	77	100	227*
	26	100	98	189	226	264*	100	105	88	104	264**
	52	100	105	178	273*	359**	100	116	96	125	336**
γ-Glutamyltranspeptidase	13	2	2	3	3	4	2	3	3	2	3
	26	3	5	3	5	3	2	2	4	3	4
	52	3	3	4	4	4	3	3	3	4	5
Albumin	26	100	97	94	97	92	100	97	92	95	87*
	52	100	100	94	106	94	100	95	95	92	87**
Total cholesterol	52	100	85	95	105	92	100	81	73*	79	72*

Data presents relative values when control value is shown as 100. #: Data is scored as number of animals exhibiting findings out of total number of animals examined. *: p<0.05, **: p<0.01. Shadow presents biologically significant change. "na" means data not available.

V-4. Assessment for toxicological relevance of thyroid hypertrophy observed in general toxicity and/or carcinogenicity studies with S-2200TG

Thyroid abnormality may relate to the endocrine disrupting issue. Since the perturbation of homeostasis of the hypothalamus-pituitary-thyroid axis by an extrathyroidal mechanism appears not to be relevant to humans as discussed later, therefore, determination of MOA for possible induction of thyroid follicular-cell hypertrophy by S-2200TG in rat is important for assessment of its toxicological relevance to humans. Structured frameworks are extremely useful in promoting transparent, harmonized approaches to the risk assessment of chemicals. One area where this has been particularly successful is in the analysis of MOAs for chemical carcinogens and other forms of toxicity in experimental animals and their relevance to humans. The International Programme on Chemical Safety (IPCS) recently published an updated version of its MOA framework in animals to address human relevance (cancer human relevance framework, or HRF)(Boobis et al., 2006). This work has now been extended to noncancer effects, with the eventual objective of harmonizing framework approaches to both cancer and noncancer endpoints (Boobis et al., 2008; Carmichael et

al., 2011). Using this framework, we evaluate potential hazard of S-2200TG to humans based on MOA on thyroid.

A. Postulated mode of action for the induction of thyroid follicular-cell hypertrophy in rat

The postulated MOA for possible induction of thyroid follicular-cell hypertrophy by S-2200TG involves the perturbation of homeostasis of the hypothalamus-pituitary-thyroid axis by an extrathyroidal mechanism. Specifically, S-2200TG induces hepatic T4-UGT activity, leading to enhanced catabolism of T4 by conjugation and increased biliary excretion of the conjugated hormone. The result of this enhanced liver metabolism is a decrease in serum T4 (and sometimes T3) half-life. The pituitary gland responds to a decrease in circulating serum levels of T4 by enhancing the output and serum level of TSH. Prolonged elevation of circulating TSH levels stimulates the thyroid gland to deplete its stores of thyroid hormone and continues to induce hormone production. Thus, the thyroid follicular-cells enlarge (hypertrophy). This MOA is often associated with follicular cells proliferating at an increased rate and to increase in number (hyperplasia), and thyroid hyperplasia eventually progresses to neoplasia with chronic exposure. However, chronic exposure with S-2200TG did not induce thyroid hyperplasia or neoplasia.

B. Key events in experimental animals

The sequence of key events in the MOA for effects of S-2200TG on thyroid includes: induction of hepatic UGT activity, increase in hepatic metabolism and biliary excretion of T4, decrease in serum T4 half-life and concentration, increase in circulating TSH concentration, and thyroid follicular-cell hypertrophy.

To determine whether S-2200TG works via disruption of thyroid-pituitary status by increasing hepatic clearance of circulating thyroid hormone, several parameters were evaluated in rats in a short term study (Tables 1 and 2).

i. Enzyme induction

UGT isoform induction has been shown to be associated with thyroid hypertrophy and tumour formation in rodents for phenobarbital and related compounds (Finch et al., 2006; Hiasa, et al., 1982; McClain, et al., 1988; Whysner, et al., 1996) and involves activation of nuclear receptors, particularly the CAR (Qatanani and Moore, 2005; Qatanani, et al., 2005). Hepatic CYP2B and UGT were both induced by phenobarbital *via* CAR (Deguchi, et al., 2009; Holsapple, et al., 2006; Qatanani and Moore, 2005; Qatanani, et al., 2005; Yamamoto, et al., 2004). Increases of hepatic T4-UGT activity were observed in male rat treated with 400 ppm and higher and in female rats treated with 7000 and higher of S-2200TG for 7 days (Tables 1 and 2, Figure 1).

Additional evidence that microsomal enzyme induction is occurring at biologically relevant levels is supported by the observation that there was hepatocellular hypertrophy and proliferation of SER (Tables 1 and 2, Figure 1). These are characteristic changes of microsomal enzyme inducers, such as phenobarbital and related compounds. Thus, there is both direct and indirect evidence for microsomal enzyme induction by S-2200TG administration with biologically important consequences.

ii. Circulating levels of thyroid hormone and TSH

Induction of hepatic T4-UGT activity results in increase in hepatic metabolism and biliary excretion of T4, and consequently decreases in serum T4 half-life and concentration. While hepatic metabolism and biliary excretion of T4 were not determined, reduced serum levels of T4 and T3 and increase of serum TSH level were observed in S-2200TG-treated rats (Tables 1 and 2, Figure 2).

iii. Thyroid morphology

Increased thyroid follicular-cell hypertrophy was observed at 7000 ppm and higher in females after 7- and 14-day treatment although the findings were not clearly increased but were observed in some treated male animals (Tables 1 and 2). Increased thyroid follicular-cell hypertrophy was also observed at longer treatments such as in the 90-day study (4000 ppm and higher), in the 1-year study (7000 ppm and higher), and the 2-year study (15000 ppm). Thus, there is direct and strong evidence to support the key event of increased thyroid follicular-cell hypertrophy by S-2200TG. Especially, time course alteration of the thyroid morphology is similar to the T4-UGT inducer, PB. In contrast to PB, however, no increased incidence of thyroid follicular-cell hyperplasia or tumours was observed in rats treated with S-2200TG even at doses of the MTD (15000 ppm) for 2 years. This is most likely related to the lower potency of S-2200TG compared to PB.

Thus, based on the key events listed, biological indicators of MOA by S-2200TG should include changes in liver metabolism, alterations in hormone levels, increases in thyroid growth, and follicular-cell hypertrophy in the thyroid.

C. Dose-response relationships

To evaluate the dose-response relationship (and time-course of the response) in rats, the data for males and females treated with S-2200TG are re-summarized in Tables 9 and 10, respectively.

i. Enzyme induction

In the MOA study (Asano, 2012), after 7-day treatment, the increased T4-UGT activity was observed in a dose related manner; statistically significant increase at 400 ppm and higher in males, and slight increase at 7000 ppm and significant increase at 15000 ppm in females,.

ii. Circulating levels of thyroid hormone and TSH

For male, decreased serum levels of T4 were observed in a dose related manner; statistically significant decrease was observed at 15000 ppm after 7- and 14-day treatment. No apparent alteration was observed in T3 and TSH after 7-day treatment. However, after 14-day treatment, 15000 ppm of S-2200TG revealed statistically significant increase of TSH accompanied with significant decrease of T4.

For females, decreased serum T4 and increased serum TSH were observed in a dose related manner; statistically significant change was not observed on T4 but observed on TSH at 15000 ppm. However, after 14-day treatment, 15000 ppm of S-2200TG significantly decreased T4 and significantly increased TSH.

iii. Thyroid morphology

For males, a small number of animals (3-5 out of 10) revealed thyroid follicular-cell hypertrophy after 7- or 14-day treatment at 15000 ppm but the incidence was not statistically significant from control (1-2 out of 10). However, after 90-day treatment, the finding was increased at 4000 ppm (6 out of 12, without statistical significance), 10000 ppm (9 out of 12, with statistical significance), and 20000 ppm (7 out of 12, without statistical significance). After longer treatment, significant increase was observed at 7000 and 15000 ppm after 1-year treatment and at 15000 ppm after 2-year treatment. Overall, the Lowest Observable Effect Level (LOEL) on thyroid follicular-cell hypertrophy was considered to be 4000 ppm in males.

For females, thyroid follicular-cell hypertrophy was significantly increased at 7000 ppm and higher after 7-day treatment. Small number of animals (4-6 out of 11-12) revealed thyroid follicular-cell hypertrophy at 7000 ppm and higher but the incidence was not statistically significant from control incidence (2 out of 12) after 90-day treatment. Regarding thyroid follicular-cell hypertrophy observed in small number of animals (4 out of 11) at 4000 ppm, it is difficult to consider it as biologically relevant because liver hypertrophy (increased liver weight and

hepatocellular hypertrophy) at 4000 ppm was much less potent than those at 10000 ppm. After longer treatment, a significant increase was observed at 7000 and 15000 ppm after 1-year treatment, and no significant increase was observed after 2-year treatment (only 4 of 50 animals had the finding at 15000 ppm). Overall, the LOEL on thyroid follicular-cell hypertrophy was considered to be 7000 ppm in females.

The effects on liver enzymes/weight and pituitary–thyroid hormone concentrations would be anticipated to occur at doses at least as low as those that produce thyroid weight changes and increases in thyroid follicular-cell hypertrophy incidence, given that this thyroid disruption MOA is a threshold phenomenon (Dellarco et al., 2006). Although we should consider phase of treatment as discussed in “*Temporal association*” Section, the various parameters described above that are related to the key events in the development of thyroid follicular-cell hypertrophy were generally observed at or below the dose levels producing hypertrophy (Tables 9 and 10). In addition, as discussed later, the alterations of the biochemical key events preceded to the morphological alterations, as evidenced by early phase of treatment in males.

Taken together, there are strong parallels in the dose response for the key events and thyroid follicular-cell hypertrophy. Again, it is noteworthy that the degree of change observed in each of the parameters was relatively mild, consistent with no increase of hypertrophy incidences. However, the increased incidence of thyroid follicular-cell hyperplasia and neoplasia was not observed in rats treated with S-2200TG at 15000 ppm for 2 years.

Table 9. Dose-response relationship of alteration on thyroid and liver of male rats treated with S-2200TG

Treatment period	Dose levels (ppm)									
	0	400	800	2000	4000	7000	10000	15000	20000	PB
Hepatocellular hypertrophy (number of animals with finding /number of animals examined)										
7 days	0/10	0/10	na	1/10	na	8/10**	na	9/10**	na	10/10**
14 days	2/10	na	na	na	na	na	na	10/10**	na	10/10**
90 days	0/12	na	0/12	na	12/12	na	12/12	na	12/12	na
1 year	0/19	0/19	na	0/19	na	15/20**	na	20/20**	na	na
2 years	7/50	11/50	na	13/50	na	30/50**	na	37/50**	na	na
Relative liver weight (g/100gBW)										
7 days	3.65±0.22	3.61±0.177	na	3.77±0.214	na	4.03±0.215**	na	4.53±0.342**	na	4.61±0.278**
	100	99	na	103	na	110	na	124	na	124
14 days	3.47±0.223	na	na	na	na	na	na	4.52±0.232**	na	4.68±0.201**
	100	na	na	na	na	na	na	130	na	135
90 days	2.262±0.0711	na	2.368±0.1446	na	2.607±0.0993**	na	2.885±0.1368**	na	3.541±0.2167**	na
	100	na	105	na	115	na	128	na	157	na
1 year	2.014±0.1662	2.157±0.1906	na	2.218±0.2171*	na	2.350±0.159**	na	2.816±0.3355**	na	na
	100	107	na	110	na	117	na	140	na	na
2 years	2.051±0.1704	2.029±0.187	na	2.042±0.2167	na	2.215±0.2068	na	2.334±0.2452	na	na
	100	99	na	100	na	108	na	114	na	na
Hepatic T4 UGT induction (pmol/min/mg S9 protein)										
7 days	0.41±0.054	0.51±0.024*	na	0.54±0.075	na	0.62±0.051*	na	0.81±0.046*	na	0.79±0.122*
	100	124	na	132	na	151	na	149	na	193
Serum T3 levels (ng/mL)										
7 days	0.5±0.07	0.5±0.1	na	0.5±0.08	na	0.4±0.08	na	0.5±0.1	na	0.5±0.11
	100	100	na	100	na	80	na	100	na	100
14 days	0.5±0.11	na	na	na	na	na	na	0.4±0.1	na	0.5±0.08
	100	na	na	na	na	na	na	80	na	100
Serum T4 levels (µg/dL)										
7 days	5.04±0.722	5.35±0.848	na	5.36±0.71	na	4.56±0.897	na	4.09±0.869*	na	4.45±1.305
	100	106	na	106	na	90	na	81	na	88
14 days	5.7±0.744	na	na	na	na	na	na	4.08±0.978**	na	4.03±0.872**
	100	na	na	na	na	na	na	72	na	71
Serum TSH levels (ng/mL)										
7 days	8.6±2.76	7.3±2.33	na	7.5±2.5	na	6.7±2.12	na	7.5±2.96	na	10.6±3.42
	100	85	na	87	na	78	na	87	na	123
14 days	8.0±3.75	na	na	na	na	na	na	11.7±3.6*	na	12.7±3.9*
	100	na	na	na	na	na	na	146	na	159
Relative thyroid weight (mg/100g BW)										
7 days	5.2±0.74	5.6±0.98	na	5.8±1.58	na	5.4±0.88	na	6.2±0.92	na	6.2±1.43
	100	108	na	112	na	104	na	119	na	119
14 days	6.4±0.83	na	na	na	na	na	na	7.5±1.23*	na	8.1±0.9**
	100	na	na	na	na	na	na	117	na	127
90 days	na	na	na	na	na	na	na	na	na	na
	na	na	na	na	na	na	na	na	na	na
1 year	na	na	na	na	na	na	na	na	na	na
	na	na	na	na	na	na	na	na	na	na
2 years	na	na	na	na	na	na	na	na	na	na
	na	na	na	na	na	na	na	na	na	na
Thyroid follicular-cell hypertrophy; diffuse (number of animals with finding /number of animals examined)										
7 days	1/10	1/10	na	2/10	na	2/10	na	3/10	na	5/10
14 days	2/10	na	na	na	na	na	na	5/10	na	6/10
90 days	2/12	na	2/12	na	6/12	na	9/12*	na	7/12	na
1 year	1/19	0/19	na	1/19	na	9/20**	na	18/20**	na	na
2 years	1/50	2/50	na	0/50	na	0/50	na	11/50**	na	na
Thyroid follicular-cell adenoma (number of animals with finding /number of animals examined)										
2 years	7/50	4/50	na	7/50	na	7/50	na	7/50	na	na
Thyroid follicular-cell carcinoma (number of animals with finding /number of animals examined)										
2 years	0/50	0/50	na	2/50	na	1/50	na	0/50	na	na

Data presents mean ± SD and relative values when control value is shown as 100. *: p<0.05, **: p<0.01. Shadow presents biologically significant change. “na” means data is not available.

Table 10. Dose-response relationship of alteration on thyroid and liver of female rats treated with S-2200TG

Treatment period	Dose levels (ppm)									
	0	400	800	2000	4000	7000	10000	15000	20000	PB
Hepatocellular hypertrophy (number of animals with finding /number of animals examined)										
7 days	1/10	1/10	na	5/10	na	6/10*	na	8/10**	na	10/10**
14 days	0/10	na	na	na	na	na	na	5/10*	na	10/10**
90 days	0/12	na	0/12	na	4/11*	na	9/12**	na	12/12**	na
1 year	0/20	1/20	na	1/19	na	17/19**	na	15/20**	na	na
2 years	16/50	22/50	na	32/50**	na	43/50**	na	42/50**	na	na
Relative liver weight (g/100gBW)										
7 days	3.44±0.125	3.41±0.241	na	3.63±0.12*	na	3.79±0.095**	na	4.17±0.118**	na	4.07±0.232**
	100	99	na	106	na	110	na	121	na	118
14 days	3.24±0.197	na	na	na	na	na	na	4.14±0.187**	na	4.12±0.304**
	100	na	na	na	na	na	na	128	na	127
90 days	2.550±0.2289	na	2.510±0.1422	na	2.760±0.1770	na	3.047±0.2271**	na	3.81±0.2856**	na
	100	na	98	na	108	na	119	na	150	na
1 year	2.328±0.2131	2.335±0.2476	na	2.439±0.2162	na	2.826±0.4013**	na	3.150±0.3017**	na	na
	100	100	na	105	na	121	na	135	na	na
2 years	2.153±0.3228	2.038±0.2232	na	2.472±0.2223*	na	2.446±0.209*	na	2.753±0.1907*	na	na
	100	95	na	115	na	114	na	128	na	na
Hepatic T4 UGT induction (pmol/min/mg S9 protein)										
7 days	0.46±0.069	0.43±0.033	na	0.46±0.071	na	0.53±0.057	na	0.62±0.074**	na	0.56±0.06*
	100	93	na	100	na	115	na	135	na	122
Serum T3 levels (ng/mL)										
7 days	0.6±0.19	0.6±0.12	na	0.6±0.21	na	0.6±0.15	na	0.6±0.08	na	0.6±0.14
	100	100	na	100	na	100	na	100	na	100
14 days	0.7±0.15	na	na	na	na	na	na	0.5±0.88**	na	0.5±0.11**
	100	na	na	na	na	na	na	71	na	71
Serum T4 levels (µg/dL)										
7 days	4.7±1.412	4.7±1.124	na	4.52±1.331	na	4.34±1.24	na	3.95±1.311	na	3.17±0.641**
	100	100	na	96	na	92	na	84	na	67
14 days	4.68±1.152	na	na	na	na	na	na	3.5±0.718*	na	2.52±0.572**
	100	na	na	na	na	na	na	75	na	54
Serum TSH levels (ng/mL)										
7 days	6.1±1.71	6.3±2.13	na	6.7±2.14	na	9.1±4.99	na	15.1±6.22**	na	10.2±3.8**
	100	103	na	110	na	149	na	248	na	167
14 days	6.1±1.97	na	na	na	na	na	na	12.3±6.55*	na	10.6±4.48*
	100	na	na	na	na	na	na	202	na	174
Relative thyroid weight (mg/100g BW)										
7 days	6.5±1.38	6.9±1.09	na	6.7±1.17	na	8.6±1.12**	na	8.5±1.38**	na	7.9±1.04*
	100	106	na	103	na	132	na	131	na	122
14 days	6.7±1.01	na	na	na	na	na	na	8.9±1.05**	na	8.1±1**
	100	na	na	na	na	na	na	133	na	121
90 days	na	na	na	na	na	na	na	na	na	na
	na	na	na	na	na	na	na	na	na	na
1 year	na	na	na	na	na	na	na	na	na	na
	na	na	na	na	na	na	na	na	na	na
2 years	na	na	na	na	na	na	na	na	na	na
	na	na	na	na	na	na	na	na	na	na
Thyroid follicular-cell hypertrophy; diffuse (number of animals with finding /number of animals examined)										
7 days	0/10	1/10	na	1/10	na	4/10*	na	6/10**	na	3/10
14 days	0/10	na	na	na	na	na	na	5/10*	na	3/10
90 days	2/12	na	1/12	na	4/11	na	5/12	na	6/12	na
1 year	0/20	0/20	na	0/19	na	9/19**	na	15/20**	na	na
2 years	0/50	1/50	na	2/50	na	0/50	na	4/50	na	na
Thyroid follicular-cell adenoma (number of animals with finding /number of animals examined)										
2 years	2/50	2/50	na	2/50	na	5/50	na	1/50	na	na
Thyroid follicular-cell carcinoma (number of animals with finding /number of animals examined)										
2 years	0/50	0/50	na	0/50	na	1/50	na	0/50	na	na

Data presents mean ± SD and relative values when control value is shown as 100. *: p<0.05, **: p<0.01. Shadow presents biologically significant change. "na" means data is not available.

D. Temporal association

It is critical in the evaluation of the MOA that early key events occur before the appearance of thyroid follicular-cell hypertrophy, and this is clearly the case with S-2200TG. Multiple exposure time data at 7, 14, and 90 days, and 1 and 2 years are available in which Wistar rats were administered diets containing 15000 ppm (Tables 9 and 10). Liver effects, including weight and hepatocellular hypertrophy, were increased at all observation times from the earliest time of assessment on day 7. Hepatic T4-UGT activity was also increased at the earliest observation time examined (day 7). Biliary excretion of conjugated T4 was not measured in this experiment;

Generally, the follicular-cell hyperplasia and tumour occurred only after chronic administration of UDP inducers (Finch et al., 2006; Hurley et al., 1998; IARC, 2001; Whysner, 1996). But in the case of S-2200TG, hyperplastic and neoplastic changes were not observed even after 2-year treatment.

Strength, consistency, and specificity of the association can be established from the studies described earlier. The quantifiable precursor events, fundamental to the proposed MOA, are relatively consistent with the emergence of thyroid follicular-cell hypertrophy. Observation of liver weight increase and induction of hepatic T4-UGT in rats receiving S-2200TG in the diet would be consistent with perturbation of homeostasis of the pituitary–thyroid axis by an extrathyroidal mechanism. An increase in hepatic T4-UGT activity is a step occurring before the other key biochemical changes and before thyroid follicular-cell hypertrophy. S-2200TG treatment clearly results in a decrease in circulating T4 and an increase in TSH following enhanced liver metabolism of T4. Furthermore, the development of thyroid hypertrophy was shown to be increased under the same conditions of dose and time as the appearance of changes in thyroid hormone levels.

As mentioned earlier in this document, there is an excellent review of thyroid biology (Dellarco, et al., 2006; IARC, 2001). The published data are consistent with increased TSH concentrations alone causing thyroid follicular-cells of rats to enter a state of pre-hypertrophy. Therefore, the suggestion that high dose of S-2200TG causes thyroid follicular-cell hypertrophy in rats by initially inducing hepatic T4-UGT is coherent with the known physiology of the hypothalamus–pituitary–thyroid dynamic control system, at least to the stage of hypertrophy. The S-2200TG-induced key events summarized above are similar to those seen with PB (Dellarco, et al., 2006; Finch, et al., 2006), and we can therefore conclude that S-2200TG has a similar MOA to that of CAR activators such as PB for rat thyroid hypertrophy formation.

As described in the earlier discussion, it can be concluded that S-2200TG has no genotoxic potential, suggesting that the MOA for thyroid follicular-cell hypertrophy is non-genotoxic.

Additional effects on the hypothalamic–pituitary–thyroid axis or disruption of other pathways of thyroid hormone metabolism (ex., blockade of T4 synthesis, receptor blockade etc) are other possibilities of altering thyroid homeostasis. These variations would not differ in any fundamental way from the one that has been proposed for S-2200TG; all would lead to prolonged TSH stimulation with continuous exposure. Furthermore, we should recognize that S-2200TG did not induce direct cytotoxicity on the thyroid as evidenced by histopathology.

H. Uncertainties, inconsistencies, and data gaps

For the most part, the data are consistent with the proposed key events at several time points and at several doses, as noted in Tables 7 and 8. However, there are some apparent gaps and inconsistencies.

The increased thyroid weight is a key event in the MOA for thyroid hypertrophy induced by enhancement of thyroid hormone metabolizing enzyme (Dellarco, et al., 2006). However, the thyroid weight was not fully examined in all studies with S-2200TG; it was not examined in the rat 90-day and chronic/carcinogenicity studies. Data of thyroid weight is sometimes unreliable because of its small size. Thyroid histopathology tends to be a more reliable indicator than thyroid weight (DeVito et al., 1999; Hood, et al., 1999; Yamada et al., 2004). Therefore, we do not believe that this is an essential data gap because direct evidence of histopathological alterations of thyroid, such as hypertrophy, suggesting increased thyroid weight, was observed.

Although data for T4 biliary elimination is not collected, we believe that this is not essential because the direct evidence for reduced serum T4 and of increased hepatic T4-UGT by S-2200TG administration was obtained.

Lastly, compared to the typical rodent thyroid carcinogens (thyroid follicular-cell tumour inducer such as CAR activators, PB and pyrethrin) (Finch et al., 2006; IARC, 2001), a thyroid tumour response was not elicited by S-2200TG although S-2200TG has CAR activation evidenced by CYP2B induction and proliferation of SER in hepatocytes (Asano, 2012). Although CAR activators often induce liver tumour in rodents (IARC, 2001; Osimitz and Lake, 2009; Yamada, et al., 2009), S-2200TG did not induce liver tumours in rat or mouse. Therefore, these findings suggest that CAR activation by S-2200TG may attenuate at later phases of treatment or may lack unknown key event(s) which are necessary for cell transformation. Most likely, the low potency of S-2200TG compared to PB and that reported for other CAR activators is the basis for its mild effect on liver thyroid and the lack of tumorigenicity in either organ. While details are unknown, the lack of tumorigenicity decreases toxicological concern of the thyroid and liver hypertrophy caused by S-2200TG.

I. Assessment of postulated mode of action

As described above, the key events in the MOA for S-2200TG have been well documented, with a strong dose and temporal consistency. In addition, this is a well known MOA, and the various parameters essential for documenting this MOA have been presented for S-2200TG. Thus, we consider that the level of confidence in the postulated MOA is high.

J. Human applicability of the proposed mode of action

Human applicability of the proposed MOA for thyroid follicular-cell hypertrophy by S-2200TG is also evaluated by the IPCS Human Relevance Framework (Boobis, et al., 2008; Carmichael, et al., 2011).

1. *Is the weight of evidence sufficient to establish a mode of action in animals?*

As described in detail earlier, there is clear evidence that S-2200TG alters thyroid homeostasis by UDP induction causing reduced serum T4 (and maybe T3) levels and consequently elevating serum TSH.

2. *Can human relevance of the mode of action be reasonably excluded on the basis of fundamental, qualitative differences in key events between experimental animals and humans?*

The current understanding of the regulation of thyroid hormone homeostasis in humans and of the role of increased TSH levels (as a result of altered thyroid homeostasis) as a risk factor for

thyroid abnormality including cancer was considered in order to assess the human relevance of the key events in animal MOA by S-2200TG. Although there are substantial quantitative dynamic differences (discussed later), the fundamental mechanisms involved in the function and regulation of the hypothalamic–pituitary–thyroid axis in rats are qualitatively similar to those in humans (Bianco et al., 2002). UGT activation by CAR activators is also operated in human (Sugatani, et al., 2001). Therefore, an agent that decreases T4 levels in rats could likewise reduce T4 in humans; this, in turn, could potentially lead to an increase in TSH levels. There are data showing that rodents and humans respond in a similar fashion to perturbations of pituitary–thyroid function. For example, it is well known that iodine deficiency, which readily leads to decreased thyroid hormone levels, stimulates thyroid cell proliferation in humans, leading to goiter. If left untreated, iodine deficiency may lead to tumour formation, albeit rarely (Thomas and Williams, 1999). Although there is no evidence of increased susceptibility to thyroid cancer, a number of pharmaceuticals (e.g., propylthiouracil, lithium, amiodarone, iopanoic acid) that disrupt thyroid homeostasis by acting directly on the thyroid gland (for example, by inhibiting hormone synthesis or release or by blocking the conversion of T4 to T3) are known to lead to hypothyroidism and increases in TSH in humans (Ron et al., 1987).

In contrast to rats, no increases in TSH levels have been found in humans following exposure to agents that induce hepatic microsomal enzymes and reduce circulating T4 levels (Meek, et al., 2003). For example, the pharmaceutical compounds phenytoin, rifampin, and carbamazepine induce hepatic microsomal enzymes, including UGT, and reduce circulating T4 levels, but TSH levels are unchanged (Curran and DeGroot, 1991); agents that produce thyroid tumours in rats by increasing glucuronidation and biliary excretion of T4 at high experimental doses (e.g., omeprazole, lansoprazole, and pantoprazole) produce no changes in thyroid hormones at clinical doses in humans (Masubuchi et al., 1997). Thus, there appears to be a substantial difference in the dose-response relationship for altered homeostasis of the pituitary–thyroid axis in rats compared to humans. As discussed next, this observation is due to quantitative dynamic differences between rats and humans in the basic physiological processes underlying pituitary–thyroid function.

3. Can human relevance of the mode of action be reasonably excluded on the basis of quantitative differences in either kinetic or dynamic factors between experimental animals and humans?

The primary effect of S-2200TG is on hepatic metabolizing enzymes, and the increase in metabolic activity indirectly increases the systemic clearance of T4 (and maybe T3), leading to the hypothyroid state and the compensatory increase in TSH found in rats. Although there are no chemical-specific data on the potential for S-2200TG to disrupt thyroid hormone homeostasis in humans, a number of other microsomal enzyme inducers have been extensively studied, such as phenobarbital (Meek, et al., 2003). UGT activation by CAR activators is operated in human (Sugatani, et al., 2001). However, as discussed earlier, agents that produce hypothyroidism by altering hepatic clearance of T4 do not appear to result in elevated TSH levels in humans. Presumably, TSH is not increased because a critical reduction of T4 is not reached. There are several important physiological and biochemical differences between rats and humans related to thyroid function. Rats have a smaller reserve capacity of thyroid hormones when compared with humans. The rat has a much shorter thyroid hormone half-life than humans. The half life of T4 is about 12 h in rats, compared to 5–9 days in humans (Dohler et al., 1979). The shorter half-life in rats is likely related to the absence of a high-affinity binding globulin for T4 that is present in humans (Hill, et al., 1989). In rats, the increased clearance contributes to the need for a higher rate of production of T4 (per unit of body weight) to maintain normal levels of T4. In contrast, in humans, the binding of thyroid hormone to this globulin accounts for a slower metabolic degradation and clearance that in turn results in the thyroid gland being less active than in rats. The constitutive TSH levels are approximately 25 times higher in rats than in humans, reflecting the higher activity of the pituitary–thyroid axis in rats (Dohler, et al., 1979; McClain, 1992). Therefore,

humans are quantitatively less sensitive than rats to agents that reduce T4 and lead to elevated TSH. There is no increased risk of thyroid tumour development if TSH is not elevated.

Another difference of rats compared to humans is the histological appearance of the thyroid. This histological difference is related to the higher rate of production of T4 to maintain a consistent serum concentration, thus making the rat thyroid more “functionally active” than primates including humans (McClain, 1995). More of the follicular epithelium in the rat is stimulated to synthesize thyroglobulin, and therefore more of the follicular-cells are tall cuboidal and appear to be active in synthesis. In contrast, more of the follicular-cells in humans tend to be short cuboidal or almost squamous in appearance, suggesting they are quiescent. Because rat follicular-cells are already generally active, under stimulation from TSH, they will respond with hyperplasia more readily than human follicular-cells. Because of the greater storage capability of the human thyroid and the greater numbers of cells in a quiescent state, human thyroid follicular-cells will be roused from their quiescent state to synthesize and secrete additional thyroid hormone without the need for a hyperplastic response to reestablish homeostasis. Therefore, the primary response in the human thyroid gland would be thyroglobulin reabsorption and cellular hypertrophy rather than hyperplasia. In short, there is much greater buffering capacity in the biochemistry of the human than the rat thyroid.

Even though certain agents can cause a reduction in thyroid hormone levels in humans, there is no clear evidence that these agents increase susceptibility to thyroid cancer (Ron, et al., 1987). For example, epidemiologic studies with phenobarbital do not show any increased risk of thyroid cancer (Friedman et al., 2009; Olsen, et al., 1989; Olsen et al., 1993). Studies of individuals with conditions that would lead to elevated TSH (patients with Graves’ disease or goiter) indicate the occurrence of thyroid cancer is rare in these circumstances (Gabriele et al., 2003; Mazzaferri, 2000). A study of environmental and heritable causes of cancer among 9.6 million individuals, using the Nationwide Swedish Family Cancer Database, found that the environment did not appear to play a principal causative role in thyroid cancer (Czene et al., 2002). The only known human thyroid carcinogen is radiation, a mutagenic exposure. As summarized in Table 11, there is sufficient evidence in the general literature on the biochemical and physiological differences in thyroid function to indicate differences in toxicity susceptibility including tumour induction between rats and humans. In contrast to humans, rats are very susceptible to thyroid abnormality secondary to hypothyroidism. In particular, modest changes in thyroid hormone homeostasis will induce hypertrophy and promote tumour formation in rats. Thus, thyroid follicular-cell hypertrophy observed in rats treated with 7000 ppm of S-2200TG involving increased hepatic clearance of hormone and altered homeostasis of the pituitary–thyroid axis in rodents are considered not relevant to humans, based on quantitative dynamic differences. Even if S-2200TG induce thyroid follicular-cell hypertrophy in human, no tumour is developed in human.

Table 11. A comparison of key events by S-2200TG in rats and human

Key event	Evidence in rats	Evidence in humans
Increase hepatic clearance of T4.	In short-term and chronic rat studies, the liver is found to be the most sensitive target, and evidence of increased T4 hepatic clearance is provided by studies on T4-hepatic UGT activity, and liver weights and hepatocellular hypertrophy.	No data available for S-2200TG, but microsomal enzyme induction is plausible.
Decreased serum T4.	Direct experimental evidence.	No data available for S-2200TG, but plausible given that other microsomal enzyme inducers have been shown to reduce T4 in humans.

Key event	Evidence in rats	Evidence in humans
Increased TSH levels.	Direct experimental evidence.	No data available for S-2200TG, but other microsomal enzyme inducers have not been shown to increase TSH levels even when T4 is decreased.
Increased TSH increases thyroid cell hypertrophy formation.	Direct experimental evidence.	Induction of thyroid follicular-cell hypertrophy secondary to hypothyroidism is remote in humans, given the quantitative differences in thyroid function/homeostasis.
Increased TSH increases thyroid cell proliferation (hyperplasia) and tumour formation.	Direct experimental evidence showing no thyroid hyperplasia and tumour development.	Induction of thyroid follicular-cell tumours secondary to hypothyroidism is remote in humans, given the quantitative differences in thyroid function/homeostasis. Occurrence of thyroid cancer is rare even in severely hypothyroid individuals.

K. Conclusion: statement of confidence, analysis, and implications

The data available for S-2200TG are considerable, and despite some data gaps such as the lack of thyroid weight data and direct data regarding T4 biliary elimination, it is clear that the MOA for S-2200TG-induced rat thyroid follicular-cell hypertrophy is secondary to enhanced metabolism of T4 (and maybe T3) leading to hormone imbalance. Although the possibility that S-2200TG may potentially result in hypothyroidism in humans cannot be ruled out, there is sufficient quantitative evidence on the basic physiological processes in the general literature to conclude that thyroid abnormality including tumours induced by a process involving increased hepatic clearance of thyroid hormone and altered homeostasis of the hypothalamus–pituitary–thyroid axis in rodents is not likely to lead to an increase in susceptibility to thyroid abnormality development (including tumour) in humans. Based on the above evidence, it is reasonable to conclude that S-2200TG will not have any hazard on thyroid in humans.

VI. Conclusions

In this assessment, we firstly conclude that S-2200TG is a hepatic enzyme inducer via at least CAR activation in rat, similar to PB, evidenced by CYP2B and UGT induction and SER proliferation. Therefore, the liver hypertrophy (i.e., increased liver weight and/or hepatocellular hypertrophy) caused by S-2200TG is judged to be an adaptive response by enzyme induction at least via CAR and not adverse. This activity also appears to be plausible in mouse and dog. Furthermore, this activity would theoretically operate in humans, as demonstrated by CYP2B inducers. S-2200TG at much higher dose levels induced adverse effects on the liver as some additional functional changes or additional pathological findings to the hypertrophy were observed. However, the adverse effects occurred in a dose related manner and there was a threshold at relatively high exposure levels; and most importantly, S-2200TG did not induce liver tumours in rat and mouse which further reduces concern in human risk assessment.

Secondly, we obtained data indicating that S-2200TG increased T4-UGT and indirectly perturbed the hypothalamus-pituitary-thyroid hormone axis, and then increased thyroid follicular-cell hypertrophy in rats, which is also similar to PB, a CAR activator. The postulated MOA for possible induction of thyroid follicular-cell hypertrophy in rats was tested against the modified Bradford Hill criteria, and was found to satisfy the conditions of dose and temporal concordance, biological plausibility, coherence, strength, consistency, and specificity that fit with a well-established MOA for thyroid follicular-cell hypertrophy. Although the postulated MOA could

theoretically qualitatively operate in humans, marked quantitative differences in the inherent susceptibility for thyroid abnormality, especially tumour induction, to thyroid hormone imbalance in rats.

Therefore, even through liver and/or thyroid hypertrophy were induced by S-2200TG treatment in experimental animals, the findings from MOA analysis allow for the conclusion that S-2200TG does not pose a hazard to humans.

VII. References

- Asano, K. (2012). Short-term study for mode of action analysis for rat liver and thyroid findings by S-2200TG-Dose response, time-course, and reversibility-. Sumitomo Chemical Company Ltd., Study No. S1560, Technical report ROT-0067, Unpublished data.
- Bayer, I., Mitmaker, B., Gordon, P. H. and Wang, E. (1992). Modulation of nuclear statin expression in rat thyroid follicle cell following administration of thyroid stimulating hormone. *J. Cell. Physiol.* **150**, 276-282.
- Bianco, A. C., Salvatore, D., Gereben, B., Berry, M. J. and Larsen, P. R. (2002). Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. *Endocr. Rev.* **23**, 38-89.
- Boobis, A. R., Cohen, S. M., Dellarco, V., McGregor, D., Meek, M. E., Vickers, C., Willcocks, D. and Farland, W. (2006). IPCS framework for analyzing the relevance of a cancer mode of action for humans. *Crit. Rev. Toxicol.* **36**, 781-792.
- Boobis, A. R., Doe, J. E., Heinrich-Hirsch, B., Meek, M. E., Munn, S., Ruchirawat, M., Schlatter, J., Seed, J. and Vickers, C. (2008). IPCS framework for analyzing the relevance of a noncancer mode of action for humans. *Crit. Rev. Toxicol.* **38**, 87-96.
- Capen, C. C. (1997). Mechanistic data and risk assessment of selected toxic end points of the thyroid gland. *Toxicol. Pathol.* **25**, 39-48.
- Carmichael, N., Bausen, M., Boobis, A. R., Cohen, S. M., Embry, M., Fruijtier-Polloth, C., Greim, H., Lewis, R., Meek, M. E., Mellor, H., Vickers, C. and Doe, J. (2011). Using mode of action information to improve regulatory decision-making: An ECETOC/ILSI RF/HESI workshop overview. *Crit. Rev. Toxicol.* **41**, 175-186.
- Cohen, S. M. and Arnold, L. L. (2011). Chemical Carcinogenesis. *Toxicol. Sci.* **120**, S76-S92.
- Curran, P. G. and DeGroot, L. J. (1991). The effect of hepatic enzyme-inducing drugs on thyroid hormones and the thyroid gland. *Endocr. Rev.* **12**, 135-150.
- Czene, K., Lichtenstein, P. and Hemminki, K. (2002). Environmental and heritable causes of cancer among 9.6 million individuals in the swedish family-cancer database. *Int. J. Cancer* **99**, 260-266.
- Deguchi, Y., Yamada, T., Hirose, Y., Nagahori, H., Kushida, M., Sumida, K., Sukata, T., Tomigahara, Y., Nishioka, K., Uwagawa, S., Kawamura, S. and Okuno, Y. (2009). Mode of action analysis for the synthetic pyrethroid metofluthrin-induced rat liver tumours: Evidence for hepatic CYP2B induction and hepatocyte proliferation. *Toxicol. Sci.* **108**, 69-80.
- Dellarco, V. L., McGregor, D., Berry, S. C., Cohen, S. M. and Boobis, A. R. (2006). Thiazopyr and

Masubuchi, N., Hakusui, H. and Okazaki, O. (1997). Effects of proton pump inhibitors on thyroid hormone metabolism in rats : A comparison of UDP-glucuronyltransferase induction. *Biochem. Pharmacol.* **54**, 1225-1231.

Mazzaferri, E. L. (2000). Thyroid cancer and Graves' disease: The controversy ten years later. *Endocr Pract* **6**, 221-225.

McClain, R. M. (1992). Thyroid gland neoplasia: Non-genotoxic mechanisms. *Toxicol. Lett.* **64-65** Spec No, 397-408.

McClain, R. M. (1995). Mechanistic considerations for the relevance of animal data on thyroid neoplasia to human risk assessment. *Mutat. Res.* **333**, 131-142.

McClain, R. M., Posch, R. C., Bosakowski, T. and Armstrong, J. M. (1988). Studies on the mode of action for thyroid gland tumour promotion in rats by phenobarbital. *Toxicol. Appl. Pharmacol.* **94**, 254-265.

Meek, M. E., Bucher, J. R., Cohen, S. M., Dellarco, V., Hill, R. N., Lehman-McKeeman, L. D., Longfellow, D. G., Pastoor, T., Seed, J. and Patton, D. E. (2003). A framework for human relevance analysis of information on carcinogenic modes of action. *Crit. Rev. Toxicol.* **33**, 591-653.

Olsen, J. H., Boice, J. D., Jr., Jensen, J. P. and Fraumeni, J. F., Jr. (1989). Cancer among epileptic patients exposed to anticonvulsant drugs. *J. Natl. Cancer Inst.* **81**, 803-808.

Olsen, J. H., Wallin, H., Boice, J. D., Jr., Rask, K., Schulgen, G. and Fraumeni, J. F., Jr. (1993). Phenobarbital, drug metabolism, and human cancer. *Cancer Epidemiol. Biomarkers Prev.* **2**, 449-452.

Osimitz, T. G. and Lake, B. G. (2009). Mode-of-action analysis for induction of rat liver tumours by pyrethrins: Relevance to human cancer risk. *Crit. Rev. Toxicol.* **39**, 501-511.

Qatanani, M. and Moore D. D. (2005). CAR, the continuously advancing receptor, in drug metabolism and disease. *Curr Drug Metab* **6**, 329-339.

Qatanani, M., Zhang, J. and Moore, D. D. (2005). Role of the constitutive androstane receptor in xenobiotic-induced thyroid hormone metabolism. *Endocrinology* **146**, 995-1002.

Ron, E., Kleinerman, R. A., Boice, J. D., Jr., LiVolsi, V. A., Flannery, J. T. and Fraumeni, J. F., Jr. (1987). A population-based case-control study of thyroid cancer. *J. Natl. Cancer Inst.* **79**, 1-12.

Sugatani, J., Kojima, H., Ueda, A., Kakizaki, S., Yoshinari, K., Gong, Q. H., Owens, I. S., Negishi, M. and Sueyoshi, T. (2001). The phenobarbital response enhancer module in the human bilirubin UDP-glucuronosyltransferase UGT1A1 gene and regulation by the nuclear receptor CAR. *Hepatology* **33**, 1232-1238.

Thomas, G. A. and Williams, E. D. (1999). Thyroid stimulating hormone (TSH)-associated follicular hypertrophy and hyperplasia as a mechanism of thyroid carcinogenesis in mice and rats. *IARC Sci. Publ.*, 45-59.

Wei, P., Zhang, J., Egan-Hafley, M., Liang, S. and Moore, D. D. (2000). The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism. *Nature* **407**, 920-923.

Whysner, J., Ross, P. M. and Williams, G. M. (1996). Phenobarbital mechanistic data and risk assessment: Enzyme induction, enhanced cell proliferation, and tumour promotion. *Pharmacol. Ther.* **71**, 153-191.

Yamada, T. (2012). Short-term study for mode of action analysis for mouse liver findings by S-2200TG. Sumitomo Chemical Company Ltd., Study No. S1618, Technical report ROT-0068, Unpublished data.

Yamada, T., Kunimatsu, T., Miyata, K., Yabushita, S., Sukata, T., Kawamura, S., Seki, T., Okuno, Y. and Mikami, N. (2004). Enhanced rat herberger assay appears reliable for detection of not only (anti-)androgenic chemicals but also thyroid hormone modulators. *Toxicol. Sci.* **79**, 64-74.

Yamada, T., Uwagawa, S., Okuno, Y., Cohen, S. M. and Kaneko, H. (2009). Case study: An evaluation of the human relevance of the synthetic pyrethroid metofluthrin-induced liver tumours in rats based on mode of action. *Toxicol. Sci.* **108**, 59-68.

Yamamoto, Y., Moore, R., Goldsworthy, T. L., Negishi, M. and Maronpot, R. R. (2004). The orphan nuclear receptor constitutive active/androstane receptor is essential for liver tumour promotion by phenobarbital in mice. *Cancer Res.* **64**, 7197-7200.

(Completed)

ROT-0075

Document Title

Updated interpretation of higher incidence of ovarian sex-cord stromal tumour in female rats treated with S-2200TG in a 2-year carcinogenicity study

Author

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

Date

October 2, 2013

Title: Updated interpretation of higher incidence of ovarian sex-cord stromal tumour in female rats treated with S-2200TG in a 2-year carcinogenicity study

Facility:

[REDACTED]
[REDACTED]
[REDACTED]
[REDACTED]

Personnel

Author:

[REDACTED] **Date :** _____

Approved by:

[REDACTED] **Date :** _____
Group Manager, Toxicology group

CONTENTS

	Page
1. Summary	4
2. Introduction	4
3. Genotoxicity	5
4. Metabolism in rats	5
5. Carcinogenicity study in mouse	6
6. Carcinogenicity study in rat	6
6-1. Study design	6
6-2. Incidence of ovary tumours	7
6-3. Assessment of significance	9
(a) Tumour type and background incidence	10
(b) Multi-site responses	12
(c) Progression of lesions to malignancy	12
(d) Reduced tumour latency	12
(e) Whether responses are in single or both sexes	13
(f) Whether responses are in a single species or several species	13
(g) Structural similarity to a substance(s) for which there is good evidence of carcinogenicity	13
(h) Routes of exposure	13
(i) Comparison of absorption, distribution, metabolism and excretion between test animals and humans	13
(j) The possibility of a confounding effect of excessive toxicity at test doses	14
(k) Mode of action (MOA) and its relevance for humans	17
7. Determination of criteria for classification and labeling for carcinogenicity	21
8. References	22-24

1. Summary

S-2200 Technical Grade (abbreviated as S-2200TG in this document) is a candidate novel strobilurin fungicide developed by Sumitomo Chemical Co., Ltd. S-2200TG was not genotoxic in a battery of *in vitro* and *in vivo* assays. The tumourigenic potential of S-2200TG was studied in male and female rats and mice in standard bioassays under the guidelines of Good Laboratory Practice and the test protocols designated by authorities.

An increased incidence of ovary sex-cord stromal tumour (SCST) was observed in female rats; the increased incidence exceeded the historical control ranges for this strain of rats; therefore, a causal relationship between S-2200TG administration and the ovary tumour induction was not ruled out. In the mouse study, the number of tumours in any tissue did not increase by exposure to S-2200TG. Therefore, one tumour type (benign) in one sex (female) of one species (the rat) occurred in one study. Four and six cases of benign ovarian SCST occurred in female rats exposed to 7000 and 15000 ppm (475 and 1016 mg/kg/day) S-2200TG, respectively. These were dose levels at which body weight gain reduced by > 20%, indicating that the physiology of the rat was sufficiently stressed so that endocrine status may be abnormal. Sex-cord stromal hyperplasia is quite common in aged Wistar rats, and the animals used in the 2-year study with S-2200TG appear to be derived from a susceptible batch. The incidences of sex-cord stromal proliferative lesions were well within historical controls for all groups, and there was no statistical difference between groups for hyperplasia, tumours, or hyperplasia plus tumours. Higher survival rates in the two higher groups may contribute to the higher number of ovarian tumours. The known modes of action via endocrine imbalance are unlikely, evidenced by no interaction with the estrogen receptor and steroidogenesis by *in vitro* assays, no direct ovarian toxicity, and no reproductive abnormality. Furthermore, there was no accumulation or persistence of S-2200 and its metabolites in the ovary. Thus, the sex-cord stromal lesions are unlikely to be direct effects by treatment with S-2200TG.

Based on these considerations, the increased incidence of the SCST observed in the rat 2-year study is not toxicologically significant. Therefore, the overall conclusion is that the data do not suggest a carcinogenic effect of S-2200TG and thus its classification is not warranted.

2. Introduction

S-2200TG is a candidate novel strobilurin fungicide developed by Sumitomo Chemical Co., Ltd. The tumourigenic potential of S-2200TG has been studied in male and female rats and mice in standard bioassays under the guidelines of Good Laboratory Practice and the test protocols designated by the European Community (EC), Organisation for Economic Co-operation and Development (OECD), US Environmental Protection Agency (US EPA), and Ministry of Agriculture, Forestry and Fisheries of Japan (Japan MAFF). These studies have been conducted at Covance Laboratories Ltd, Harrogate, North Yorkshire, England. No tumorigenic findings were observed in mice. An increased incidence of benign ovarian sex-cord stromal tumour (abbreviated as SCST) was observed at the terminal sacrifice in the rat 2-year study.

In this document, the toxicological significance of the slightly higher incidence of benign ovarian SCST in rats is discussed based on existing data of S-2200TG, the usual background of ovarian proliferative change present in elderly rats, and published information. Based on these considerations, the increased incidence of the SCST observed in the rat 2-year study is not toxicologically significant. Therefore, the overall conclusion is that the data do not suggest a carcinogenic effect and thus classification is not warranted.

3. Genotoxicity

S-2200TG was not genotoxic in a battery of *in vitro* and *in vivo* assays: reverse mutation test in a bacterial system, gene mutation test in Chinese Hamster V79 cells, chromosomal abbreviation test in Chinese Hamster lung cells (CHL/IU), and micronucleus test in CD-1 mice.

4. Metabolism in rats

The absorption, metabolism, distribution and excretion of [benzyl-¹⁴C]- and [phenoxy-¹⁴C]S-2200 have been investigated in rats. S-2200 is well absorbed (>90%) following oral administration, and is extensively metabolized with effective first pass effect, without any differences in metabolic profiles between [benzyl-¹⁴C]- and [phenoxy-¹⁴C]S-2200. Subsequent distribution of metabolites of S-2200 is widespread, with relatively high ¹⁴C concentration in liver, kidney, pancreas, fat, uterus, ovaries, lung, and adrenals. However, elimination of total radioactivity was rapid, with >95% of the administered dose being recovered in excreta within 24 hours, and elimination of radioactivity from the plasma and these tissues was almost complete at 168 hours post-administration. Faecal elimination, via the bile, was the primary route of elimination from the body of the rat. In addition, renal elimination was also important for the excretion of metabolites. There was no marked gender-related difference in absorption, distribution, metabolism or excretion.

Therefore, these findings suggest that accumulation and persistence of S-2200 or its metabolites in the ovary inducing ovarian dysfunctions is unlikely to occur in the rat 2-year bioassay.

5. Carcinogenicity study in mouse

Male and female Crl:CD1(ICR) mice were fed 0 (control), 700, 2000, or 7000 ppm S-2200TG (purity, 93.4%) in the diet for 78 weeks (average chemical intakes: 83, 239, and 824 mg/kg/day for males; 99, 280, and 994 mg/kg/day for females, respectively). There were no adverse effects observed. Therefore, the No Observed Adverse Effect Level (NOAEL) for this study was considered to be 7000 ppm (824 mg/kg/day for males and 994 mg/kg/day for females) following 78 weeks of treatment. There were no effects on survival/mortality or on the incidence or morphology of tumours to indicate any oncogenic potential.

6. Carcinogenicity study in rat

6-1. Study design

Male and female Crl:WI(Han) rats were fed 0 (control), 400, 2000, 7000, or 15000 ppm S-2200TG (purity, 93.4%) in the diet for 104 weeks (average chemical intakes: 21, 105, 376, and 804 mg/kg/day for males; 27, 135, 475 and 1016 mg/kg/day for females, respectively). In a previous GLP study "S-2200 Technical Grade: 13 Week Oral (Dietary) Administration Toxicity Study in the Rat (Covance Study Number 0333/290; Dose levels of 0, 800, 4000, 10000 and 20000 ppm; 12 animals/group/sex), there was an overall decrease in body weight gain for males and females given 20000 ppm and an increase in total plasma cholesterol for males and females given 10000 and 20000 ppm, respectively. Liver weights increased in males given ≥ 4000 ppm and females given ≥ 10000 ppm. Microscopically, liver hepatocyte hypertrophy was recorded for animals given 4000, 10000 or 20000 ppm, and the incidence of thyroid follicular cell hypertrophy was higher in animals given 4000, 10000 or 20000 ppm. In addition, the severity of hyaline droplets was higher in the kidney of males given 10000 or 20000 ppm. Due to the decrease in body weight gain, the magnitude of liver weight changes, a higher degree of hepatocyte hypertrophy and increased blood biochemistry parameters (total cholesterol and gamma glutamyltransferase) at 20000 ppm, the overall No-Observed-Adverse-Effect-Level (NOAEL) for the study was considered to be 10000 ppm.

Based on consultation with US.EPA and the Health Canada Pest Management Regulatory Agency (PMRA) regarding MTD (Maximum Tolerated Dose) determination for long-term studies, 15000 ppm was selected to be the highest dose level for the 2-year bioassay. To examine the dose response and no-adverse effect level, 400, 2000 and 7000 ppm were selected for the lower dose levels according to a common ratio of approximately 2 to 5.

6-2. Incidence of ovary tumours

As shown in Figure 1 and Table 1, the incidence of benign ovarian SCST was higher at 7000 and 15000 ppm than control; however, pair wise comparisons were not statistically significant by

the Fisher exact test. For tests of increasing dose response by Peto analysis, benign SCST in the ovary of females were statistically significant in treated animals vs. controls ($P=0.005$). These incidences exceeded the historical background range (0-3.1%, Table 2). Malignant SCST was not observed in any group.

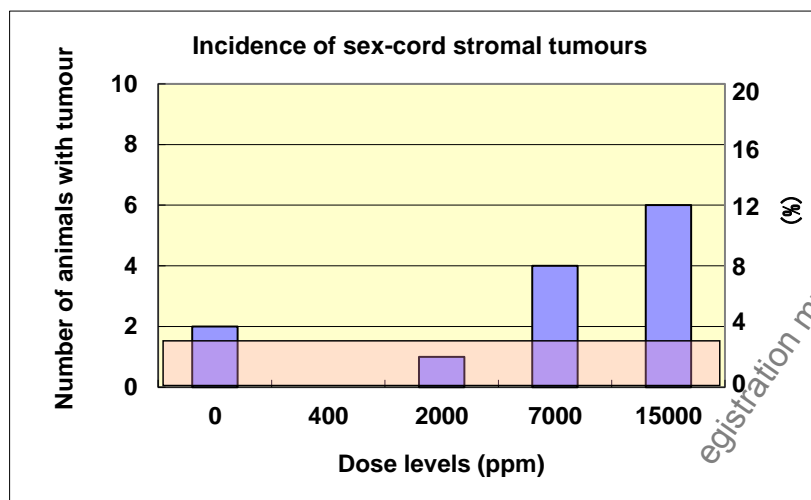


Figure 1. Incidence of sex-cord stromal tumours in the rat 2-year study with S-2200TG.

Highlighted area presents historical background range (0-3.1%).

Fisher exact test; Not significant ($p>0.05$), Peto test; Significant ($p=0.005$).

Table 1. Incidence of microscopic findings in the rat 2-year study with S-2200TG

Finding	Level (ppm)	0	400	2000	7000	15000
	No. examined:	50	50	50	50	50
Benign sex-cord stromal tumour (SCST)		2	0	1	4	6
	(%)	4	0	2	8	12
Malignant sex-cord stromal tumour		0	0	0	0	0
	(%)	0	0	0	0	0
Sex-cord stromal hyperplasia		3	8	5	6	5
	(%)	6	16	10	12	10
Combined sex-cord stromal tumour and hyperplasia#		5	8	6	8	9
	(%)	10	16	12	16	18

Fisher exact test; Not significant ($p > 0.05$), Peto test; Significant ($p = 0.005$).

#: the number of animal exhibiting both hyperplasia and tumour is counted as 1.

Historical control data at Covance UK is shown Table 2.

■ Sex-cord stromal hyperplasia: 27.1% [120/442]; range 2 - 48% [1/50 - 31/64].

■ Sex-cord stromal tumour - benign: 0.68% [3/442]; range 0 – 3.1% [0/100 - 2/64]

(This tumour was only recorded in two studies out of seven; the incidence was 1/70 and 2/64).

Table 2. Historical background values in females of the rat 2-year studies at Covance UK.

Study ID	1	2	3	4	5	6	7	Total Average
Completed year	2000	2001	2003	2004	2005	2007	2008	2000-08
Pathologist	A	B	B	C	A	D	D	A, B, C, D
Number of animals examined	70	50	50	50	100	60	100	480
Survival rate (%)	78	74	72	72	79	58	45	68
Sex-cord stromal hyperplasia#	31/70	14/49	1/50	1/50	31/64	16/59	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

#: Data represent the number of animal exhibiting findings compared to the total number of animals examined.

Pathologist of the rat 2-year study with S-2200TG is E but not A~D.

Figure 2 and Table 3 show incidence and severity of sex-cord stromal hyperplasia with gradable differentiation. The number of animals with sex-cord stromal hyperplasia was increased in all treatment groups, but not in a dose-related manner. All of these incidences were within the historical background range (2-48%, Table 2).

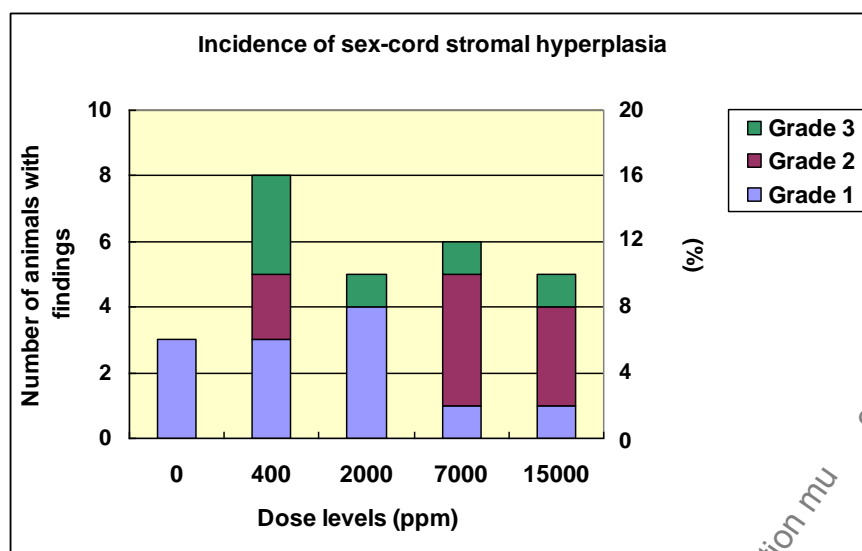


Figure 2. Incidence and severity of sex-cord stromal hyperplasia in the rat 2-year study with S-2200TG.

Table 3. Incidence and severity of sex-cord stromal hyperplasia in the rat 2-year study with S-2200TG

Finding	Level (ppm)	0	400	2000	7000	15000
	No. examined:	50	50	50	50	50
Sex-cord stromal hyperplasia		3	8	5	6	5
Grade 1		3	3	4	1	1
Grade 2		0	2	0	4	3
Grade 3		0	3	1	1	1
	(%)	6	16	10	12	10

Grade 1, minimal; Grade 2, slight; Grade 3, moderate.

Mann-Whitney U-test; Not significant ($p > 0.05$).

6-3. Assessment of significance

When assessing the overall level of concern of the ovarian SCST observed in the rat S-2200TG carcinogenicity study, some important factors need to be taken into consideration for evaluation of carcinogenic potential.

- Tumour type and background incidence;**
- Multi-site responses;**
- Progression of lesions to malignancy;**
- Reduced tumour latency;**
- Whether responses are in single or both sexes;**
- Whether responses are in a single species or several species;**
- Structural similarity to a substance(s) for which there is good evidence of carcinogenicity;**
- Routes of exposure;**
- Comparison of absorption, distribution, metabolism and excretion between test animals and humans;**
- The possibility of a confounding effect of excessive toxicity at test doses;**
- Mode of action and its relevance for humans, such as cytotoxicity with growth stimulation, mitogenesis, immunosuppression, mutagenicity.**

(a) Tumour type and background incidence;

Ovarian tumours in rodents can be subdivided into five broad categories including epithelial tumours, sex-cord/stromal-cell-derived tumours, germ cell tumours, tumours derived from nonspecialized soft tissues of the ovary, and tumours metastatic to the ovary from distant sites. Sex-cord/stromal cell-derived tumours include granulosa cell tumour, Sertoli cell tumour, thecoma and SCST (mixed). Ovarian SCST is recognized as a rare tumour amongst human ovary tumours (Scully, *et al.*, 1998) but is the most common spontaneous and induced rat ovarian neoplasm (Tsubota *et al.*, 2004). Because ovarian SCST are morphologically heterogeneous neoplasms that are relatively infrequently encountered, their diagnosis can be difficult. The diagnostic features of ovarian SCST are defined by the International Agency for Research on Cancer (IARC) (Mohr, 1997) as follows:

- Tumour consists of a mixture of granulosa, luteal, theca, Sertoli, and stromal cells, which may show various degrees of differentiation. No cell type dominates (>70%).
- Discrete, well demarcated focal lesions, which are bigger than one large corpus luteum.
- Included in this category are also extremely large, diffuse, mixed-type lesions which encompass the whole ovary and are in size/diameter markedly larger than a normal ovary (old age type sex-cord stromal hyperplasia).

Therefore, focal discrete lesions larger than a large corpus luteum are, in the absence of any other neoplastic morphological criteria, considered to be a tumour. Diffuse mixed-type lesions occasionally become very large. They may encompass the major part of the ovary and have a size larger than a normal ovary. In these cases they are arbitrarily registered as tumour rather than hyperplasia. Hyperplasia of the sex-cord-stromal cells is quite common in aged Wistar rats and probably other strains of rats but tends not to be diagnosed by some pathologists because it is viewed as a normal aging change. It is seen most commonly in 2-year carcinogenicity studies (Dixon *et al.*, 1999).

In the rat carcinogenicity study with S-2200TG, ovarian SCST were observed in 4/50 (8%) and 6/50 (12%) females of the 7000 and 15000 ppm groups (see Tables 1-3, Figure 1). The incidences (8 and 12%) were higher than that from the historical control range (0-3.1%) derived from several studies of 24-months duration. However, the incidence of the ovarian SCST in the control group of this study (2/50=4%) was higher than the highest level of the historical control range (3.1%; but zero in the most studies, see Table 2), suggesting that animals used in this study were derived from a batch susceptible for ovarian SCST.

The incidences of sex-cord stromal hyperplasia were similar in all groups and within the range of historical controls for this lesion (see Tables 1-3, Figure 2). The historical control data for sex-cord stromal hyperplasia and SCSTs in Table 2, indicates that SCSTs occur only in the presence of the highest rates of sex-cord stromal hyperplasia; which would imply some sort of progressive mechanism from hyperplasia to tumour. In contrast, the absence of a clear dose relationship in incidence or severity of hyperplasia in the current study (Figure 2) provides further support for the tumours to have arisen from a mechanism not involving hyperplasia, and thus are likely to be a chance occurrence.

Since the distinction between hyperplasia and tumour is somewhat arbitrary, based on size, the overall number of sex cord stromal proliferative lesions (hyperplasia plus tumour) provides a more reasonable assessment of a possible effect. For S-2200TG, the combined incidences of SCST and hyperplasia (Figure 3) were within the historical control incidences for hyperplasia alone (average, 120/442=27.1%; range, 2 - 48%; see Table 2), there is no increase in incidence of the combined sex-cord stromal proliferative lesions, and the incidences in all groups are well within the historical control range (actually less than the mean). The lack of statistical significance for hyperplasia, tumours, or hyperplasia plus tumours further supports the conclusion that these are not treatment related.

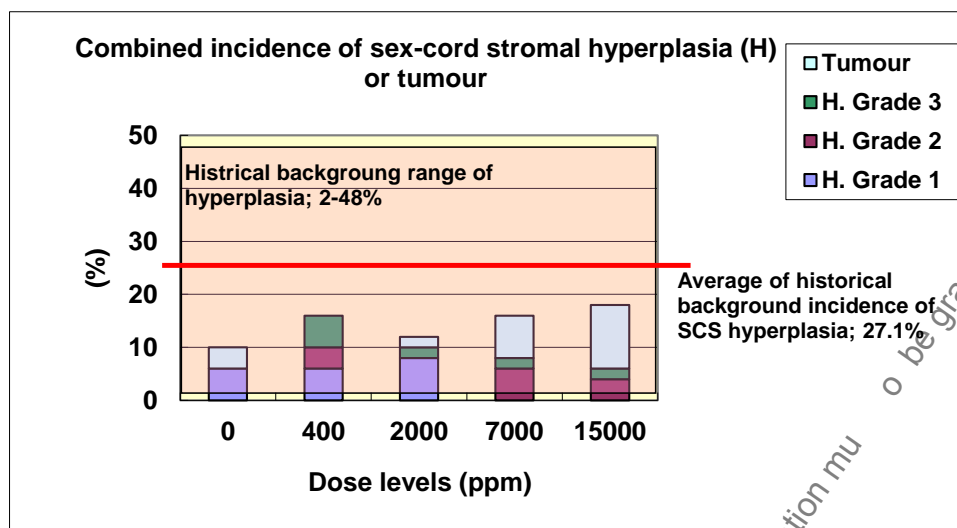


Figure 3. Combined incidence of sex-cord stromal hyperplasia and tumour in the rat 2-year study with S-2200TG.

The animal exhibiting both hyperplasia and tumour is counted as animals exhibiting tumour.
Mann-Whitney U-test; Not significant ($p > 0.05$).

(b) Multi-site responses;

The increase of tumour incidence was only observed in a single-site (ovary). There was no evidence of tumour multiplicity observable in the ovary in the rat 2-year study with S-2200TG.

(c) Progression of lesions to malignancy;

The ovarian SCSTs observed in the rat 2-year study with S-2200TG were all benign tumours. No malignant SCST was observed. Therefore, there was no evidence of progression to malignant tumour.

(d) Reduced tumour latency;

Since most SCSTs were observed in animals sacrificed after two years of treatment with S-2200TG but not observed at an interim sacrifice after one year of treatment, the tumours appeared at a late stage of treatment, after 1.5 years of treatment based on unscheduled death/sacrifice animals: in the 15000 ppm group, no SCSTs were observed in any of the 9 unscheduled death/sacrificed animals during Weeks 57 to 98. At 7000 ppm, one unscheduled sacrificed animal at Week 98 had ovarian SCS hyperplasia and tumour; no SCSTs were observed in the other 9 unscheduled death/sacrificed animals during Weeks 57 to 102. Therefore, reduced tumour latency was not observed in the rat 2-year study with S-2200TG.

(e) Whether responses are in single or both sexes;

A higher incidence of tumour with possible treatment-related effect was observed in the ovary but not in the testis; three animals bearing testicular Leydig cell tumour were observed at 15000 ppm ($3/50 = 6\%$), but this was within the historical background range in the laboratory (0-6%) and there was no increased incidence of interstitial cell hyperplasia of the testis. Therefore, the response was observed only in a single sex (female).

(f) Whether responses are in a single species or several species;

A higher incidence of the ovarian SCST was observed in rat but not in mouse. There were no increased incidences of any tumour type in the mouse. Therefore, the response was observed

in a single species (rat). Thus, this is a slight increase in the incidence of a benign tumour at a single site in a single sex in a single species.

- (g) Structural similarity to a substance(s) for which there is good evidence of carcinogenicity;
S-2200TG is a candidate novel strobilurin fungicide. Of known strobilurin chemicals, none increase ovarian tumours in rats or mice.

- (h) Routes of exposure;

In the carcinogenicity study, rats were treated with S-2200TG fed in the diet. The oral route was selected because it is one of the potential exposure routes for humans.

- (i) Comparison of absorption, distribution, metabolism and excretion between test animals and humans;

Three metabolites (5-CH₂OH-S-2200, 2-CH₂OH-S-2200, and 4-OH-S-2200) were formed in liver S9 fraction of humans, rats and mice. No species related difference was observed in the three metabolites. Data for absorption, distribution and excretion of S-2200 are not available in humans.

- (j) The possibility of a confounding effect of excessive toxicity at test doses;

Four and six cases of benign ovarian SCST were observed in female rats exposed to 7000 and 15000 ppm of S-2200TG, respectively. As shown in Figure 4 and Table 4, body weight gain was suppressed by more than 10% after Week 40 at 7000 ppm, and throughout the treatment period at 15000 ppm. Maximal suppression reached more than 20%, indicating that these doses considerably exceeded the maximum tolerated dose. 2000 ppm of S-2200TG also showed weight suppression of more than 10%, but it was only observed at a later period of treatment (i.e., after Week 68).

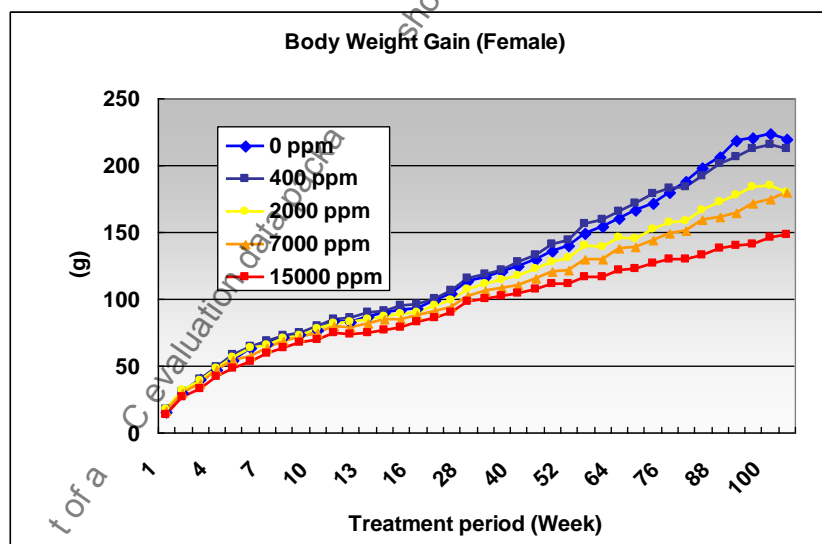


Figure 4. Alteration of body weight gain in female rats treated with S-2200TG. Data present group mean value.

Table 4. Relative suppression of body weight gain in female rats treated with S-2200TG.

Week	Dose levels of S-2200TG			
	400 ppm	2000 ppm	7000 ppm	15000 ppm
1	12	18	6	-12
2	2	5	-3	-13
3	1	-2	-7	-17
4	6	2	1	-10
5	8	4	0	-11
6	2	0	-9	-16
7	4	0	-4	-10
8	1	-1	-3	-12
9	2	-1	-3	-8
10	4	1	-2	-10
11	2	-3	-5	-11
12	3	-1	-5	-12
13	3	-3	-7	-14
14	1	-3	-6	-15
15	3	-4	-7	-14
16	3	-3	-5	-11
20	1	-4	-8	-13
24	2	-5	-10	-15
28	2	-5	-10	-13
32	2	-4	-8	-14
36	1	-4	-10	-15
40	2	-6	-11	-17
44	3	-6	-11	-17
48	3	-6	-11	-18
52	3	-7	-14	-21
56	5	-7	-13	-22
60	3	-10	-15	-24
64	3	-9	-14	-24
68	3	-13	-17	-26
72	4	-11	-16	-26
76	1	-13	-17	-28
80	-2	-16	-20	-31
84	-3	-16	-20	-33
88	-2	-16	-22	-33
92	-5	-19	-25	-36
96	-4	-17	-22	-36
100	-4	-18	-22	-35
104	-3	-18	-18	-33

Values represent percent change from control value.

Highlighted values indicate more than 10% suppression of body weight gain.

The highest dose in the bioassay needs to induce minimal toxicity, such as characterized by an approximately 10% reduction in body weight gain (maximal tolerated dose, MTD) (ECHA, 2012; Rhomberg *et al.*, 2007). The MTD is the highest dose of the test agent during the bioassay that can be predicted not to alter the animal's normal longevity from effects other than carcinogenicity. Excessive toxicity, for instance toxicity at doses exceeding the MTD, can affect the carcinogenic responses in bioassays. Tumours occurring only at excessive doses associated with severe toxicity generally have a more doubtful potential for carcinogenicity in humans (ECHA, 2012; Rhomberg *et al.*, 2007).

Survival rate also appears to affect interpretation of results in the bioassay. The common practice of scheduling a terminal sacrifice after 2 years on test reflects a judgment that this is optimal for detecting potential carcinogenic effects (Rhomberg *et al.*, 2007). However, shorter survival risks missing induced tumours and longer survival tends to induce an increasing number of background tumours. Figure 5 presents the survival rate in female rats of the 2-year study with S-2200TG. Survival rate was relatively higher at 7000 and 15000 ppm than control; especially, in the last 6 months of treatment period (Figure 5B).

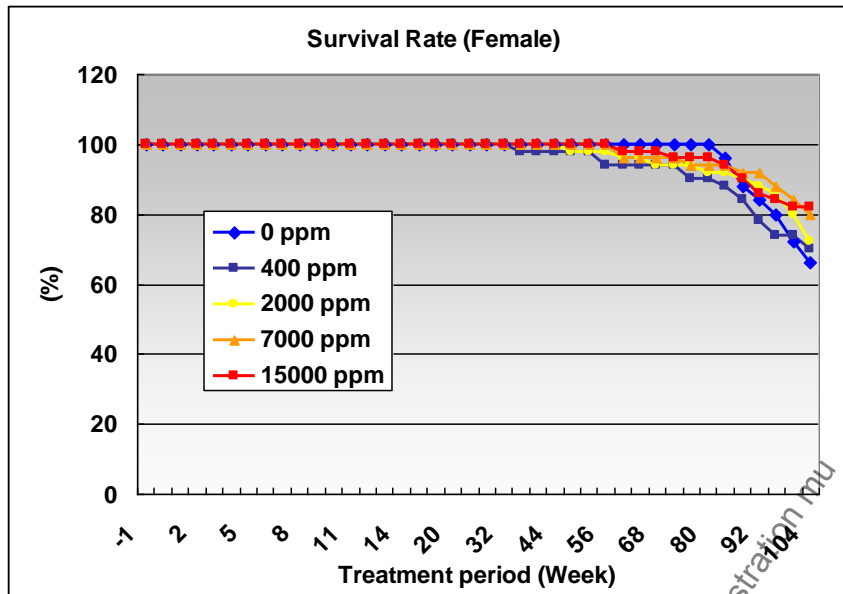


Figure 5A. Survival rate in females of the rat 2-year study with S-2200TG throughout 2-year treatment

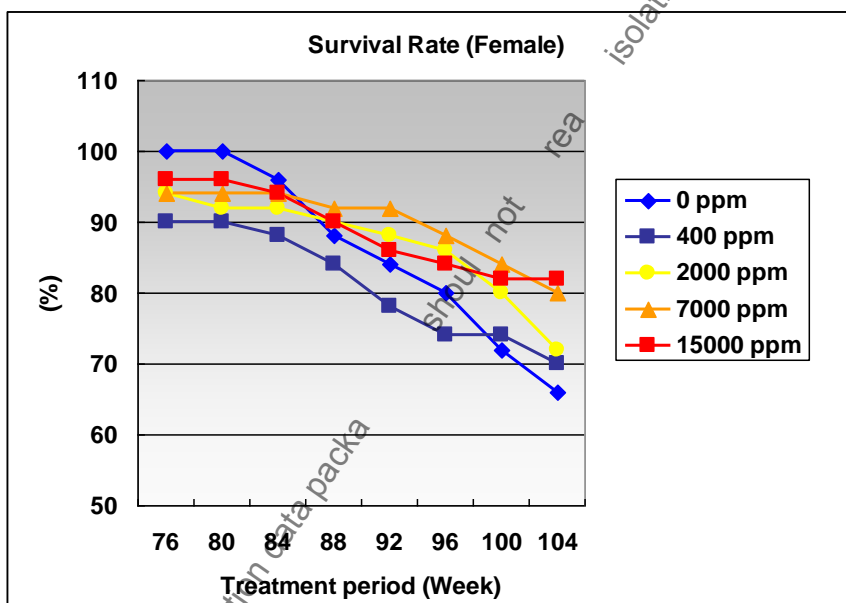


Figure 5B. Survival rate in females of the rat 2-year study with S-2200TG during last 6-month treatment

As shown in Table 5, the number of surviving rats of the 7000 ppm and 15000 ppm female groups at terminal sacrifice (40 and 41 rats, respectively) was higher than those of the control female group (33 rats). These values were not statistically significant; p-values of 7000 and 15000 ppm groups by the Fisher exact test were 0.088 and 0.055, respectively. In comparison with historical background data at Covance UK (see Table 2), the survival rate of 7000 and 15000 ppm groups (80 and 82%, respectively) were slightly higher than the control range (45-79%). The higher number of surviving animals may affect the incidence of the SCST as a confounding factor because animals used in this study were derived from a susceptible batch for ovarian SCST as discussed in Section (a).

Table 5. Survival rate in the rat carcinogenicity study with S-2200TG

	Male	Female
--	------	--------

Dose levels (ppm)	0	400	2000	7000	15000	0	400	2000	7000	15000
Survivals	32	42*	32	41*	36	33	35	36	40#	41##
Survival rate (%)	64	84	64	82	72	66	70	72	80	82

*, p<0.05, #, p=0.088, ##, p=0.055 by Fisher exact test.

(k) Mode of action (MOA) and its relevance for humans

(k-1). Negative findings of genotoxicity studies suggest a non-genotoxic MOA

(k-2). Ovarian function and dysfunction are intimately linked with the hypothalamus, the pituitary, the uterus, and other endocrine organs. It is known that there are two kinds of MOA for induction of ovarian tumour including SCST in rodents; they involve direct and secondary effects on the ovary. In both cases, increased gonadotropin is the causative factor for ovary tumour development. The transplantation of ovarian tissue under the splenic capsule in ovariectomized rats caused ovarian tumours, including SCST (Biskind and Biskind, 1949; Biskind *et al.*, 1950; Capen, 2008; Dittrich *et al.*, 2001; Jager *et al.*, 1995). Transplantation in ovariectomized rats caused estrogens produced by the transplanted ovary to drain to the liver. As sex hormone globulin levels are low in adult rats (Dittrich *et al.*, 2001), estradiol is metabolized directly and completely during first passage through the liver so that no estradiol reaches the pituitary gland to down-regulate gonadotropin secretion. The presence of a single functioning gonad prevented the development of the proliferative lesions in the ovary. Furthermore, administration of exogenous estrogen or testosterone after transplantation completely suppressed development of the proliferative changes in the ovarian cortex. Taken together, as shown in Figure 6, lack of negative feedback from estrogen and a high level of gonadotropin stimulation are necessary for the development of these tumours in rats (Biskind and Biskind, 1949; Capen, 2008; Jager *et al.*, 1995).

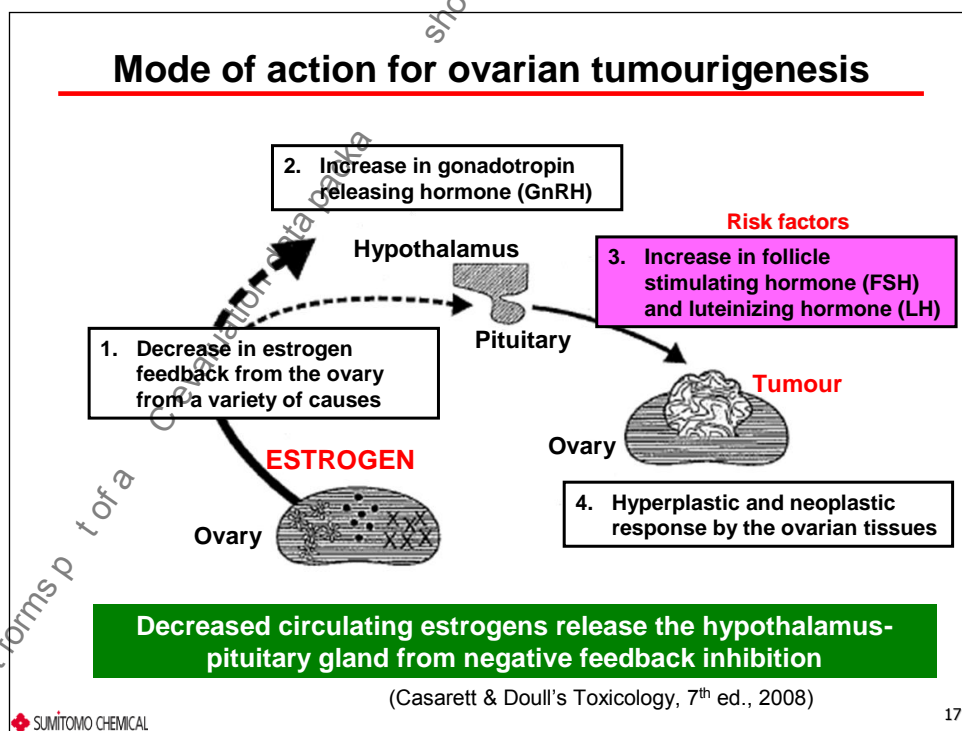


Figure 6. Mode of action for ovarian tumourigenesis in rodents. Figure modified from Casarett & Doull's Toxicology: The basic Science of Poisons (Capen, 2008).

■ No interaction with estrogen receptor and no effects on steroidogenesis

Selective estrogen receptor modulators (SERMs) (e.g., tamoxifen, toremifene, and raloxifene) have been reported to increase the incidence of ovarian tumours when administered chronically to rodents (Capen, 2008; Long *et al.*, 2001). In fact, raloxifene binding to the estrogen receptor induced an elevation of serum Luteinizing hormone (LH) and ovarian tumour in rats (Long *et al.*, 2001).

S-2200TG was negative in an estrogen receptor reporter gene assay (Suzuki, 2012) and assay for steroidogenesis (Kubo, 2012). Unfortunately, hormone levels in peripheral blood could not be determined in the 2-year study. Taken together with no abnormalities in reproductive function in rats or in endocrine organs in general toxicity studies in rats, mice and dogs, it is suggested that secondary effects via endocrine disruption by S-2200TG are unlikely. Especially, in the case of LH, though, the absence of an increase in Leydig cell tumours in the current study is important in supporting the position that LH was not elevated, and thus the SCSTs did not arise by a hormonal mechanism, rather being most likely due to chance. This is particularly likely since the incidences of hyperplasia plus benign tumours (total proliferative lesions of the ovary) were within historical controls.

■ No indication of endocrine disruption in the toxicity-studies

Apart from the secondary effect via endocrine disruption, direct effect (cytotoxic effect) on the ovary also involves the lack of negative feedback from estrogen and a consequent high level of gonadotropin. Maronpot (1987) demonstrated that the ovarian lesions documented in the National Toxicology Program (NTP) studies covered in his paper provide supportive evidence that alterations noted in ovaries of treated rodents at the conclusion of a 90-day study may herald the ultimate development of ovarian neoplasia upon continued treatment (Maronpot, 1987). S-2200TG did not provide any evidence indicating ovary lesions or toxicity in any studies in rat, mouse and dog, except for the hyperplasia and tumour at the terminal sacrifice in the rat 2-year study. Furthermore, no abnormality was observed in the rat reproduction study. Therefore, a direct cytotoxic effect of S-2200TG on the ovary is also unlikely.

■ Severe suppression of body weight may relate to induction of ovary tumours

A number of studies showed that life-long restriction in caloric intake improves 2-year survival, controls adult body weight and delays the onset of diet- and age-related spontaneous diseases and tumours (Molon-Noblot *et al.*, 2003). Pituitary function is also reduced during fasting or food restriction, and life-long food restriction induced a decline of the incidence of pituitary tumours in rat (Molon-Noblot *et al.*, 2003). Unfortunately, the incidence of ovary tumours was not determined in the study by Molon-Noblot *et al.* (2003). However, Rehm *et al.* (1984) demonstrated that all animals subjected to food restriction live longer and developed more ovarian neoplasms than those fed ad libitum in a mouse study. This finding suggests that a decrease of tumour appearance by life-long dietary restriction did not prove to be true for the present type of ovarian neoplasms, even though the secretion of gonadotropins is impaired under limited feeding conditions (Rehm *et al.*, 1984). Interestingly, McShane and Wise (1996) have shown that moderate life-long caloric restriction, resulting in average body weights that are approximately 76% those of controls, delays reproductive senescence in rats without causing a cessation of regular estrous cycles earlier in life. Furthermore, they demonstrated that LH secretion is enhanced by caloric restriction. Unfortunately, serum LH levels were not able to be determined in the rat 2-year study of S-2200TG, but significant reduction in the incidence of pituitary tumours was observed in females

treated with 15000 ppm of S-2200TG together with more than 20% suppression of body weight.

Therefore, the existing data do not support that the higher incidence of the SCST observed in rats treated with S-2200TG is via this well-known MOA involving excess gonadotropin. Thus, the slightly higher incidence of the SCST is not toxicologically significant at most, especially when viewed in the perspective of a high background of proliferative lesions (stromal hyperplasia), a higher survival at dose levels where severe body weight suppression (which may induce abnormal endocrine status) was observed, and that this represents a slight increase of a benign lesion at a single site in a single sex in a single species. Taken together, the evidence supports the conclusion that the ovarian lesions in female rats were not toxicologically significant.

(k-3). No correlation between the ovarian cancer in women and increased serum gonadotropin.

A positive result in a rodent carcinogenicity study should not automatically preclude further development of a compound; future progress in this field should increase the accuracy of the rodent carcinogenicity study as a tool in human safety assessment (Alison *et al.*, 1994). In humans, the concentration of sex hormone-binding globulins is high enough to prevent the fast metabolism of estradiol in the liver (Mueller *et al.*, 2005). Therefore, this suggests that it is difficult to elevate gonadotropin levels in human peripheral blood. Furthermore, although hormonal imbalances in rodents have been shown to alter ovarian morphology and function and are correlated with an increased incidence of ovarian neoplasia, these preclinical models are not predictive of ovarian cancer in women. To date, there is no correlation between the occurrence of ovarian cancer in women and increased serum gonadotropin levels. In fact, women with a personal or family history of ovarian cancer have significantly lower serum LH concentrations than women without such a history. Thus, the relationship between elevated circulating gonadotropin levels and an increased incidence of ovarian tumours in rodents does not appear to be true in humans (Capen, 2008; Long *et al.*, 2001). Stromal tumours are rare in the human ovary (Scully *et al.*, 1998). Furthermore, epidemiology demonstrated that a positive association between ovarian cancer and obesity is well known (Olsen *et al.*, 2007), but not associated with lower body weight. Rather, ovarian cancer is associated with height and, among never-users of hormone therapy, with body mass index (Collaborative Group on Epidemiological Studies of Ovarian Cancer, 2012), and moderate energy restriction during adolescence was associated with a decrease in ovarian cancer risk (Schouten *et al.*, 2011).

Therefore, even if the higher incidence of the SCST in rats treated with S-2200TG is treatment-related, the toxicological significance of this alteration for human safety is questionable.

7. Determination of criteria for classification and labeling for carcinogenicity

Our proposed classification and labeling of S-2200TG for carcinogenicity is as follows:

One tumour type (benign) in one sex (female) of one species (the rat) occurred in one study with S-2200TG. S-2200TG is non-genotoxic, which lowers the level of concern for classification. Four and six cases of benign ovarian sex-cord stromal tumours occurred in female rats exposed to 7000 and 15000 ppm (475 and 1016 mg/kg/day) S-2200TG, respectively. These were dose levels at which body weight gain was reduced by > 20%, indicating that the physiology of the rat was sufficiently stressed so that endocrine status may be abnormal. The sex-cord stromal hyperplasia is quite common in aged Wistar rats (Dixon *et al.*, 1999), and the animals used in the 2-year study

with S-2200TG appear to be derived from a susceptible batch. The incidences of sex-cord stromal proliferative lesions were well within historical controls for all groups, and there was no statistical difference between groups for hyperplasia, tumours, or hyperplasia plus tumours. Higher survival rate in the two higher groups may contribute to the higher number of ovarian tumours. The known modes of action via endocrine imbalance are unlikely, evidenced by no interaction of estrogen receptor and steroidogenesis by *in vitro* assays, no direct ovarian toxicity, and no reproductive abnormality. There was no accumulation or persistence of S-2200 and its metabolites in the ovary. Thus, the sex-cord stromal lesions in female rat are not toxicologically significant.

Given the uncertainties over the significance of the finding, the overall conclusion is that the data do not suggest a carcinogenic effect and thus classification is not warranted.

8. References

- Alison, R. H., Capen, C. C. and Prentice, D. E. (1994). Neoplastic lesions of questionable significance to humans. *Toxicol. Pathol.* **22**, 179-186.
- Biskind, G. R. and Biskind, M. S. (1949). Experimental ovarian tumors in rats. *Am. J. Clin. Pathol.* **19**, 501-521.
- Biskind, G. R., Kordan, B. and Biskind, M. S. (1950). Ovary transplanted to spleen in rats: The effect of unilateral castration, pregnancy, and subsequent castration. *Cancer Res.* **10**, 309-318.
- Capen, C. C. (2008). Unit 4. Target organ toxicity. 21. Toxic responses of the endocrine system. 7th ed. (C. D. Klaassen, Ed.). McGraw-Hill Companies, Inc., New York.
- Collaborative Group on Epidemiological Studies of Ovarian Cancer. (2012). Ovarian cancer and body size: Individual participant meta-analysis including 25,157 women with ovarian cancer from 47 epidemiological studies. *PLoS Med* **9**, e1001200.
- Dittrich, R., Recabarren, S., Mitze, M. and Jaeger, W. (2001). Role of gonadotropins in malignant progression of sex cord stromal tumors produced by sequential auto- and isogenic transplantation of ovarian tissue in ovariectomized rats. *J. Cancer Res. Clin. Oncol.* **127**, 495-501.
- Dixon, D., Leininger, J., Valerio, M., Johnson, A., Stabinski, L. and Frith, C. (1999). Proliferative lesions of the ovary, uterus, vagina, cervix and oviduct in rats. STP/ARP/AFIP, Washington, DC.
- ECHA (2012). Guidance on the application of the CLP criteria, guidance to regulation (EC) no 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, version 2.0.
- Jager, W., Dittrich, R., Recabarren, S., Wildt, L. and Lang, N. (1995). Induction of ovarian tumors by endogenous gonadotropins in rats bearing intrasplenic ovarian grafts. *Tumour Biol.* **16**, 268-280.
- Kubo, H. (2012). In vitro steroidogenesis assay of S-2200TG in H295R cells. Study no. HK001, Sumitomo Chemical Company Ltd., technical report, unpublished data.
- Long, G. G., Cohen, I. R., Gries, C. L., Young, J. K., Francis, P. C. and Capen, C. C. (2001). Proliferative lesions of ovarian granulosa cells and reversible hormonal changes induced in rats by a selective estrogen receptor modulator. *Toxicol. Pathol.* **29**, 719-726.
- Maronpot, R. R. (1987). Ovarian toxicity and carcinogenicity in eight recent national toxicology program studies. *Environ. Health Perspect.* **73**, 125-130.

- McShane, T. M. and Wise, P. M. (1996). Life-long moderate caloric restriction prolongs reproductive life span in rats without interrupting estrous cyclicity: Effects on the gonadotropin releasing hormone/luteinizing hormone axis. *Biol. Reprod.* **54**, 70-75.
- Mohr, U. (1997). International Classification of Rodent Tumours. Part I-The Rat. 9.Female Genital System., The International Agency for Research on Cancer., Lyon.
- Molon-Noblot, S., Laroque, P., Coleman, J. B., Hoe, C.-M. and Keenan, K. P. (2003). The effects of ad libitum overfeeding and moderate and marked dietary restriction on age-related spontaneous pituitary gland pathology in sprague-dawley rats. *Toxicol. Pathol.* **31**, 310-320.
- Mueller, A., Maltaris, T., Dimmler, A., Hoffmann, I., Beckmann, M. W. and Dittrich, R. (2005). Development of sex cord stromal tumors after heterotopic transplantation of cryopreserved ovarian tissue in rats. *Anticancer Res.* **25**, 4107-4111.
- Olsen, C. M., Green, A. C., Whiteman, D. C., Sadeghi, S., Kolahdoost, F. and Webb, P. M. (2007). Obesity and the risk of epithelial ovarian cancer: A systematic review and meta-analysis. *Eur. J. Cancer* **43**, 690-709.
- Rehm, S., Dierksen, D. and Deerberg, F. (1984). Spontaneous ovarian tumors in han:Nmri mice: Histologic classification, incidence, and influence of food restriction. *J. Natl. Cancer Inst.* **72**, 1383-1395.
- Rhomberg, L. R., Baetcke, K., Blancato, J., Bus, J., Cohen, S., Conolly, R., Dixit, R., Doe, J., Ekelman, K., Fenner-Crisp, P., Harvey, P., Hattis, D., Jacobs, A., Jacobson-Kram, D., Lewandowski, T., Liteplo, R., Pelkonen, O., Rice, J., Somers, D., Turturro, A., West, W. and Olin, S. (2007). Issues in the design and interpretation of chronic toxicity and carcinogenicity studies in rodents: Approaches to dose selection. *Crit. Rev. Toxicol.* **37**, 729-837.
- Schouten, L. J., van Dijk, B. A. C., Lumey, L. H., Goldbohm, R. A. and van den Brandt, P. A. (2011). Energy restriction during childhood and early adulthood and ovarian cancer risk. *PLoS ONE* **6**, e27960.
- Scully, R. E., Young, R. H. and Clement, P. B. (1998). Tumors of the Ovary, Maldeveloped Gonads, Fallopian Tube, and Broad Ligament. Atlas of Tumor Pathology, Fascicle 23, Third Series, Armed Forces Institute of Pathology, Washington, DC, p.169-226.
- Suzuki, N. (2012). Evaluation of effects of S-2200TG and its metabolites on human estrogen receptor alpha and human androgen receptor using *in vitro* reporter gene assays. Study no. RGA-130, Sumitomo Chemical Company Ltd., technical report, unpublished data.
- Tsubota, K., Yoshizawa, K., Fujihira, S., Okazaki, Y., Matsumoto, M., Nakatsuji, S. and Oishi, Y. (2004). A spontaneous ovarian immature teratoma in a juvenile rat. *J. Toxicol. Pathol.* **17**, 211-218.

(Completed)

B.6.16 References relied on

Annex point/ reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed Y/N-R/NR	Owner
Annex II Data and Information					
IIA, 5.1.1/01 5.1.2 /01	██████████ ██████████ ██████████	2012	Amended Final Report 1. [¹⁴ C]S-2200: Absorption, Distribution, Metabolism and Excretion following Single Oral Administration to the Rat. ██████████ (Sumitomo ROM-0033) GLP, Unpublished.	Y	SUM
IIA, 5.1.1/02 5.1.2 /02	██████████	2011	Metabolism of S-2200 R-isomer (S-2167) and S-2200 S-isomer (S-2354) in Rats. ██████████ Study No. 4136. (Sumitomo ROM-0021) GLP, Unpublished.	Y	SUM
IIA, 5.1.3 /01	██████████ ██████████	2012	Amended Final Report 1. [¹⁴ C]S-2200: Absorption, Distribution, Metabolism and Excretion following Repeat Oral Administration to the Rat ██████████ (Sumitomo ROM-0036) GLP, Unpublished.	Y	SUM
IIA, 5.2.1 / 01	██████████	2010a	Acute Oral Toxicity Study of S-2200TG in Rats ██████████ Study No. 4161 (Sumitomo ROT-0010) GLP, Unpublished.	Y	SUM
IIA, 5.2.2 / 01	██████████	2010b	Acute Dermal Toxicity Study of S-2200TG in Rats ██████████ Study No. 4162 (Sumitomo ROT-0011) GLP, Unpublished.	Y	SUM
IIA, 5.2.3 / 01	██████████	2010	Acute Inhalation Toxicity Study of S-2200 TG in Rats ██████████ Study No. 4160 (Sumitomo ROT-0020) GLP, Unpublished.	Y	SUM
IIA, 5.2.4 / 01	██████████	2010a	Primary Skin Irritation test of S-2200TG in Rabbits ██████████, Study No. 4152 (Sumitomo ROT-0015) GLP, Unpublished.	Y	SUM
IIA, 5.2.5 / 01	██████████	2010b	Amended Final Report: Primary Eye Irritation Test of S-2200TG in Rabbits ██████████ Study No. 4153 (Sumitomo ROT-0016) GLP, Unpublished.	Y	SUM

Annex point/ reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed Y/N-R/NR	Owner
IIA, 5.4.3 / 01	Wollny, H-E.	2010	Gene Mutation Assay in Chinese Hamster V79 Cells <i>in vitro</i> (V79/HPRT) with S-2200TG Harlan Cytotest Cell Research GmbH, Study No. 1289600 (Sumitomo ROT-0021) GLP, Unpublished.	Y	SUM
IIA, 5.4.4 / 01	██████████	2010c	Micronucleus Test on S-2200TG in CD-1 Mice. ██████████ Study No. 4154 (Sumitomo ROT-0014) GLP, Unpublished.	Y	SUM
IIA, 5.5.2 / 01	██████████	2012b	S-2200 Technical Grade: 104 Week Oral (Dietary) Administration Combined Toxicity/Carcinogenicity Study in the Rat. ██ (Sumitomo ROT-0072) GLP, Unpublished.	Y	SUM
IIA, 5.5.3 / █	██████████	2012c	S-2200 Technical Grade: 78 Week Oral (Dietary) Administration Carcinogenicity Study in the Mouse. ██ (Sumitomo ROT-0073) GLP, Unpublished.	Y	SUM
IIA, 5.5.4.1 / 01	██████████	2012a	The toxicological relevance of the liver and thyroid alterations observed in rats treated with S-2200TG based on mode of action ██████████ Study No. none (Sumitomo ROT-0070) Non-GLP, Unpublished.	Y	SUM
IIA 5.5.4.1 / 02	██████████	2012e	Short-Term Study for Mode of Action Analysis for Rat Liver and Thyroid Findings by S-2200TG – Dose Response, Time-Course and Reversibility. ██████████ Study No. S1560 (Sumitomo ROT-0067) Non-GLP, Unpublished.	Y	SUM
IIA, 5.5.4.1 / 03	██████████	2012b	Short-Term Study for Mode of Action Analysis for Mouse Liver Findings by S-2200TG. ██████████, Study No. S1618 (Sumitomo ROT-0068) Non-GLP, Unpublished.	Y	SUM
IIA, 5.5.4.2 / 01	██████████ ██████████	2012	Interpretation of Higher Incidence of Ovarian Sex-cord Stromal Tumour in Female Rats treated with S-2200TG in a 2-year carcinogenicity study. ██████████, Study No. none (Sumitomo ROT-0069) Non-GLP, Unpublished.	Y	SUM

Annex point/ reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed Y/N-R/NR	Owner
IIA, 5.5.4.2 / 02	Kubo, H.	2012	<i>In vitro</i> Steroidogenesis Assay of S-2200TG in H295R Cells Sumitomo, Study No. HK001 (Sumitomo ROT-0065) Non-GLP, Unpublished.	Y	SUM
IIA, 5.5.4.2 / 03	Suzuki, N.	2012	Evaluation of Effects of S-2200TG and its Metabolites on Human Estrogen Receptor alpha and Human Androgen Receptor using <i>in vitro</i> Reporter Gene Assays Sumitomo, Study No. RGA-130 (Sumitomo ROT-0066) Non-GLP, Unpublished.	Y	SUM
IIA, 5.5.4.2 / 04	██████████	2013	Up dated interpretation of higher incidence of ovarian sex-cord stromal tumour in female rats treated with S-2200TG in a 2-year carcinogenicity study ██████████ Study No. none (Sumitomo ROT-0075) Non-GLP, Unpublished.	Y	SUM
IIA, 5.6.1 / 01	██████████	2010	Dose Range-finding Study for Two Generation Reproduction Study of S-2200 TG in Rats. ██ ██████████ (Sumitomo ROT-0018) Non-GLP, Unpublished.	Y	SUM
IIA, 5.6.1 / 02	██████████	2012	Two-Generation Reproduction Toxicity Study of S-2200TG in Rats ██ ██████████ (Sumitomo ROT-0064) GLP, Unpublished.	Y	SUM
IIA, 5.6.10 / 01	██████████	2009a	S-2200 TG: Oral (Gavage) Range-Finding Study of Prenatal Development in the Rat ██ (Sumitomo ROT-0009) Non- GLP, Unpublished.	Y	SUM
IIA, 5.6.10 / 02	██████████	2012a	S-2200 TG: Oral (Gavage) Prenatal Development Toxicity Study in the Rat ██ (Sumitomo ROT-0051) GLP, Unpublished.	Y	SUM
IIA, 5.6.11 / 01	██████████	2009b	S-2200 TG: Oral (Gavage) Range-Finding Study of Prenatal Development in the Rabbit ██ (Sumitomo ROT-0008) Non-GLP, Unpublished.	Y	SUM

Annex point/ reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed Y/N-R/NR	Owner
IIA, 5.6.11 / 02	[REDACTED]	2012b	S-2200 TG: Oral (Gavage) Prenatal Development Toxicity Study in the Rabbit [REDACTED] (Sumitomo ROT-0052) GLP, Unpublished.	Y	SUM
IIA, 5.7.1 / 01	[REDACTED]	2011a	An Oral (Gavage) Dose Range-Finding Acute Neurotoxicity Study of S-2200TG in Wistar Rats [REDACTED] (Sumitomo ROT-0036) GLP, Unpublished.	Y	SUM
IIA, 5.7.1 / 02	[REDACTED]	2011b	An Oral (Gavage) Acute Neurotoxicity Study of S-2200TG in Wistar Rats. [REDACTED] (Sumitomo ROT-0037) GLP, Unpublished.	Y	SUM
IIA, 5.7.4 / 01	[REDACTED]	2012	A 90-Day Oral Dietary Neurotoxicity Study of S-2200TG in Wistar Rats [REDACTED] (Sumitomo ROT-0050) GLP, Unpublished.	Y	SUM
IIA, 5.8 / 01	[REDACTED]	2012a	Acute Oral Toxicity Study of 2-COOH-S-2200 in Rats [REDACTED] Study No. 4234 (Sumitomo ROT-0043) GLP, Unpublished.	Y	SUM
IIA, 5.8 / 02	[REDACTED]	2012a	Reverse Mutation Test of 2-COOH-S-2200 in Bacterial Systems [REDACTED] Study No. 4221. (Sumitomo ROT-0041) GLP, Unpublished.	Y	SUM
IIA, 5.8 / 03	Kitamoto, S.	2012b	<i>In vitro</i> Chromosomal Aberration Test on 2-COOH-S-2200 in Chinese Hamster Lung Cells (CHL/IU) Sumitomo, Study No. 4219. (Sumitomo ROT-0046) GLP, Unpublished.	Y	SUM
IIA, 5.8 / 04	Wollny, H-E.	2011a	Gene Mutation Assay in Chinese Hamster V79 Cells <i>in vitro</i> (V79/HPRT) with 2-COOH-S-2200 Harlan Cytotest Cell Research GmbH, Study No. 1394801. (Sumitomo ROT-0033) GLP, Unpublished.	Y	SUM

Annex point/ reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed Y/N-R/NR	Owner
IIA, 5.8 / 05	████████	2012	Micronucleus Assay of 2-COOH-S-2200 in Mice ████████ (Sumitomo ROT-0040) GLP, Unpublished.	Y	SUM
IIA, 5.8 / 06	████████	2012b	Acute Oral Toxicity Study of 5-COOH-S-2200 in Rats ████████ Study No. 4233 (Sumitomo ROT-0044) GLP, Unpublished.	Y	SUM
IIA, 5.8 / 07	Kitamoto, S.	2012c	Reverse Mutation Test of 5-COOH-S-2200 in Bacterial Systems Sumitomo, Study No. 4222. (Sumitomo ROT-0042) GLP, Unpublished.	Y	SUM
IIA, 5.8 / 08	Kitamoto, S.	2012d	<i>In vitro</i> Chromosomal Aberration Test on 5-COOH- S-2200 in Chinese Hamster Lung Cells (CHL/IU) Sumitomo, Study No. 4220. (Sumitomo ROT-0047) GLP, Unpublished.	Y	SUM
IIA, 5.8 / 09	Wollny, H-E.	2011b	Gene Mutation Assay in Chinese Hamster V79 Cells <i>in vitro</i> (V79/HPRT) with 5-COOH-S-2200 Harlan Cytotest Cell Research GmbH, Study No. 1394802. (Sumitomo ROT-0034) GLP, Unpublished.	Y	SUM
IIA, 5.8 / 10	████████	2012c	Acute Oral Toxicity Study of 2-CH ₂ OH-S-2200 in Rats ████████ Study No. 4242 (Sumitomo ROT-0053) GLP, Unpublished.	Y	SUM
IIA, 5.8 / 11	Kitamoto, S.	2012e	Reverse Mutation Test of 2-CH ₂ OH-S-2200 in Bacterial Systems Sumitomo, Study No. 4226. (Sumitomo ROT-0048) GLP, Unpublished.	Y	SUM
IIA, 5.8 / 12	████████	2012d	Acute Oral Toxicity Study of 4-OH-S-2200 in Rats ████████ Study No. 4241 (Sumitomo ROT-0054) GLP, Unpublished.	Y	SUM
IIA, 5.8 / 13	Kitamoto, S.	2012f	Reverse Mutation Test of 4-OH-S-2200 in Bacterial Systems Sumitomo, Study No. 4227. (Sumitomo ROT-0049) GLP, Unpublished.	Y	SUM

Annex point/ reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed Y/N-R/NR	Owner
IIA, 5.8 / 14	██████████	2011	Acute Oral Toxicity Study of De-Xy-S-2200 in Rats ██████████ Study No. 4225 (Sumitomo ROT-0031) GLP, Unpublished.	Y	SUM
IIA, 5.8 / 15	Kitamoto, S.	2011	Reverse Mutation Test of De-Xy-S-2200 in Bacterial Systems Sumitomo, Study No. 4223. (Sumitomo ROT-0038) GLP, Unpublished.	Y	SUM
IIA 5.9/ 01	Nishioka, K.	2012	Statement from S-2200 Manufacturer Sumitomo, Study No. none (Sumitomo ROT-0074) Non-GLP, Unpublished	Y	SUM
IIA, 5.10.1 / 01	██████████	2011a	S-2200TG – A 28-Day Dietary Dose Range- Finding Study in Wistar Han Rats ██ ██████████ (Sumitomo ROT-0035) Non-GLP, Unpublished.	Y	SUM
IIA, 5.10.1 / 02	██████████	2011b	S-2200TG – A 28-Day Oral (Dietary) Immunotoxicity Study in Female Wistar Han Rats ██ ██████████ (Sumitomo ROT-0039) GLP, Unpublished.	Y	SUM
Annex III Data and Information					
IIIA, 7.1.1/01	██████████	2011a	Acute oral toxicity study of S-2200 25SC in rats ██ ██ Sumitomo Chemical Co., Ltd., Report No. ROT- 0029 GLP, Unpublished	Y	SUM
IIIA, 7.1.2/01	██████████	2011b	Acute dermal toxicity study of S-2200 25SC in rats ██ ██ Sumitomo Chemical Co., Ltd., Report No. ROT- 0030 GLP, Unpublished	Y	SUM
IIIA, 7.1.3/01	██████████	2011	S-2200 25SC: An acute inhalation toxicity study in Sprague-Dawley rats ██ ██ Sumitomo Chemical Co., Ltd., Report No. ROT- 0032 GLP, Unpublished	Y	SUM

Annex point/ reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed Y/N-R/NR	Owner
IIIA, 7.1.4/01	██████	2011a	A skin irritation study of S-2200 25SC in rabbits ██ ██ Sumitomo Chemical Co., Ltd., Report No. ROT-0026 GLP, Unpublished	Y	SUM
IIIA, 7.1.5/01	██████	2011b	An eye irritation study of S-2200 25SC in rabbits ██ ██ Sumitomo Chemical Co., Ltd., Report No. ROT-0027 GLP, Unpublished	Y	SUM
IIIA, 7.1.6/01	██████	2011c	A skin sensitization study of S-2200 25SC in guinea pigs (Buehler test) ██ ██ Sumitomo Chemical Co., Ltd., Report No. ROT-0028 GLP, Unpublished	Y	SUM
IIIA, 7.6.2/01	Hadfield N.	2011	S-2200 25SC: <i>In vitro</i> absorption through human epidermis using [¹⁴ C]S-2200 Dermal Technology Laboratory Ltd., UK; Study No. JV2146 Sumitomo Chemical Co., Ltd., Report No. ROM-0024 GLP, Unpublished	Y	SUM