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VOLUME 3 – Annex B (AS)

- Flutolanil -

B.6 Toxicology and metabolism

Rapporteur Member State: The Netherlands

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Draft Assessment Report and Proposed decision of the Netherlands prepared in the context of the possible approval of Flutolanilunder Regulation (EC) 1107/2009

Version history page

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B.6 Toxicology and metabolism data

This document supports the application for renewal of registration of the active substance flutolanil, currently approved under Commission Regulation (EU) 1107/2009.

Data that has been reviewed under Uniform Principles (UP) for the first approval of flutolanil under Directive 91/414/EEC and inclusion in Annex I, are considered relevant for this renewal. The evaluations of these data were presented in the Draft Assessment Report (DAR) for flutolanil (Finland, May 2005) and in an Addendum thereto (January 2008). New studies that were not included in the DAR are also summarized here, where necessary.

For a detailed overview of analysis methods please refer to section B.5.

B.6.1 Absorption, distribution, metabolism and excretion in mammals

B.6.1.1 Absorption, distribution, metabolism and excretion by oral route

B.6.1.1.1 Absorption, distribution, metabolism and excretion by oral route by single and exposure, study 1

Previous evaluation	DAR (May 2005) and addendum 1 to DAR (October 2006)
Evaluation of the RMS	No comments on the original evaluation.

reference	:	Murakami N, *et al.* 1983	exposure	:	Single dose administered by gavage
Report number	:	56-076-(1) (T-3021); also published in J. Pesticide Science 8, 483-491 (1983)	doses	:	20 and 100 mg/kg bw
test substance	:	^{14}C-flutalonil	GLP	:	no
species	:	Sprague-Dawley rats	guideline	:	In general complies with OECD 417
group size	:	3/ dose	acceptability	:	acceptable

Test guideline and GLP

The study is a published scientific paper. The study was not performed using an official guideline or test method, and it was not done under GLP. The study was published in 1983 before GLP was required and, in general the method used complies with OECD guideline 417 (Directive 67/548/EEC, Annex V, Method B.36). However, only three animals in each dose group were used and distribution and metabolism studies were performed with low dose group at 20 mg/kg bw only.

Study design

[Aniline ring-U-^{14}C] flutolanil (Specific activity: 11 mCi/mmol, Radiochemical purity: >99%) in 0.5 ml olive oil was orally administered by gavage to fasted male Sprague-Dawley rats at the dose levels of 20 and 100 mg/kg bw. Three animals were allocated to each group. Urine and faeces were separately collected for 5 days. Blood was collected from the tail vein of the animals at various times between 15 min and 48 hr after dosing. For analysis of radiocarbon distribution in tissues and organs, rats were sacrificed at 0.5, 2, 12 or 72 hr after the 20 mg bw dose administration and 10 major tissues were obtained. Radioactivity and metabolites in blood, urine, faeces and tissues was investigated, after

appropriate extraction and processing, by comparison with standards by thin-layer chromatography, autoradiography and liquid scintillation counting.

Results

Absorption: Radioactivity concentrations in blood reached their maximum at 2 hr post dose in both low and high dose groups. The maximum concentrations were 4.18 and 12.48 µg flutolanil equivalent/ml by 20 and 100 mg/kg dosing, respectively. Afterwards, elimination of radioactivity from blood was found to be rapid. At 6-8 hr post dose, blood radioactivity decreased to about half of the maximum levels described above. Much lower levels, 0.06 and 0.31 µg flutolanil equivalent/ml for 20 and 100 mg/kg dosing, were detected at 48 hr post dose.

Distribution: In parallel to the change of blood radioactivity, highest radioactivity levels in tissues and organs were detected at 2 hr post dose then decreased to below or around detection minimum at 72 hr post-dose. Among tissues and organs examined, liver and kidney showed the highest radioactivity levels throughout the study (table B.6.1.1.1-1). A similar result was obtained by whole-body autoradiography.

Table B.6.1.1.1-1: Radiocarbon distribution in tissues (or organs) after oral administration of ^{14}C-flutolanil at the dose of 20 mg/kg to rats (Murakami *et al.* 1983)

Tissue (or ogan)	µg ^{14}C-flutonlanil equivalent/g wet tissue (or organ) [a]			
	Hours after administration			
	0.5	2	12	72
Adrenal	0.4	0.9 ±0.4	0.3 ±0.1	<0.2[b]
Brain	≤0.05	0.08±0.04	≤0.04	<0.04
Fat	0.22±0.05	0.77±0.28	0.30±0.10	<0.15
Heart	0.34±0.11	0.51±0.16	0.25±0.15	<0.04
Kidney	4.14±1.76	10.17±3.17	3.44±1.61	0.05±0.02
Liver	8.50±2.14	15.38±2.79	7.78±1.76	0.85±0.15
Lung	0.39±0.06	1.43±0.74	0.48±0.22	<0.04
Muscle	0.10±0.01	0.31±0.14	0.12±0.06	<0.04
Spleen	0.23±0.04	0.47±0.14	0.19±0.07	<0.04
Testis	0.12±0.09	0.47±0.15	0.25±0.09	<0.04

[a] Each value represents the mean ± SD of 3 rats. [b] Lower than detection limit

Excretion: No significant radioactivity excretion into expired air was observed. More than 80% of the administrated radioactivity was excreted within 24 hr. The majority of radioactivity was excreted via urine. Excretion of radioactivity into urine and faeces during the first 72 hr post-dose is summarized in table B.6..1.1.1-2.

Table B.6.1.1.1-2: Mean radioactivity in excretes of rats fed radiolabelled flutolanil after 72 h

DOSE (MG/KG)	MEAN RADIOACTIVITY EXCRETED (% OF ADMINISTERED DOSE)		
	Urine	Faeces	Total
20	69.1	26.4	95.5
100	66.5	29.6	96.0

Table B.6.1.1.1-3: Cumulative ^{14}C excretion into bile from rats orally administered 20 mg/kg of ^{14}C-flutolanil (Murakami *et al.,* 1983)

Hours after administration	Cumulative excretion (% of dose)*
1	0.28
2	0.95
3	2.29
4	4.99
6	9.34
8	14.74
24	34.34

*Each value represents the mean of two experiments

Metabolism: Glucuronide and sulphate conjugates of α,α,α-trifluoro-3'-hydroxy-o-toluanilide (DIP/M-4) were found in urine as major metabolites (see Table B.6.1.1.1-4). Conjugates of α,α,α-trifluoro-4'-hydroxy-3'-isopropoxy-o-toluanilide (HFT/M-2) and α,α,α-trifluoro-4'-hydroxy-3'-methoxy-o-toluanilide (HMD/M-7) were also detected as urinary metabolites. There was no evidence for cleavage of the anilide bond. In faeces, on the other hand, free metabolites without conjugation such as unchanged flutolanil, M-2 and M-4 were found. In addition to those metabolites mentioned above, 2-[3-(α,α,α-trifluoro-o-toluoylamino)phenoxy]propionic acid (M-11) was assumed to be present both in urine and faeces at lower but significant levels. None of the unidentified metabolite in urine and faeces exceeded 5% of the administered dose. From the urine, 2.3% of the administered 14C was recovered as unchanged flutolanil, which was more than that in the faeces (1.22%).

Table B.6.1.1.1-4: urinary and feacal metabolites in rat exposued to 20 mg/kg bw flutolanil, 72 hours following exposure

Compounds	% of administered radioactivity (0-72 hours)								
	urine				feces				Sum of free, glucuronide and sulfate metabolites
	free	glucuronide	sulfate	HCl hydrolyzed	free	glucuronide	sulfate	HCl hydrolyzed	
Flutolanil	2.3	-	-	-	1.2	-	-	-	3.5
M-1	-	-	-	-	0.8	-	-	-	0.8
M-2	-	-	0.2	-	-	-	-	-	0.2
HFT	0.1	1.2	0.5	0.5	2.1	-	-	-	3.9
M-3	-	-	0.3	-	0.3	-	-	-	0.6
M-4	-	0.2	-	-	-	-	-	-	0.2
DIP	1.2	12.1	37.3	46.6	5.2	0.3	0.8	0.4	56.9
HMD	0.2	0.6	1.4	4.3	-	-	0.1	0.1	2.3
M-5	-	-	-	0.2	-	-	-	-	-
M-6	0.8	-	0.3	0.4	0.8	0.1	-	0.1	2.0
M-7	-	-	-	-	-	-	-	0.2	-
M-8	-	-	-	-	2.4	0.1	-	0.2	2.5
M-9	-	0.3	-	-	-	-	-	-	0.3
origin	1.1	1.3	1.9	1.3	2.3	1.8	1.5	2.7	9.9
total	5.7	15.7	41.9	53.3	15.1	2.3	2.4	3.7	83.1

Conclusion

Flutolanil was rapidly excreted into urine and faeces as metabolites and it was not retained in specific tissue or organ. The study is acceptable.

B.6.1.1.2 Absorption, distribution, metabolism and excretion by oral route by single and repeated exposure, study 2

Previous evaluation	DAR (May 2005)
Evaluation of the RMS	No comments on the original evaluation.

reference	:	[redacted] 1992 (original report) and [redacted], 1994 (addendum)	exposure	:	Single and repeated exposure by oral gavage
Report number	:	[redacted] 90076 (MRID Nos. 42606602 and 43452301) (T-3068)	doses	:	20 mg/kg and 1000 mg/kg
test substance	:	^{14}C flutalonil	GLP	:	yes
species	:	Sprague Dawley rats (CD strain)	guideline	:	Complies with OECD guideline 417
group size	:	3 groups/ 10 rats per group/ 5 per sex	acceptability	:	yes

Test guideline and GLP

The study was done under GLP. It was not performed using any official guideline or test method, but the method used complies with OECD guideline 417 (Directive 67/548/EEC, Annex V, Method B.36).

Study design

[Aniline ring-U-14C] flutolanil (Specific activity: 20.3 mCi/mmol, Radiochemical purity: >97.6%) suspended in vehicle containing 1% Tween 80 and 0.5% carboxymethyl cellulose was orally administered to both male and female CD (Sprague-Dawley derived) rats. Five animals of each sex were allocated to each group. The first group received a single dose of 20 mg/kg, the second group received consecutive doses of non-radiolabelled flutolanil (purity 97.6 or 99.9%) for 14 days followed by a single 14C- flutolanil dose on the 15th day, and the third group received a single dose of 1000 mg/kg. Following 14C- flutolanil administration, urine, faeces and cage wash were collected at selected intervals for 7 days. Exhaled 14C was not measured, as a previous study had indicated that it accounted <0.1% of the administered dose. After the sample collection at 7th day, the animals were sacrificed to obtain blood and tissue samples (12).

Composite urine samples were diluted and extracted three times with ether, the aqueous phases being subjected to acid and alkaline treatment plus acid and enzyme (glucuronidase and sulphatase) hydrolysis. Lyophilized faecal samples were extracted into methanol and then with cold and boiling water; the water-soluble fractions were hydrolysed chemically (with HCL) or enzymically (with glucuronidase and sulphatase) and re-extracted. The presence of metabolites was investigated by comparison with standards by thin-layer chromatography, autoradiography and liquid scintillation counting.

Results

More than 70% of the administered radioactivity was excreted within 24 hr post dose. For rats administered a single dose of 20 mg/kg flutolanil, mean radioactivity in excreta following 24 hours were 77% and 78% in males and females, respectively. For rats administered a single dose of 1000 mg/kg this was 83% and 74%, respectively. Although the mean total radioactivity excreted following administration of 20 mg/kg and 1000 mg/kg is comparable, significant differences can be indicated between the levels excreted in urine and faeces. During the first 24 hours, flutolanil was found to remain mostly unconverted in faeces, while in urine it is converted into M4. Taking into account that the proportion of flutolanil excreted as parent is higher at the top dose level and that the extent of absorption varies with dosing, it is likely that saturation occurs at the top dose level. Recovered radioactivity in urine, faeces and cage wash after 7 days post dose is summarised in table B.6.1.1.2-1. Most of the radioactivity was excreted within 24 h, approximately half of the urinary excretion occurring within 12 h, indicating relatively rapid absorption.

Table B.6.1.1.2-1: Mean radioactivity in excretes of rats fed radiolabelled flutolanil

SEX/DOSE (MG/KG)	MEAN RADIOACTIVITY EXCRETED (% OF ADMINISTERED DOSE)			
	Urine	Faeces	Cage wash	Total
Male/20	44.94	42.02	3.17	90.18
Female/20	40.83	40.74	6.73	88.36
Male/20*	70.27	28.78	3.47	102.55
Female/20*	70.94	31.63	5.88	108.50
Male/1000	6.97	78.28	0.77	86.02
Female/1000	9.83	66.20	2.88	78.92

*Consecutive dosing of non-radiolabelled flutolanil for 14 days followed by single dosing of radiolabelled flutolanil

Radioactivity concentration and distribution in blood and tissues at 7 days post dose was minimal, averaging 0.04-0.06% and 0.01 µg/g, ml, respectively for 20 mg/kg groups and 0.01% and 0.15 µg/g, ml for 1000 mg/kg group. The only tissue in which consistent concentrations were found was the liver, in which the mean concentration was about 10 times higher than those found in blood. Bone (femur, and by inference the marrow) contained similar absolute levels as found in other tissues apart from the liver. Analysis of urine and faeces for metabolites revealed that flutolanil remains mostly unchanged in the faeces and converts to M-4 (α,α,α-trifluoro-3'-hydroxy-o-toluanilide) in the urine during the first 24 hours following dose administration. In the urine, M-4 appears to be present in conjugated form. The concentrations of M-4 were significantly higher in animals dosed repeated at 20 mg/kg bw/day than in animals dosed once. All other metabolites represented <0.3% of the administered dose. There was no evidence of cleavage at the amido bridge. Comparison of the results by sex and dose revealed that females conjugated flutolanil more extensively than males and that repeated dosing increased the extent of metabolism to M-4 and conjugates. The proposed metabolic pathway is presented in figure B.6.1.1.2-1.

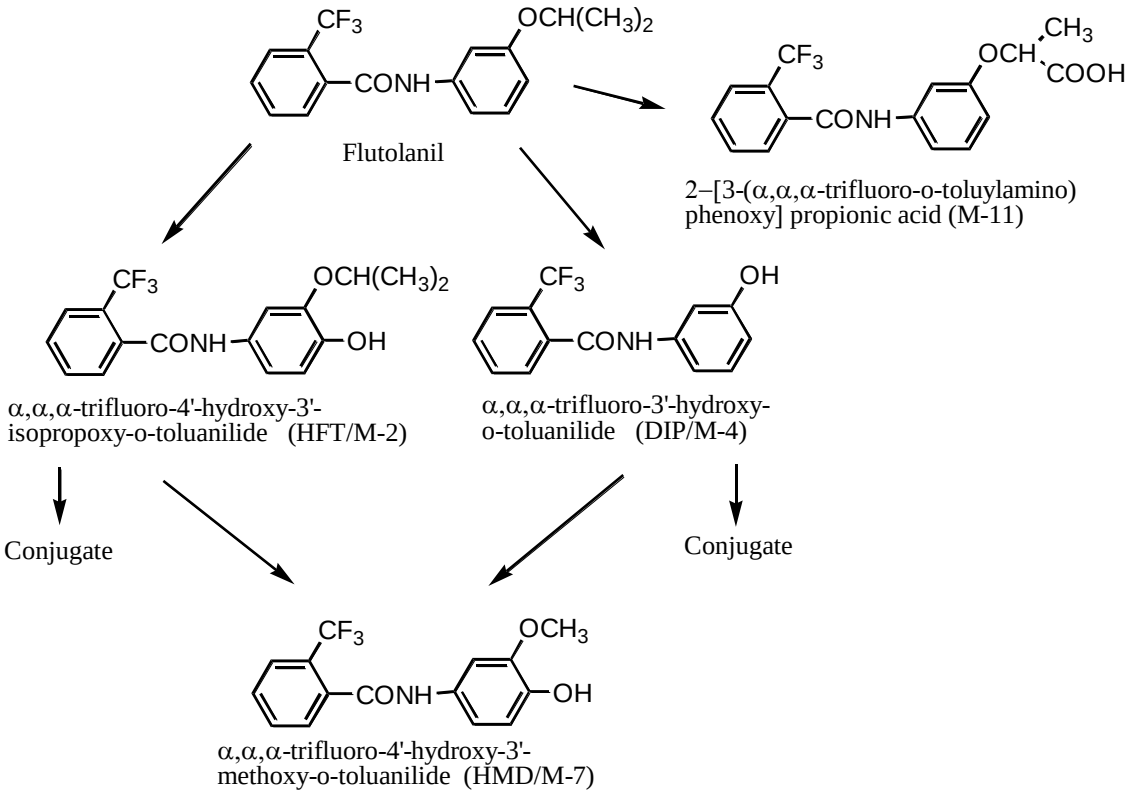


Figure B.6.1.1.2-1: The proposed metabolic pathways of flutolanil ([redacted] 1992)

Table B.6.1.1.2-2: urinary and feacal metabolites in male rats exposed to 20 mg/kg bw flutolanil, 72 hours following exposure – single exposure

Compounds	% of administered radioactivity (0-72 hours)							
	urine				feces			
	free	glucuronide	sulfate	HCl hydrolyzed	free	glucuronide	sulfate	HCl hydrolyzed
Flutolanil	-	-	-	<0.01	31	0.098	0.10	0.34
M-1	-	-	-	0.086	-	-	-	-
M-2	0.07	-	-	-	-	-	-	-
HFT	-	-	-	-	-	-	-	-
M-3	-	-	-	-	-	-	-	-
M-4	1.1	1.0	0.86	16	2.8	0.10	-	0.14
DIP	-	-	-	-	-	-	-	-
HMD	-	-	-	-	-	-	-	-
M-5	-	-	-	0.21	-	-	-	-
M-6	-	-	-	0.043	-	-	-	-
M-7	-	-	-	0.52	-	-	-	-
M-9	-	-	-	-	-	-	-	0.16
M-11	-	-	-	0.13	-	-	-	-
origin	0.67	2.7	2.3	0.98	<0.1	0.10	0.30	0.26

unidentified	1.4	0.2	-	-	0.2	-	-	-
total	3.2	3.9	3.2	18	34	0.30	0.40	0.90

Table B.6.1.1.2-3: urinary and feacal metabolites in female rats exposed to 20 mg/kg bw flutolanil, 72 hours following exposure – single exposure

Compounds	% of administered radioactivity (0-72 hours)							
	urine				feces			
	free	glucuronide	sulfate	HCl hydrolyzed	free	glucuronide	sulfate	HCl hydrolyzed
Flutolanil	-	-	-	0.21	36	0.16	0.14	0.26
M-1	-	-	-	-	-	-	-	-
M-2	-	-	-	-	-	-	-	-
HFT	-	-	-	-	-	-	-	-
M-3	-	-	-	-	-	-	-	-
M-4	0.48	-	1.5	16	1.4	-	-	-
DIP	-	-	-	-	-	-	-	-
HMD	-	-	-	-	-	-	-	-
M-5	-	-	-	0.088	-	-	-	-
M-6	-	-	-	-	-	-	-	-
M-7	0.19	-	-	1.6	-	-	-	-
M-11	-	-	-	-	-	-	-	-
M-9	-	-	-	-	-	-	-	-
origin	1.6	8.8	3.0	2.6	0.00	0.14	0.060	0.24
unidentified	-	-	-	-	0.3	-	-	0.10
total	2.3	8.8	4.5	20	37	0.30	0.20	0.60

Table B.6.1.1.2-3: urinary and feacal metabolites in male rats exposed to 20 mg/kg bw flutolanil, 72 hours following exposure – repeated exposure

Compounds	% of administered radioactivity (0-72 hours)							
	urine				feces			
	free	glucuronide	sulfate	HCl hydrolyzed	free	glucuronide	sulfate	HCl hydrolyzed
Flutolanil	-	-	-	-	24	0.185	0.22	-
M-1	-	-	-	-	-	-	-	-
M-2	-	-	-	-	-	-	-	-
HFT	-	-	-	-	-	-	-	-
M-3	-	-	-	-	-	-	-	-
M-4	1.8	-	5.6	15	1.24	0.045	-	-
DIP	-	-	-	-	-	-	-	-
HMD	-	-	-	-	-	-	-	-
M-5	<0.01	-	-	-	-	-	-	-
M-6	0.028	-	-	-	-	-	-	-
M-7	0.023	-	-	<0.01	-	-	-	-
M-11	-	-	-	-	-	-	-	-

M-9	-	-	-	-	1.1	-	-	-
origin	2.0	0.50	0.50	0.23	<0.01	0.070	0.083	0.50
unidentified	-	1.2	-	-	-	-		0.10
total	3.8	1.7	6.1	15	27	0.30	0.30	0.60

Table B.6.1.1.2-4: urinary and feacal metabolites in female rats exposed to 20 mg/kg bw flutolanil, 72 hours following exposure – repeated exposure

Compounds	% of administered radioactivity (0-72 hours)							
	urine				feces			
	free	glucuronide	sulfate	HCl hydrolyzed	free	glucuronide	sulfate	HCl hydrolyzed
Flutolanil	-	-	-	-	19	0.081	0.17	0.54
M-1	-	-	-	-	-	-	-	-
M-2	-	-	-	-	-	-	-	-
HFT	-	-	-	-	-	-	-	-
M-3	-	-	-	-	-	-	-	-
M-4	3.0	-	11.0	22	4.0	-	-	0.36
DIP	-	-	-	-	-	-	-	-
HMD	-	-	-	-	-	-	-	-
M-5	0.26	-	-	-	-	-	-	-
M-6	-	-	-	-	-	-	-	-
M-7	2.1	-	-	2.1	-	-	-	-
M-11	-	-	-	-	-	-	-	-
M-9	-	-	-	-	-	-	-	-
origin	2.6	1.9	1.3	0.33	<0.01	0.12	-	0.10
unidentified	-	1.1	-	-	0.30	-	0.13	-
total	8.0	3.0	12.3	24	23	0.20	0.30	1.0

Table B.6.1.1.2-5: urinary and feacal metabolites in male rats exposed to 1000 mg/kg bw flutolanil, 72 hours following exposure – single exposure

Compounds	% of administered radioactivity (0-72 hours)							
	urine				feces			
	free	glucuronide	sulfate	HCl hydrolyzed	free	glucuronide	sulfate	HCl hydrolyzed
Flutolanil	-	-	-	-	51	0.37	0.50	0.50
M-1	-	-	-	-	-	-	-	-
M-2	-	-	-	-	-	-	-	-
HFT	-	-	-	-	-	-	-	-
M-3	-	-	-	-	-	-	-	-
M-4	0.52	-	1.0	2.0	0.71	0.090	-	-
DIP	-	-	-	-	-	-	-	-
HMD	-	-	-	-	-	-	-	-
M-5	-	-	-	-	-	-	-	-
M-6	-	-	-	-	-	-	-	-

M-7	-	-	0.019	1.2	-	-	-	-
M-11	-	-	-	-	-	-	-	-
M-9	-	-	-	-	-	-	-	-
origin	0.18	<0.01	<0.01	<0.01	<0.01	0.14	<0.01	<0.01
unidentified	0.10	0.40	-	-	0.10	-	-	-
total	0.80	0.40	1.0	3.2	52	0.60	0.50	0.50

Table B.6.1.1.2-5: urinary and feacal metabolites in female rats exposed to 1000 mg/kg bw flutolanil, 72 hours following exposure – single exposure

Compounds	% of administered radioactivity (0-72 hours)							
	urine				feces			
	free	glucuronide	sulfate	HCl hydrolyzed	free	glucuronide	sulfate	HCl hydrolyzed
Flutolanil	-	-	-	-	36	-	0.087	0.77
M-1	-	-	-	-	-	-	-	-
M-2	-	-	-	-	-	-	-	-
HFT	-	-	-	-	-	-	-	-
M-3	-	-	-	-	-	-	-	-
M-4	0.22	-	1.9	2.3	0.64	-	-	-
DIP	-	-	-	-	-	-	-	-
HMD	-	-	-	-	-	-	-	-
M-5	-	-	-	-	-	-	-	-
M-6	-	-	-	-	-	-	-	-
M-7	0.44	-	-	0.41	-	-	-	-
M-11	<0.01	-	-	-	-	-	-	-
M-9	-	-	-	-	-	-	-	-
origin	0.53	<0.01	0.13	0.14	<0.01	<0.01	0.013	0.030
unidentified	0.20	1.5	0.2	-	0.10	0.20	-	-
total	1.4	1.5	2.2	3.2	36	0.20	0.10	0.80

Conclusion

Flutolanil was rapidly excreted into urine as metabolites and into faeces mainly as unchanged flutolanil, and only a small amount was retained in the tissue at 7 days post dose. The primary metabolite was M-4 (α,α,α-trifluoro-3'-hydroxy-o-toluanilide), which appears to be further conjugated and excreted into urine. The study is acceptable.

B.6.1.1.3 Absorption, distribution, metabolism and excretion by oral route by single and repeated exposure, study 3

Previous evaluation	New study submitted for the purpose of the renewal
Evaluation of the RMS	The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study. The administered dose is relatively low (20 mg/kg),

	but high enough to allow for metabolite identification in excreta and dose selection was justified. The study is considered acceptable.

reference	:	[redacted] (2012)	exposure	:	Oral administration by gavage
Report number	:	LSRC-M11-083A (T-3233)	doses	:	20 mg/ kg
test substance	:	flutalonil	GLP	:	yes
species	:	Sprague dawley rats	guideline	:	In accordance with OECD 417 (2010)
group size	:	4/ sex/ dose	acceptability	:	Acceptable

Executive summary

Sprague-Dawley rats (4/sex) received a single oral dose of ^{14}C-flutolanil (>98% purity) at 20 mg/kg body weight (NOAEL in 90-day repeat oral dosing was approximately 40 mg/kg/day, and the previous ADME study ([redacted] 1992, above) indicated no difference in metabolism dependent on dosage). Urine and faeces were collected every 24 h until 7 days after the final treatment, when the animals were sacrificed. Exhaled ^{14}C-CO_2 was sampled at 24 h after administration.

Recovery of >95% of radioactivity was achieved. Excretion was mainly into urine (male: 50.2%, female 54.6%) and faeces (male 45.8 %, female 45.4%). No significant exhaled ^{14}C-CO_2 was observed up to 24 h after administration.

In urine the major metabolites were:

M-4 (sulphate and glucuronate conjugates), 41% males, 42% females

M-2 and M-7 (sulphate and glucuronate conjugates), to a lesser extent

M-2, M-4, M-7, M-11 and sulfate conjugates of M-5

Trace amounts of M-101 and M-102 produced by cleavage at the amide bond

In faeces, major metabolites were unchanged: flutolanil (27% male, 32% female) followed by M-2 and M-4. Minor metabolites found in faeces were M-5, M-7 and M-11. No bioaccumulation was observed, with rapid excretion of radioactivity into urine and faeces and no significant sex-related differences in metabolic fate. Metabolism was by breakdown of the isopropyl group, hydroxylation of the 4-position in the aniline ring and sulphate or glucuronide-conjugation of the 3,4 position of the aniline ring. There was only limited cleavage of the molecule's backbone. After 7 days, only ≤0.05% of the dose was found in all the organs and tissues.

Materials and methods

Test item:	Flutolanil technical (unlabelled)
Batch Nos.:	8AE0010P
Purity:	99.5%
Expiry:	27 October 2011
Radiochemical:	[phenyl –(U)-^{14}C]-flutolanil, Lot 0AE0002S-R, purity >99.7%, activity 2.37 GBq/mmol
Vehicle:	0.5% aqueous carboxymethylcellulose (CMC) with 1% Tween 80
Animals:	Sprague-Dawley rat, 7 weeks old ([redacted])
Study start and completion:	04 April 2011 to 13 March 2012

[Phenyl-(U)-^{14}C] flutolanil suspended in aqueous 0.5% w/v sodium carboxymethylcellulose solution containing 0.1% w/v Tween 80 was orally administered to 4 male and 4 female rats at the dose level

of 20 mg/kg body weight. The concentration and homogeneity of the dosing suspensions, the achieved dosages and stability, were confirmed by measuring the radioactivity in individual formulation dose aliquots and in residual formulation remaining after each dose.

The rats were fasted for 15-16 h prior to administration and for 3 h afterwards. All animals were observed immediately after dosing, one hour after dosing and at the time of sample collection, for any clinical signs of toxicity. Urine and faecal samples were collected every 24 h thereafter until 168 h after administration, when the animals were sacrificed for collection of blood, organs and tissue. Blood was taken using heparinized syringes and the samples centrifuged immediately to obtain the plasma. Expired air was sampled at 24 h after administration only, as excretion of radioactivity had not previously been observed after that period.

Prior to counting the study samples, blank vials were used to determine background levels in the liquid scintillation counter (LSC) system, the mean plus four standard deviations was defined as the detection minimum. The radioactivity measurement method was validated, with detection of 98.8 to 102.2% being demonstrated (≥95% for bone). Measurement was performed in duplicate for each test sample.

Metabolites were identified by co-chromatography with analytical standards, using TLC. After development, radioluminography (TLC-RLG) was performed against the relevant reference standard. Confirmation for the main metabolites was with HPLC (retention time) and quantification with plate counting.

Radioactivity concentration was calculated as flutolanil equivalent (µg eq./g or mL):

$(A-B)/W_S/R$

A = Measured value (dpm) by LSC

B = Background calculated value (dpm)

W_S = Weight or volume of sample (g or mL)

R = Specific activity of dose formulation (dpm/µg)

Excretion index was calculated as the percentage of the radioactivity administered:

$(A-B)/W_S \times W_T/C \times 100$

A = Measured value (dpm) by LSC

B = Background calculated value (dpm)

C = Amount of radioactivity administered (dpm)

W_S = Weight or volume of sample (g or mL)

W_T = Total weight or volume of sample (g or mL)

Excretion of each metabolite was calculated as the percentage of radioactivity administered:

$D \times E/100$

D = Excretion rate of radioactivity into fractions subjected to analysis (% of dose)

E = Percentage of each metabolite on the chromatogram (%)

Results

Radiochemical purity, based on the TLC and HPLC results, after purification was 98.35 to 99.96%. For the test item in dosing formulation after administration it was 98.71 to 99.87%.

The distribution of radioactivity in the organs and tissues at 168 h after administration is shown in the table below. The amounts found were only trace, lower than 0.05% of the amount administered, irrespective of gender. No accumulation was indicated.

Table B.6.1.1.3-1: distribution of radioactivity in organs and tissues 168 h post-administration

Organ/tissue	Radioactivity concentration (µg eq./g) [A]			
	Male		Female	
	Mean	S.D.	Mean	S.D.
Blood	<0.01	N.A. [B]	0.02	<0.01
Plasma	N.D. [C]	N.A.	N.D.	N.A.
Brain	N.D. (N.D.)	N.A. (N.A.)	N.D. (N.D.)	N.A. (N.A.)
Pituitary	N.D. (N.D.)	N.A. (N.A.)	N.D. (N.D.)	N.A. (N.A.)
Eye ball	<0.01 (<0.01)	N.A. (N.A.)	<0.01 (<0.01)	N.A. (N.A.)
Thyroid	N.D. (N.D.)	N.A. (N.A.)	0.04 (<0.01)	N.A. (N.A.)
Salivary gland	N.D. (N.D.)	N.A. (N.A.)	<0.01 (<0.01)	N.A. (N.A.)
Heart	N.D. (N.D.)	N.A. (N.A.)	<0.01 (<0.01)	N.A. (N.A.)
Lung	<0.01 (<0.01)	<0.01 (<0.01)	<0.01 (<0.01)	N.A. (N.A.)
Thymus	N.D. (N.D.)	N.A. (N.A.)	<0.01 (<0.01)	N.A. (N.A.)
Liver	0.19 (0.05)	0.05 (0.02)	0.14 (0.04)	0.04 (0.02)
Kidney	0.02 (<0.01)	0.01 (<0.01)	0.03 (<0.01)	0.01 (<0.01)
Adrenal	0.01 (<0.01)	N.A. (N.A.)	N.D. (N.D.)	N.A. (N.A.)
Spleen	N.D. (N.D.)	N.A. (N.A.)	N.D. (N.D.)	N.A. (N.A.)
Pancreas	N.D. (N.D.)	N.A. (N.A.)	<0.01 (<0.01)	N.A. (N.A.)
Stomach	N.D. (N.D.)	N.A. (N.A.)	<0.01 (<0.01)	N.A. (N.A.)
Small intestine	<0.01 (<0.01)	N.A. (N.A.)	<0.01 (<0.01)	N.A. (N.A.)
Large intestine	<0.01 (<0.01)	N.A. (N.A.)	<0.01 (<0.01)	<0.01 (<0.01)
Urinary bladder	N.D. (N.D.)	N.A. (N.A.)	0.01 (<0.01)	N.A. (N.A.)
Testis/uterine	N.D. (N.D.)	N.A. (N.A.)	<0.01 (<0.01)	N.A. (N.A.)
Prostate/ovary	<0.01 (<0.01)	N.A. (N.A.)	<0.01 (<0.01)	N.A. (N.A.)
Fat	N.D.	N.A.	<0.01	N.A.
Muscle	N.D.	N.A.	<0.01	N.A.
Bone marrow	N.D.	N.A.	0.02	N.A.
Bone	N.D.	N.A.	N.D.	N.A.
Gastrointestinal Content	<0.01 (<0.01)	N.A. (N.A.)	<0.01 (<0.01)	N.A. (N.A.)

[A]: Figures are mean of 4 animals. Figure in parentheses are expressed as percentage toward dosed radioactivity (%)

[B]: Not applicable.

[C]: Not detected.

Cumulative excretion into urine, faeces and expired air are shown in the tables below. The values were closely similar for both males and females, and there was no excretion into expired air at 24 h.

Table B.6.1.1.3-2: cumulative excretion into urine, faeces and expired air

Time after administration (hr)	Cumulative excretion (% of dosed radioactivity) [A]					
	20 mg/kg					
	Male			Female		
	Urine	Faeces	Expired air	Urine	Faeces	Expired air
0- 24	46.89	41.78	N.D. [B]	53.52	44.65	N.D.
- 48	49.69	45.56	N.S. [C]	54.31	45.34	N.S.
- 72	50.04	45.74	N.S.	54.47	45.39	N.S.
- 96	50.14	45.78	N.S.	54.55	45.39	N.S.
-120	50.19	45.79	N.S.	54.59	45.39	N.S.
-144	50.22	45.79	N.S.	54.62	45.39	N.S.
-168	50.24	45.79	N.S.	54.64	45.39	N.S.
Cage wash [D]	0.01			0.02		
Carcass	N.E. [E]			N.E.		
Total	96.04			100.05		

[A]: Figures are mean of 4 animals
[B]: Not detected.
[C] : Due to no radioactivity found during 24 hrs after the treatment, no sample are corrected from this time point.
[D]: Corrected at 168 hrs after the treatment.
[E]: Due to high cumulative recovery rate (>95%) during 168 hrs after the treatment, not measured.

Almost all the radioactivity was excreted into urine and faeces. The metabolites in urine and faeces are shown in the following table, those in urine being mostly sulphate and glucoronate conjugates of M-4, while in feces it was mostly unchanged flutolanil. There were no essential differences between the genders.

Table B.6.1.1.3-3: metabolites excreted into urine and faeces

Metabolites	Metabolites excreted (% of dosed radioactivity)[A]			
	Male		Female	
	Urine	Faeces	Urine	Faeces
Flutolanil	N.D. [B]	26.56	N.D.	32.18
M-2	0.10	4.83	0.03	2.26
M-4	0.08	4.28	0.10	3.15
M-5	N.D.	0.42	N.D.	1.44
M-7	0.10	0.41	0.28	1.01
M-11	0.87	0.41	1.20	0.26
M-101	0.06	N.D.	0.06	N.D.
M-102	0.08	N.D.	0.05	N.D.
M-2 sulfate/glucuronate conjugation [C]	2.52 (0.20/2.32)	N.E. [D]	1.60 (0.31/1.29)	N.E.
M-4 sulfate/glucuronate conjugation [C]	40.93 (37.73/3.19)	N.E.	41.56 (36.11/5.45)	N.E.
M-5 sulfate conjugation	0.57	N.E.	0.42	N.E.
M-7 sulfate/glucuronate conjugation [C]	2.65 (1.92/0.73)	N.E.	7.22 (4.71/2.51)	N.E.
Other un-identified metabolites Total	0.40	3.26	0.19	0.71
TLC origin	0.62	0.94	0.80	0.89
Non-extractable fraction	0.01	3.68	0.01	2.75
Total	48.99	44.79	53.52	44.65

[A]: Figures are mean of 4 animals
[B]::Not detected.
[C]: Sum of sulfate conjugate and glucuronate conjugation. Figure in parentheses are detected amount of sulfate conjugate and glucuronate conjugation (sulfate conjugate / glucuronate conjugation).

[D)]: In faeces , radioactivity retained at TLC origin is <5%, so, enzyme-digestion was not conducted.

The proposed metabolic pathway is shown in the following figure. Metabolism was mainly by breakdown of the isopropyl group, hydroxylation of the 4-position in the aniline ring, and by sulphate- or glucuronide-conjugation of the 3,4-position in the aniline ring. Metabolites from oxidation of the isopropoxy-terminal methyl group of the 3-position in the aniline ring, methylation of the hydroxyl group produced by breakdown of the isopropyl group, and cleavage of the flutolanil molecule's backbone, were also detected.

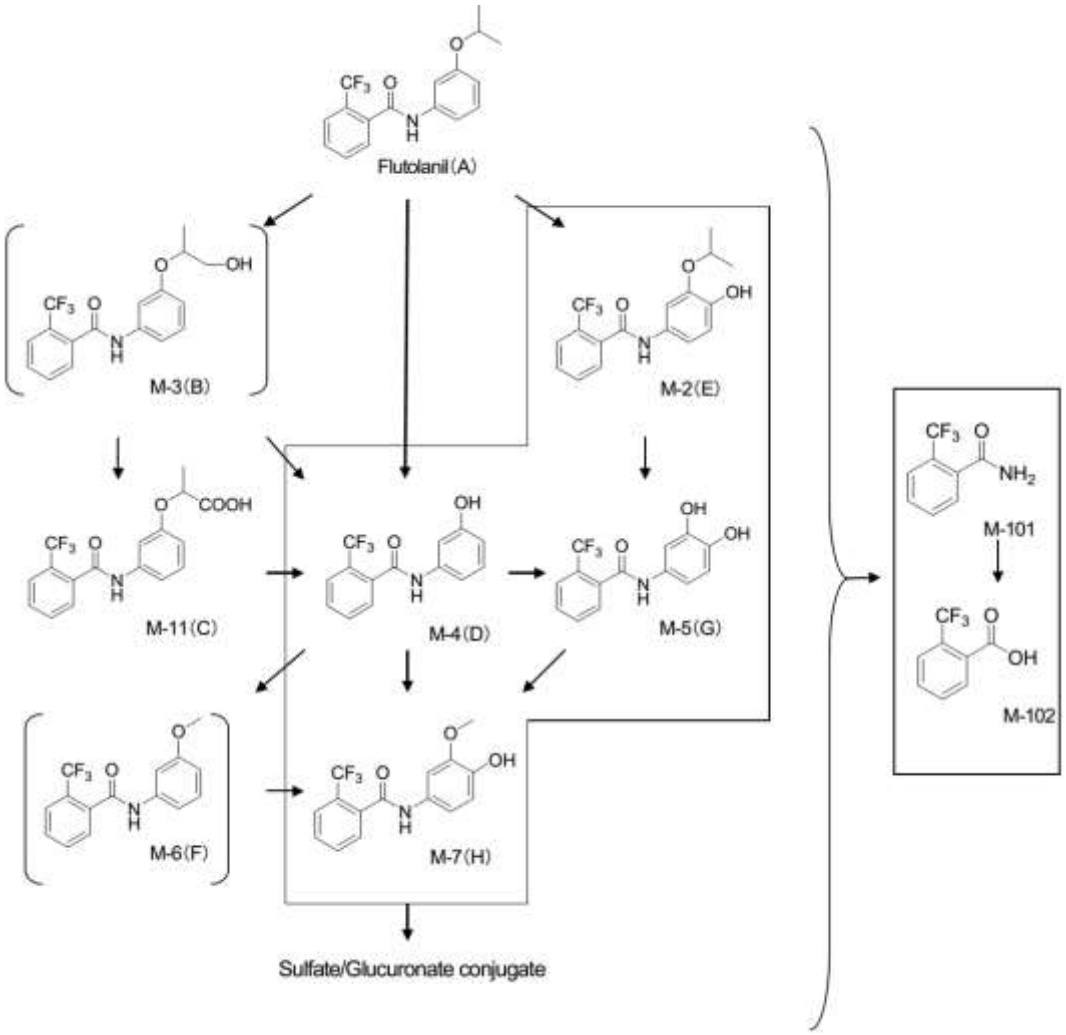


Figure B.6.1.1.3-1: Metabolites in parentheses are proposed intermediates.

Conclusions

The metabolic profile of flutolanil in Sprague-Dawley rats was characterized by:

No bioaccumulation in organs and tissues;

Rapid excretion into urine and feces;

Metabolism by breakdown of the isopropyl group, hydroxylation of the 4-position in the aniline ring, and sulphate- or glucuronide-conjugation of the 3,4-position in the aniline ring;

Limited cleavage of the flutolanil molecule's backbone;

No significant sex-related differences

B.6.1.2 Absorption, distribution, metabolism and excretion by other routes

in vitro comparative metabolism study

Previous evaluation	New study submitted for the purpose of the renewal
Evaluation of the RMS	The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study. The study is considered to provide additional information on the ADME characteristics determined in the in vivo studies.

reference	:	Yasunaga, R (2016)	vehicle	:	acetonitrile
Report number	:	LSRC-M16-031A (T-3243)	doses	:	1 or 50 µM
test substance	:	flutalonil	GLP	:	Yes
test system	:	liver microsomes from rat, mouse, rabbit, dog and the human	guideline	:	No guideline available
			acceptability	:	Acceptable

Executive summary

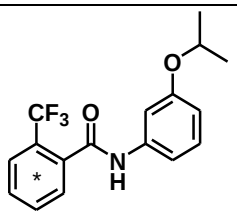
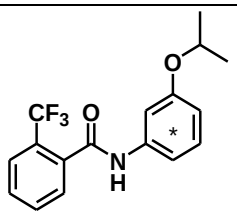
The metabolic profile of flutolanil was examined using liver microsomes from rat, mouse, rabbit, dog and the human, using [phenyl-U-^{14}C] flutolanil and [aniline ring-U-^{14}C] flutolanil. The test substance, in final concentrations of 1 or 50 µM, was incubated with liver microsomes (0.2 nmole of P450) in 0.1M potassium phosphate buffer (pH 7.4) with or without NADPH for 60 minutes at 37 ± 1°C. After the reaction, the reaction mixture was extracted with acetone/methanol (1/1, *v/v*), and then analyzed by TLC and HPLC.

Regardless of species and sex, the major metabolites were M-2 and M-4. In addition, M-3, M-5, M-101 and M-102 were also detected as minor metabolites. No significant human-specific metabolite was detected.

In conclusion, no significant species-related or sex-related difference in metabolic profile of flutolanil was recognized *in vitro*. Furthermore, the results of *in vitro* metabolism of flutolanil by rat liver microsomes was similar to those of the phase I metabolism of the rat ADME studies summarized above ([redacted] 1992 and [redacted] 2012). These results indicate that the metabolic pathway of flutolanil in the human, mouse, rabbit and dog is qualitatively comparable to that shown by the rat ADME studies.

Materials and methods

Test item: Flutolanil (radiolabelled)

Name	[phenyl-U-^{14}C]flutolanil	[aniline ring-U-^{14}C]flutolanil
Structure	 *: labelled position	 *: labelled position
Chemical name (non-labeled)	α, α, α-trifluoro-3'-isopropoxy-*o*-toluanilide	α, α, α-trifluoro-3'-isopropoxy-*o*-toluanilide

Specific activity	2.37 GBq/mmol	3.23 GBq/mmol
Lot number	0AE0002S-R	CP-3778
Storage requirement	<-20ºC, dark	<-20ºC, dark

Test item: Flutolanil standard (unlabelled)

Batch No.: 1AE0012P

Purity: 99.6%

Expiry: 05 April 2017

Storage: < 4°C, dark

Microsomes:

Species	Gender	Strain	Lot No.	Supplier
Rat	Male	Sprague-Dawley	SPA	Bioreclamation IVT (MD, USA)
	Female		TAA	
Mouse	Male	CD-1	EZG	
	Female		XWU	
Rabbit	Female	New Zealand White	LOB	
Dog	Male	Beagle	HSN	
	Female		GOV	
Human	Mixed gender	10 donor	SMX	
		10 donor	THP	
		50 donor	DNK	
		50 donor	LFJ	

All microsomes were stored at -70ºC in the dark

Co-enzyme: NADPH (Roche Diagnostics, Germany). 10 mM solution prepared for use at 1 mM final concentration

Buffer: 0.1 M potassium phosphate, pH 7.4 (0.1 M solutions of monobasic and dibasic, combined)

Study completion: 25 December 2015 to 24 March 2016

Experimental design

The test substance solution and 0.1 M potassium phosphate buffer (pH 7.4) were added to a 13×100 mm glass tube. Then the microsomes (0.2 nmole of P450) were added to each reaction tube and pre-incubated for 5 minutes at 37 ± 1°C. After the reaction was initiated by adding 100 µL of 10mM NADPH solution, the reaction mixture was incubated for 60 minutes at 37 ± 1°C. The test system without NADPH was prepared as in the same way, excepting addition of NADPH. Details of the reaction system are listed below.

Additive samples	**Additive volumes (µL)**	**Final conc.**
Test substance solution	10	1 or 50 µM
10mM NADPH solution	100	1 mM
Microsomes	Optimum volume	0.2 nmole of P450

0.1M potassium phosphate buffer (pH7.4)	Optimum volume	-
Total	1000	-

After incubation the reaction was stopped by adding 2 mL of acetone/methanol (1/1, *v/v*) in each reaction tube. The reaction mixture was then centrifuged at 2,500 rpm for 3 minutes and the supernatants were transferred to measuring flasks. The resulting precipitate was resuspended with 1 mL of acetone/methanol (1/1, *v/v*) and centrifuged at 2,500 rpm for 3 minutes. The resulting supernatants were transferred to measuring flasks and filled up to 5 mL (extracted fraction). An aliquot of extracted fraction (250 µL) was transferred into a counting vial, combined with 2 mL of scintillation cocktail and counted by LSC. The resulting residual precipitate was transferred using appropriate quantities of methanol/ distilled water (2/1, *v/v*) into a counting vial, combined with 10 mL of scintillation cocktail and counted by LSC. An aliquot of extracted fraction (4.5 mL) was dried by N_2 gas, resuspended with 500 µL of methanol/distilled water (2/1, *v/v*) and subjected to metabolite analysis under the conditions described below. Representative extracted fraction was also analyzed by RI-HPLC, also as described below.

All the quantification of radioactivity was done by LSC with 3 minutes counting time. Scintillation counting data (cpm; counts per minute) were automatically converted to amounts of radioactivity (dpm; disintegration per minute) with the installed external standard and quenching library. The quenching library was periodically renewed and accuracy of the instruments was verified daily using commercially available quenching standard. Quantification of radioactivity was done in duplicate, unless sample amounts were limited. Prior to counting the first samples for this study, 10 blank vials were counted by LSC. The mean value was used as the background. The mean plus four times the standard deviation was defined as the detection minimum.

Test substances was purified so as that radiochemical purity of those are >98% as below. Test substance dissolved in ethyl acetate was developed with one-dimensional TLC on the conditions as described below, then the UV position of flutolanil standard on TLC was scraped and extracted with ethyl acetate. Radiochemical purity of the obtained extraction was determined by TLC-RLG and RI-HPLC, also as described below.

Prior to preparation of test substance solutions, radiochemical purity of the test substance was determined by TLC, followed by radioluminography (TLC-RLG) with two different solvent conditions as described below. Analyses with radioanalysis (RI-HPLC) were also performed to confirm the results obtained with TLC-RLG, as also described below.

Test substance solutions ([phenyl-U-^{14}C]flutolanil and [aniline ring-U-^{14}C]flutolanil), concentrations 0.1 mM and 5 mM, were prepared by dilution with non-radiolabelled flutolanil, then dissolving in acetonitrile. Each test substance solution was stored in a freezer (-20°C) and used at 1 µM or 50 µM final concentration. The content of acetonitrile in the test system did not exceed 1% of total volume.

After conduction of last microsomal assay, radiochemical purity of the test substance solutions was determined as described above, to confirm stability within the period of the study.

TLC was done with silica gel $60F_{254}$ (0.25 mm x 20 cm x 20 cm). Developing solvent systems were as follows:

Solvent A: chloroform/methanol/formic acid (90/10/0.5, *v/v/v*)

Solvent B: hexane/ethyl acetate/formic acid (50/50/5, *v/v/v*)

Solvent C: hexane/ethyl acetate (7/3, *v/v*)

Metabolite analysis was done by two-dimensional development with solvent A followed by solvent B.

Determination of radiochemical purity of test substance was done by one-dimensional development with solvent A and solvent B.

Purification of test substance was done by one-dimensional development with solvent C.

TLC-radioluminograms (TLC-RLG) were obtained, analyzed and quantified. Linearity of response and LOQ for radioluminography was verified using a standardized radiation source.

The elution from the HPLC system was collected into LumaPlate® (96 well, interior of each well are coated with solid scintillator) separately for each 0.20 minutes. The obtained plates were dried and counted for radioanalysis.

Results

Radiochemical purity of [phenyl-U-^{14}C]flutolanil before preparation of test substance solutions was determined as ≥99.33% by TLC-RLG analysis and RI-HPLC analysis. Radiochemical purity of [aniline ring-U-^{14}C]flutolanil before preparation of test substance solutions was determined as ≥99.59% by TLC-RLG analysis and RI-HPLC analysis. Radiochemical stability of [phenyl-U-^{14}C]flutolanil solutions within this study was determined as ≥98.13% by TLC-RLG analysis and RI-HPLC analysis. Radiochemical stability of [aniline ring-U-^{14}C]flutolanil solutions within the study was determined as ≥98.48% by TLC-RLG analysis and RI-HPLC analysis. In both TLC and HPLC analysis, chromatographic behaviour of the radioactivity completely agreed with that of the analytical standard of flutolanil.

Recovery of radioactivity: Applied radioactivity of [phenyl-U-^{14}C]flutolanil and [aniline ring-U-^{14}C]flutolanil was quantitatively recovered from each test system. Total recoveries of radioactivity from each test system were 94.30~107.37% of applied radioactivity.

Metabolites produced by liver microsomes were identified by co-TLC with reference standards. Major metabolites were also identified by RI-HPLC with reference standards. All potential metabolites examined were separated from each other under the conditions employed in this study.

The quantification results for the various species are shown in the tables below.

Table B.6.1.2-1: In vitro metabolism of [phenyl-U-^{14}C]flutolanil by liver microsomes (with NADPH)

Metabolites	% of application [A]												
	1µM [phenyl-U-^{14}C] Flutolanil									50µM [phenyl-U-^{14}C] Flutolanil			
	Buffer ctrl	Rat		Mouse		Rabbit	Dog		Human [B]	Buffer ctrl	Rat		Human
		M	F	Male	F	F	M	F	Mixed Gender		M	F	Mixed Gender
Flutolanil	102.58	1.14	1.46	1.09	1.14	1.65	3.98	0.68	4.88	106.34	34.91	69.55	8.62
M-2	N.D. [C]	3.07	4.78	4.75	7.90	61.56	69.76	55.91	7.12	N.D.	10.83	3.62	2.52

M-3	N.D.	2.47	2.30	1.38	1.33	0.50	N.D.	N.D.	2.05	N.D.	2.13	0.91	0.69
M-4	N.D.	58.74	86.66	34.21	45.18	7.43	15.87	15.36	61.35	N.D.	43.59	30.21	66.93
M-5	N.D.	2.82	1.04	1.21	0.80	1.43	0.85	2.09	7.52	N.D.	1.68	N.D.	8.68
M-6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
M-7	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
M-11	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
M-101	N.D.	N.D.	N.D.	3.07	3.96	0.76	N.D.	1.35	N.D.	N.D.	N.D.	N.D.	N.D.
M-102	N.D.	N.D.	N.D.	1.81	1.59	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Others	N.D.	N.D.	N.D.	0.95	2.70	N.D.	1.03	1.88	N.D.	N.D.	N.D.	N.D.	N.D.
Polar metabolites	N.D.	28.87	8.10	33.93	22.61	23.14	10.08	21.49	21.03	N.D.	8.40	1.02	15.39
Un-extractable	0.14	5.31	1.28	14.52	12.02	3.23	2.99	8.61	2.39	0.14	2.05	0.21	2.17
Total	102.72	102.42	105.60	96.92	99.24	99.68	104.56	107.37	106.34	106.48	103.60	105.52	104.99

A: : All values are mean of duplicates.
B: : These values are mean of four lots.
C: : Not detected.

Table B.6.1.2-2: In vitro metabolism of [aniline ring-U-^{14}C]flutolanil by liver microsomes (with NADPH)

Metabolites	% of application [A]											
	1µM [aniline ring-U-^{14}C] Flutolanil									50µM [aniline ring-U-^{14}C] Flutolanil		
	Buffer ctrl	Rat	Mouse			Rabbit	Dog		Human [B]	Buffer ctrl	Rat		Human
		M	F	M	F	F	M	F	Mixed Gender		M	F	Mixed Gender
Flutolanil	98.07	0.85	0.99	0.39	0.28	2.30	5.41	0.63	4.77	105.46	32.89	68.00	9.98
M-2	N.D. [C]	2.52	4.03	4.97	8.39	62.24	69.91	54.58	7.03	N.D.	10.37	3.52	2.27
M-3	N.D.	2.21	2.22	1.37	1.33	0.51	N.D.	N.D.	1.92	N.D.	1.83	0.83	0.59
M-4	N.D.	52.80	78.19	35.03	47.39	7.04	16.38	13.93	58.46	N.D.	42.51	29.18	65.99
M-5	N.D.	3.31	1.82	1.02	0.96	1.70	1.08	2.51	5.43	N.D.	1.38	N.D.	9.29
M-6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
M-7	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
M-11	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Others	N.D.	N.D.	N.D.	1.15	1.55	N.D.	0.83	2.04	N.D.	N.D.	N.D.	N.D.	N.D.
Polar metabolites	N.D.	27.54	7.58	38.02	26.64	24.27	9.59	24.37	21.57	N.D.	10.09	1.03	16.52
Un-extractable	0.13	5.07	1.22	14.87	12.45	3.61	2.80	8.45	2.43	0.22	1.94	0.27	2.14
Total	98.21	94.30	96.06	96.83	98.99	101.66	105.99	106.51	101.62	105.69	101.01	102.82	106.77

A: : All values are mean of duplicates.
B: : These values are mean of four lots.
C: : Not detected.

Rat: Regardless of sex and the radiolabel employed, flutolanil was metabolized by rat liver microsomes with NADPH, and M-2 and M-4 were identified as major metabolites accounting for 2.52-10.83% and 29.18-86.66%, respectively. In addition, M-3 and M-5 were also detected as minor metabolites. Flutolanil was not metabolized in the reaction system without NADPH. In high concentration (50 µM), the metabolic profile was the same as in low concentration (1 µM), except that unchanged flutolanil increased. Polar metabolites (1.02-28.87%) detected by TLC analysis were composed of a number of metabolites.

Mouse: Regardless of sex and the radiolabel employed, flutolanil was metabolized by mouse liver microsomes with NADPH, and M-2 and M-4 were identified as major metabolites accounting for

4.75-8.39% and 34.21-47.39%, respectively. In addition, M-3, M-5, M-101 and M-102 were also detected as minor metabolites. Flutolanil was not metabolized in the reaction system without NADPH.
Rabbit: Regardless of the radiolabel employed, flutolanil was metabolized by rabbit liver microsomes with NADPH, and M-2 and M-4 were identified as major metabolites accounting for 61.56-62.24% and 7.04-7.43%, respectively. In addition, M-3, M-5 and M-101 were also detected as minor metabolites. Flutolanil was not metabolized in the reaction system without NADPH.

Dog: Regardless of sex and the radiolabel employed, flutolanil was metabolized by dog liver microsomes with NADPH, and M-2 and M-4 were identified as major metabolites accounting for 54.58-69.91% and 13.93-16.38%, respectively. In addition, M-5 and M-101 were also detected. Flutolanil was not metabolized in the reaction system without NADPH.

Human: Regardless of lot and the radiolabel employed, flutolanil was metabolized by human liver microsomes with NADPH, and M-2 and M-4 were identified as major metabolites accounting for 2.27-7.12% and 58.46-66.93%, respectively. In addition, M-3 and M-5 were also detected. Flutolanil was not metabolized in the reaction system without NADPH. In high concentration (50 µM), the metabolic profile was the same for the low concentration (1 µM), except that unchanged flutolanil increased. Polar metabolites (15.39-21.57%) detected by TLC analysis were composed of a number of metabolites, accounting for less than 5%, and all of these polar metabolites were also detected in the rat.

Conclusions
The major metabolites were M-2 and M-4. In addition, M-3, M-5, M-101 and M-102 were also detected as minor metabolites. No significant human specific metabolite was detected.
No significant species-related or sex-related difference in the metabolic profile of flutolanil were detected *in vitro*. Furthermore, the *in vitro* metabolism of flutolanil by rat liver microsomes was similar to the phase I metabolism shown in the earlier rat ADME studies summarized above. These results indicate that the metabolic pathway of flutolanil in human, mouse, rabbit and dog are qualitatively comparable to the metabolism determined for the rat.

B.6.2 Acute toxicity

B.6.2.1 Oral

B.6.2.1.1 Oral, study 1

Previous evaluation	DAR (May 2005)
Evaluation of the RMS	OECD 401 was deleted in 2002. However, since the study was carried out prior to December 2002 it is considered to be acceptable. Deviations from the guideline are that the study report does not specify if animals were fasted before dosing and that individual data were not reported. Based on the results of the study no classification is required in accordance with Regulation (EC) No. 1272/2008. This is in accordance with study 3 where it was indicated that the animals were fasted prior to dosing.

reference	:	[redacted], 1982a	exposure	:	Single administration via 4 routes: oral, intraperitoneal, subcutaneous and percutaneous
Report number	:	T-3001	doses	:	1,000 to 10,000 mg/kg
test substance		NNF-136 (Flutalonil)	GLP	:	no
species	:	Fisher rats	guideline	:	Generally complies with OECD 401 and 402
group size	:	10/ sex/ dose level/ route of administration	acceptability	:	Acceptable

Test guideline and GLP

The study was performed using the guideline of the Japanese Ministry of Agriculture,
Forestry and Fisheries (JMAFF). It was not done under GLP. Moreover, the acute toxicity of the test substance was evaluated through four routes in one study. However, the study was performed in 1982 before GLP was required and the test methods generally comply with OECD guidelines 401 and 402 (Directive 67/548/EEC, Annex V, Methods B.1 and B.3).

Study design

Acute toxicity with NNF-136 (98.2% flutolanil) was studied in Fischer rats by single administration through oral, intraperitoneal, subcutaneous and percutaneous routes. Ten males and ten females were used per treatment. For oral, intraperitoneal and subcutaneous administrations, the dose levels received by the animals were 5120, 6400, 8000 and 10 000 mg/kg bw. Oral administration was performed into the stomach with gastric tube (vehicle 1% Tween 80). Subcutaneous injection was given on the dorsocervical region of the animals. For percutaneous administration, animals received dose levels of 1000, 3000 and 5000 mg/kg bw. The test compound was applied on the centre of the dorsal region of animals. Clinical signs were observed immediately 1, 3 and 6 hours after the treatment. From the next day to the termination of the study (14 days), observations were performed twice daily. All animals were autopsied at the termination of the study and gross abnormalities were examined macroscopically.

Results

Oral administration: Sedation began to be observed from about 1 hour after the treatment and disappeared by 1 day after treatment. No animals died and no gross abnormalities were found at autopsy.

Intraperitoneal administration: Sedation and lacrymation began from about 1 hour after the treatment. Symptoms disappeared by 2 days after the treatment. No animals died. At autopsy, almost all animals showed swelling and adhesion of all lobes of the liver. A few cases at the high dose level showed swelling of the spleen.

Subcutaneous administration: No clinical signs were observed. No animals died and no gross abnormalities were found at autopsy.

Percutaneous administration: Sedation began to be observed from about 1 hour after the treatment and disappeared by 1 day after treatment. No animals died and no gross abnormalities were found at autopsy.

Conclusion

The acute oral, intraperitoneal, subcutaneous and percutaneous toxicity of flutolanil to rats was low. The LD50 is greater than 10 000 mg/kg bw via oral, intraperitoneal and subcutaneous administration and greater than 5000 mg/kg bw via percutaneous administration. The study is acceptable.

B.6.2.1.2 Oral, study 2

Previous evaluation	DAR (May 2005)
Evaluation of the RMS	OECD 401 was deleted in 2002. However, since the study was carried out prior to December 2002 it is considered to be acceptable. Deviations from OECD 401 are that the study report does not specify if animals were fasted before dosing and individual data were not reported. Based on the results of the study no classification is required in accordance with Regulation (EC) No. 1272/2008. This is in accordance with study 3 where it was indicated that the animals were fasted prior to dosing.

reference	:	[redacted] 1982b	exposure	:	Single administration via oral, intraperitoneal and subcutaneous
Report number	:	T-3002	doses	:	5,120 to 10,000 mg/kg
test substance		NNF-136 (Flutalonil)	GLP	:	No
species	:	ICR mice	guideline	:	Generally complies with OECD 401
group size	:	10/ sex/ dose level/ route of administration	acceptability	:	Acceptable

Test guideline and GLP

The study was not performed using any official guideline or test method, and it was not done under GLP. Moreover, the acute toxicity of test substance was evaluated through three routes in one study. However, the study was performed in 1982 before GLP was required and the test methods generally comply with OECD guidelines 401 (Directive 67/548/EEC, Annex V, Methods B.1).

Study design

Acute toxicity with NNF-136 (98.2% flutolanil) was studied in ICR mice by single administration through the oral, intraperitoneal and subcutaneous routes. Ten males and ten females were used per treatment. For oral, intraperitoneal and subcutaneous administrations, dose levels that animals received were 5120, 6400, 8000 and 10 000 mg/kg bw. Oral administration was given into the stomach with a gastric tube (vehicle 1% Tween 80). Subcutaneous injections were given on the dorsocervical region of the animals. Clinical signs of animals were observed immediately 1, 3 and 6 hours after the treatment. From the next day to the termination of the study (14 days), observations were performed twice daily. All animals were autopsied at the termination of the study and gross abnormalities were examined macroscopically.

Results

After oral and intraperitoneal administrations, sedation and decreased locomotor movement began to be observed immediately after the treatment. These symptoms disappeared by day 1 after treatment. No deaths were induced in any of the treated groups. At autopsy, no gross abnormalities were found following oral or subcutaneous administration. Following intraperitoneal administration, swelling of the liver was found in many cases in all dose groups. In addition, a few animals showed adhesion of the stomach, spleen and liver. The majority of the animals in the high dose group showed adhesion of lobes of the liver.

Conclusion

The acute oral, intraperitoneal and subcutaneous toxicity of flutolanil to mice was low. The LD50 is greater than 10 000 mg/kg bw via oral, intraperitoneal and subcutaneous administration. The study is acceptable.

B.6.2.1.3 Oral, study 3

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study. The study is considered acceptable. Based on the results of the study no classification is required in accordance with Regulation (EC) No. 1272/2008.

reference	:	[redacted] (2009)	exposure	:	Oral gavage
Report number	:	T-3182	doses	:	2000 mg/kg
test substance	:	Flutolanil (technical grade)	GLP	:	yes
species	:	Wistar rats (female)	guideline	:	In accordance with OECD 420 (2001)
group size	:	1/ sighting study, 4/main study	acceptability	:	Acceptable

Executive summary

The fixed dose procedure was used to assess the acute oral toxicity of flutolanil technical (98.5% purity) in Wistar rats. The test item was suspended in corn oil and administered by oral gavage. A sighting study with a single female animal at 2000 mg/kg body weight was followed by a main study with four females at the same dosage. The animals were observed for a 14-day post-dosing period and necropsied at 14 days after dosing.

There were no mortalities or abnormal clinical signs throughout, and there were no treatment related gross necropsy findings.

It was concluded that the acute oral LD_{50} for flutolanil technical in rats was >2000 mg/kg body weight. It would be 'not classified' according to current GHS criteria.

Study design

Test item: Flutolanil technical

Batch No.:	2AE0008P
Purity:	98.5%
Expiry:	17 January 2010

Description:	White powder
Storage:	Room temperature, in the dark
Vehicle:	Corn oil (Nacalai Tesque Inc., Japan)
Animals:	Female Wistar rats (RccHan:WIST), [redacted]
	8-9 weeks of age at treatment, 163-184 g
Study start and completion:	01 October 2009 to 25 December 2009

Dosing suspensions were prepared just before administration, and were visibly homogeneous at the intended concentration of 20% (w/v). The animals were deprived of food (not water) overnight (16-17 h) prior to dosing. Dosing suspension was administered in the morning on day 0, once to each animal at a volume of 10 mL/kg body weight by oral gavage, according to the body weight just before dosing, and the animals continued to be deprived of food (not water) for the subsequent 3 h.
In a sighting study, a single animal received 2000 mg/kg in a single administration, then 5 days later a further 4 females, forming the main study, received the same dosage. The animals were observed for clinical signs before and just after dosing, then at 0.25, 0.5, 1, 3 and 6 h after dosing, then once daily for the subsequent 14 days. Body weights were recorded prior to fasting, then just before administration (day 0) and days 1, 7 and 14 post dosing. The animals were humanely killed and subjected to gross necropsy after the final observation on day 14.

Results

There were no mortalities and no abnormal clinical signs, throughout. Body weight decreased consequent on fasting, then steadily increased from before dosing until termination.

Table B.6.2.3.1-1 body weight of flutolanil technical grade treated rats

Dose (mg/kg)	Animal number	Body weight (g)				
		Before fasting	Before dosing	Day 1[a)]	Day 7	Day 14
	01F01	183.8	165.8	185.9	204.6	218.7
	01F02	192.3	177.1	193.4	207.6	218.5
2000	01F03	192.3	178.3	192.5	213.3	227.2
	01F04	190.5	175.2	201.9	213.9	225.1
	01F05	181.3	167.0	191.2	194.2	208.5
	Mean	188.0	172.7	193.0	206.7	219.6
	S.D	5.1	5.9	5.8	8.0	7.3

a): Days after administration, S.D.: Standard deviation

Results in sighting study (01F01) and in main study (01F02-01F05) are combined in this table.

No abnormal/treatment related findings were recorded at necropsy for any animal.

Conclusion

The acute oral LD_{50} for flutolanil technical in rats was >2000 mg/kg body weight. It would be 'not classified' according to current GHS criteria.

B.6.2.2 Dermal

B.6.2.2.1 Dermal, study 1

Previous evaluation	DAR (May 2005)
Evaluation of the RMS	The study was evaluated in the DAR and was considered acceptable. However, with regard to the dermal exposure route several details are lacking. That is, based on the study report it remains unclear if the test substance was pulverised and moistened to ensure good contact with the skin, what the duration of exposure was, how much of the body surface was exposed and if semi-occlusive dressing was applied following percutaneous exposure. Therefore, the study is considered as supplementary for the dermal route. Based on the results of the study no classification is required in accordance with Regulation (EC) No. 1272/2008.

reference	:	[redacted], 1982a	exposure	:	Single administration via 4 routes: oral, intraperitoneal, subcutaneous and percutaneous
Report number	:	T-3001	doses	:	1,000 to 10,000 mg/kg
test substance		NNF-136 (Flutalonil)	GLP	:	no
species	:	Fisher rats	guideline	:	Generally complies with OECD 401 and 402
group size	:	10/ sex/ dose level/ route of administration	acceptability	:	supplemental

For details please refer to B.6.2.1.1 for the study of Kosaka and Saito (1982a) determining the acute toxicity of flutolanil in rats through four routes of exposure including the dermal route. In short, for percutaneous administration an LD_{50} > 5000 mg/kg bw was found.

B.6.2.2.2 Dermal, study 2

Previous evaluation	DAR (May 2005)
Evaluation of the RMS	No comments on the original evaluation. OECD 401 was deleted in 2002. However, since the study was carried out prior to December 2002 it is considered to be acceptable. Deviations from OECD 401 are that the study report does not specify if animals were fasted before dosing and individual data were not reported.

reference	:	[redacted], 1982b	exposure	:	Single administration via oral, intraperitoneal and subcutaneous
Report number	:	T-3002	doses	:	5,120 to 10,000 mg/kg
test substance		NNF-136 (Flutalonil)	GLP	:	No
species	:	ICR mice	guideline	:	Generally complies with OECD 401
group size	:	10/ sex/ dose level/ route of administration	acceptability	:	Acceptable

For details please refer to B.6.2.1.2 for the study of Kosaka and Saito (1982a) determining the acute toxicity of flutolanil in rats through four routes of exposure including the dermal route.

B.6.2.2.1 Dermal, study 3

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study. In the study an occlusive dressing was used instead of an semi-occlusive porous gauze dressing and non-irritating tape. Nevertheless, since no (local) effects were observed the study is considered acceptable. Based on the results of the study no classification is required in accordance with Regulation (EC) No. 1272/2008.

reference	:	[redacted](2009)	exposure	:	Single dermal application
Report number	:	T-3183	doses	:	2000 mg/kg
test substance		Flutolanil (technical grade)	GLP	:	yes
species	:	Wistar rats (male and female)	guideline	:	in accordance with OECD 402
group size	:	5 animals/ sex	acceptability	:	acceptable

Executive summary

Flutolanil technical (98.5% purity), moistened with distilled water, was applied dermally to each of 5 male and 5 female Wistar rats at a dosage of 2000 mg/kg body weight, under an occlusive dressing for 24 h. The animals were observed and weighed during the subsequent 14 days, and then necropsied.

There were no mortalities, clinical signs or local skin reactions.

It was concluded that the acute dermal LD_{50} for flutolanil technical in rats was >2000 mg/kg body weight. It would be 'not classified' according to current GHS criteria.

Study design

Test item:	Flutolanil technical
Batch No.:	2AE0008P
Purity:	98.5%
Expiry:	17 January 2010
Description:	White powder
Storage:	Room temperature, in the dark
Animals:	Wistar rats (RccHan:WIST), [redacted] 9 weeks of age, 261-287g (males) and 12 weeks of age, 218-229 g (females) at treatment
Study start and completion:	02 October 2009 to 10 December 2009

A single group of 5 males and 5 females was formed. The dorsal skin hair was removed using an electric clipper one day prior to dose application. The required amount of flutolanil technical for each animal was weighed (2000 mg/kg body weight) and ground in a mortar with 2.5 mL distilled water to form a paste, which was applied uniformly to a 4x5 cm piece of gauze (representing approximately 10% of the body surface area), applied to the clipped dorsal skin. The gauze was covered with gum sheet and secured with adhesive elastic tape. The animals were then returned to their individual home cages. After 24 h of exposure the gauze was removed and any remaining test item was cleansed from the skin using cotton wool moistened with warm water.

The animals were observed immediately before and after application, then at 15 min, 30 min, 1, 3, 6 and 24 h, then daily for the subsequent 14 days. Body weights were recorded prior to assignment to the group and then on days 1, 7 and 14 after dose application. The animals were humanely killed after the final observations on day 14 and subjected to gross necropsy.

Results

There were no mortalities, clinical signs or local skin reactions in any animal throughout.

Body weights were decreased at one day after the application, a change considered to have been caused by the stress involved in the dosing procedure. Thereafter, body weights increased uniformly.

Table B.6.2.2.1-1: Body weight of rats dermally treated with flutolanil

		Body weight (g)			
Group	**Anima No.**	**Initial**	**Day 1**	**Day 7**	**Day 14**
Male (2000mg/kg)	01M01	273.1	268.2	299.3	330.1
	01M02	268.0	261.6	293.0	314.1
	01M03	266.2	259.0	284.7	312.4
	01M04	260.5	257.8	273.6	294.9
	01M05	286.8	262.9	319.6	348.9
	mean ± SD	270.9 ± 10.0	261.9 ± 4.1	294.0 ± 17.3	320.1 ± 20.4
Female (2000mg/kg)	01F01	229.3	224.5	238.3	235.2
	01F02	222.0	219.4	227.1	233.6
	01F03	221.0	218.8	236.5	238.5
	01F04	218.8	207.9	227.5	229.1
	01F05	217.9	207.2	232.7	235.8
	mean + SD	221.8 ± 4.5	215.6 ± 7.7	232.4 ± 5.1	234.4 ± 3.5

There were no abnormal/treatment related findings at necropsy for any animal.

Conclusion

The acute dermal LD_{50} for flutolanil technical in rats was >2000 mg/kg body weight. It would be 'not classified' according to current GHS criteria.

B.6.2.3 Inhalation

B.6.2.3.1 Inhalation, study 1

Previous evaluation	DAR (May 2005)
Evaluation of the RMS	No comments on the original evaluation. Based on the results of the study no classification is required in accordance with Regulation (EC) No. 1272/2008.

reference	:	[redacted] 1984	exposure	:	Inhalation (aerosols, 4 h exposure)
Report number	:	T-3003	doses	:	4.50 and 5.98 mg/L
test substance	:	Flutolanil	GLP	:	no
species	:	Fisher rats	guideline	:	Generally complies with OECD 403
group size	:	2 groups of 10 rats per sex	acceptability	:	acceptable

Test guideline and GLP

The study was not performed using any official guideline or test method, and it was not done under GLP. However, the study was performed in 1983 before GLP was required and the method used complies with OECD guidelines 403 (Directive 67/548/EEC, Annex V, Methods B.2).

Study design

Fischer rats (F344/DuCrj, SPF) were exposed to aerosol of flutolanil (purity: 98.7%) in a whole-body exposure chamber for 4 hr. Actual exposure concentrations of flutolanil were 4.50 and 5.98 mg/l, the latter being the maximum attainable concentration. The mass median aerodynamic diameter (MMAD) of flutolanil was 5.8 µm for lower concentration and 6.3 µm for the higher concentration. Ten males and ten females were used per treatment. Animals were observed for signs of toxicity during the exposure and two hours after the exposure, and thereafter observed at least daily during the 14-day post exposure period. Animals were necropsied at the end of the post exposure period.

Results

No rats died during the exposure or post-exposure period. Blood-like red staining around the nose was observed in almost all the animals within one day after exposure. The staining disappeared within two days. No other clinical signs of toxicity were observed. Treatment with flutolanil had no effect on body weight. One male and one female of the higher concentration group showed changes in lungs: either or both, reddish patches and mild emphysema.

Conclusion

The acute inhalation toxicity of flutolanil to rats is low. The LC50 of flutolanil at 4 hr exposure was greater than 5.98 mg/l for males and females. The study is acceptable.

B.6.2.3.2 Inhalation, study 2

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study. The study is considered acceptable. Based on the results of the study no classification is required in accordance with Regulation (EC) No. 1272/2008.

reference	:	[redacted] (2012)	exposure	:	Inhalation (nose only)
Report number	:	T-3230	doses	:	2.51 mg/L
test substance	:	Flutolanil (technical grade)	GLP	:	yes
species	:	Sprague-Dawley rats (female)	guideline	:	In accordance with OECD 436
group size	:	3 animals/ sex	acceptability	:	acceptable

Executive summary

A limit test at 2 mg/L air of test item was conducted with 3 male and 3 female Sprague-Dawley rats, with snout-only exposure for 4 h. To obviate aggregation of flutolanil technical, pulverized mixtures with 'white carbon' ($SiO_2.nH_2O$), 20% w/w, were used. A similar white carbon exposure group served as control.

Mean achieved concentration of active ingredient was 2.151 mg/L and the mass median aerodynamic diameter (MMAD) was 3.58 µm, geometric standard deviation 1.86 µm. In the vehicle control group the corresponding values were 0.55 mg/mL, 1.40 and 4.08 µm respectively.
There were no mortalities and no clinical signs throughout. Body weight losses observed at 1 and 3 days after exposure were recovered through days 7 and 14.
It was concluded that the acute LC_{50} by 4 h snout-only inhalation in rats was concluded to be >2 mg/L air. It would be 'not classified' according to current GHS criteria.

Study design

Test item:	Flutolanil technical
Batch No.:	101141
Purity:	99.1%
Expiry:	22 July 2015
Description:	Pale yellowish/greyish powder
Vehicle:	$SiO_2.nH_2O$ ('white carbon', Carplex80D), Evonik Degussa, Lot 120313
Animals:	Sprague-Dawley rats ([redacted]), 7 weeks of age, approximately 200-240 g (males) and 160-200 g (females)
Study start and completion:	28 November 2011 to 31 July 2012

To mitigate the physical tendency for flutolanil technical to aggregate, it was mixed with 20% w/w white carbon and finely pulverized in order to meet the requirements for mass median aerodynamic diameter (MMAD) of the particles to be in the range 1 to 4 µm with geometric standard deviation in the range 1.5 to 3 µm. Homogeneity was proven by a relative standard deviation (RSD) for concentration values of 0.4% for top, middle and bottom samples, while stability was shown by a residual concentration of 100% of the initial value.
Initially, the target exposure level for the study was 5 mg/L air, based on a previous acute inhalation study (CA 5.2.3/01 above). A preliminary trial showed, however, MMADs of 4.21 to 5.25 µm and therefore, to minimize aggregation the target exposure level was reduced to 2 mg/L air. As the target level for the flutolanil/white carbon mixture (8:2 w/w) was 2.5 mg/L, the level for vehicle (white carbon) control was set at 0.5 mg/L air.
Groups of 3 male and 3 female rats were exposed continuously to the dusts at the above target levels for 4 hours, in a snout-only exposure chamber (total volume 31.2 L). The dusts were generated by a turntable type dust feeder with filtered compressed air. Homogeneity of the aerosol within the chamber was not determined during exposure, but the system had previously been validated and shown to produce homogeneous aerosols in the animals' breathing zone with a variety of test substances.
Airflow rate in the dust feeder was continuously monitored and also recorded every 30 min. Chamber equilibrium was achieved within 5 min. The total amount of dust supplied to the chamber was monitored (corrected for purity and white carbon content) in order to calculate the nominal concentration delivered. The actual concentration delivered was measured at 1, 2 and 3 h after initiation of exposure, by drawing 6-L samples of chamber air through a glass fibre filter and immediately extracting the test item into acetonitrile for analysis by HPLC. Prior to analysis a recovery

test was conducted. For the vehicle control, the quantity of white carbon trapped by the filter was determined gravimetrically. Particle size distribution was measured at 1, 2 and 3 h using an Andersen type sampler, in a 2-L sample of chamber air. The amount of test item trapped on each stage of the sampler was determined gravimetrically. Temperature and humidity in the chamber were monitored continuously and recorded every 30 min.

The animals were observed for mortality and any clinical signs at 1, 2 and 3 h after initiation of exposure, then at 1 and 4 h after exposure and once daily thereafter until termination after the 14-day observation period. Body weights were recorded shortly before exposure and on 1, 3, 7 and 14 days after exposure. The animals were euthanized after completion of the final observations on post-exposure day 14 and subjected to gross necropsy.

Results

Table B.6.2.3.2-1: exposure conditions

Exposure group	Target concentration (mg/L)	Nominal concentration (mg/L)	Actual concentration (mg/L, mean ± SD, n=3)	Particle size [d] (mean ± SD, n=3)	
				MMAD (µm)	σg
Vehicle control	-	-	-	1.40 ± 0.21	4.08 ± 0.62
		(4.75) [b]	(0.55 ± 0.20) [a]		
Test substance	2	4.82 [b]	2.151 ± 0.1104 [c]	3.58 ± 0.02	1.86 ± 0.13

a): Measured by gravimetric analysis of white carbon.
b): Measured by gravimetric analysis of active ingredient.
c): Measured by chemical analysis of active ingredient.
d): Measured by gravimetric analysis.
S.D.: Standard deviation.
MMAD: Mass median aerodynamic diameter.
σg: Geometric standard deviation of MMAD.

There were no mortalities and no clinical signs of toxicity in any animal throughout.

There were no test item related effects on body weight change. Weight losses were observed for all animals at 1 day after exposure, which were subsequently recovered.

Table B6.2.3.2-2: Body weights, males

Exposure group	Target concentration (mg/L)	Animal number	Body weight (g)									
			Before exposure		Days after exposure							
					1		3		7		14	
Vehicle control	-	1	305		287	*	310		344		390	
		2	300		290	*	316		351		399	
		3	305		303	*	321		344		381	
		Mean	303		293		316		346		390	
		N	3		3		3		3		3	
Test	2	4	310		297	*	309	*	343		385	

substance		5	307		306	*	316		355		392	
		6	311		304	*	315		352		390	
		Mean	309		302		313		350		389	
		N	3		3		3		3		3	

N: Number of animals examined
*: Body weight loss when compared with the pre-exposure value

Table B.6.2.3.2-3: Body weights, females

Exposure group	Target concentration (mg/L)	Animal number	Body weight (g)									
			Before exposure		Days after exposure							
					1		3		7		14	
Vehicle control	-	101	191		186	*	190	*	201		217	
		102	200		182	*	191	*	200		215	
		103	208		197	*	211		223		238	
		Mean	200		188		197		208		223	
		N	3		3		3		3		3	
Test substance	2	104	223		215	*	216	*	235		249	
		105	227		217	*	213	*	227		254	
		106	214		211	*	217		236		246	
		Mean	221		214		215		233		250	
		N	3		3		3		3		3	

N: Number of animals examined
*: Body weight loss when compared with the pre-exposure value

There were no abnormal macroscopic findings at necropsy for any animal.

Conclusion

The acute LC_{50} for flutolanil technical by 4 h snout-only inhalation in rats was >2 mg/L air. It would be 'not classified' according to current GHS criteria.

B.6.2.4 Skin irritation

Previous evaluation	DAR (May 2005)
Evaluation of the RMS	No comments on the original evaluation. Tabled results were included in the study summary. In the study the staged approach recommended in the current guideline (dated 2015) was not followed as six animals were used in a single phase. However, since the study was performed before the current guideline was in place, the study is considered acceptable.

	Based on the results of the study no classification is required in accordance with Regulation (EC) No. 1272/2008.

reference	:	[redacted] (1986a)	exposure	:	Dermal exposure
Report number	:	86/NHH015/212 (T-3027)	doses	:	0.5 g
test substance		Flutolanil	GLP	:	yes
species	:	New Zealand White Rabbit	guideline	:	in accordance with OECD 404
group size	:	6 animals	acceptability	:	acceptable

Test guideline and GLP

The study was performed to conform with the Japanese MAFF guidelines of 1985 and the U.S Pesticide Assessment guidelines of 1982, and was performed in compliance with GLP. The method used complies with OECD guideline 404 (Directive 94/79/EC, Directive 92/69/EEC Method B.4.).

Study design

Shaven dorsa of six male New Zealand White rabbits were exposed to 0.5 g of flutolanil (purity 97.5%, moistened with 0.2 ml water) on a shorn area of 6 cm2 for 4 hr using a semi-occlusive dressing technique. Dermal reactions were assessed 1, 24, 48 and 72 hr after removal of the dressings.

Results

No dermal irritation responses (all scores 0) were observed in any rabbit at any time during the 72-hour observation period.

Table B.6.2.4.1-1: Individual skin reactions following single dermal application of flutolanil

Observation	Observation period				Mean score 24-72 hrs
	1 h	24 hrs	48 hrs	72 hrs	
Erythema	0/0/0/0/0/0	0/0/0/0/0/0	0/0/0/0/0/0	0/0/0/0/0/0	0/0/0/0/0/0
Edema	0/0/0/0/0/0	0/0/0/0/0/0	0/0/0/0/0/0	0/0/0/0/0/0	0/0/0/0/0/0

Conclusion

Under the conditions of this test, flutolanil was non-irritant to rabbit skin. The study is acceptable.

B.6.2.5 Eye irritation

Previous evaluation	DAR (May 2005)
Evaluation of the RMS	No comments to the original evaluation. Tabled results were included in the study summary. The study deviates from the current guideline (dated 2002) since the staged approach recommended in the current guideline was not applied since six animals being used in a single phase. Moreover, no analgesia was used, but an assessment of any pain response was made at instillation. However, since the study was performed before the current guideline was in place, the study is considered acceptable. Based on the results of the study no classification is required in accordance with Regulation (EC) No. 1272/2008.

reference	:	[redacted] (1986b)	exposure	:	Eye exposure
Report number	:	86/NHH014/211 (T-3026)	doses	:	0.1 g
test substance	:	Flutolanil	GLP	:	yes
species	:	New Zealand White rabbits	guideline	:	in accordance with OECD 405
group size	:	6 animals	acceptability	:	acceptable

Test guideline and GLP

The study was performed to conform to the Japanese MAFF guidelines of 1985 and the U.S Pesticide Assessment guidelines of 1982, and was performed in compliance with GLP. The method used complies with OECD guideline 405 (Directive 94/79/EC, Directive 92/69/EEC Method B.5.). In addition, evaluations of pain responses at instillation were made.

Study design

Six male New Zealand White rabbits received ocular instillation of 0.1 g of flutolanil (purity 97.5%) into the conjunctival sac. Ocular reactions were examined 1, 24, 48 and 72 hr after treatment. Evaluations of pain responses at instillation were made.

Results

Responses following instillation of flutolanil were slight or totally absent. Slight injection of the conjunctivae blood vessels (score 1) were observed in all rabbits one hour and 24 hours after instillation of flutolanil. Resolution was complete in all animals by 72 hours post-exposure. No other responses were present. Instillation of the test material caused none or practically no initial pain response among the rabbits.

The individual scores are shown below.

Table B.6.2.5-1 grades for eye irritation following instillation of 0.1 g flutolanil

Effects	irritation per animal per hour(s) after dosing				Mean score 24-72 hrs
	1	24	48	72	
corneal opacity	0/0/0/0/0/0	0/0/0/0/0/0	0/0/0/0/0/0	0/0/0/0/0/0	0/0/0/0/0/0
iritis	0/0/0/0/0/0	0/0/0/0/0/0	0/0/0/0/0/0	0/0/0/0/0/0	0/0/0/0/0/0
conjuntival erythema	1/1/1/1/1/1	1/1/1/1/1/1	1/1/1/1/0/0	0/0/0/0/0/0	0.7/0.7/0.7/0.7/0.3/0.3
conjunctival chemosis	0/0/0/0/0/0	0/0/0/0/0/0	0/0/0/0/0/0	0/0/0/0/0/0	0/0/0/0/0/0

Conclusion

Under the conditions of this test, Flutolanil was only slightly irritant to rabbit eye, but does not require classification. The study is acceptable.

B.6.2.6 Skin sensitisation

Previous evaluation	DAR (May 2005)
Evaluation of the RMS	No comments to the original evaluation. Based on the results of the study no classification is required in accordance with Regulation (EC) No. 1272/2008.

reference	:	[redacted] (1986c)	exposure	:	Dermal
Report number	:	86/NHH016/132 (T-3028)	doses	:	Induction: 3x injection (1-2%) followed by dermal application (60%) Challenge: dermal application (60%)
test substance		Flutolanil	GLP	:	yes
species	:	Dunkin-Hartley guinea pigs	guideline	:	in accordance with OECD 406
group size	:		acceptability	:	acceptable

Test guideline and GLP

The study was performed to conform to the Japanese MAFF guidelines of 1985 and the U.S Pesticide Assessment guidelines of 1982, and was performed in compliance with GLP. The method used complies with OECD guideline 406 (Directive 94/79/EC, Directive 92/69/EEC Method B.6.).

Study design

A preliminary study was conducted to establish the irritant and non-irritant concentrations of the test material. Skin reactions to flutolanil (97.5%) were tested on 4 Dunkin-Hartley guinea-pigs by intradermal injections of 0.1 ml of 0.5%, 0.9%, 1.8%, 3%, 5% and 30% w/v of flutolanil in acetone in paraffin oil, or 0.5%, 0.9%, 1.8%, 3% or 5% flutolanil in acetone in Freunds Complete Adjuvant. Epidermal applications used were 0,03 ml of 10%, 20%, 30% 40%, 50% or 60% flutolanil in acetone.

The main study was performed according to the procedure of Magnusson and Kligman Maximization test using flutolanil purity of 97.5%. Initial induction: The shaven dorsa of twenty female Dunkin Hartley guinea-pigs were subject to intradermal injection of Freunds Complete Adjuvant (FCA), 2% w/v flutolanil in acetone/paraffin oil and 1% w/v flutolanil in acetone/FCA on day 1. Second induction: Seven days later the same skin area was treated by topical application of 60% w/v flutolanil in acetone and the test site was covered by an occlusive dressing for 48 hr. The same induction procedures were carried out on a contemporaneous control group, except that the test material was replaced by vehicle. Challenge treatment: On day 22 all animals were challenged by occluded application of acetone to the left flank and 60% w/v flutolanil in acetone to the right flank. The dressings were removed on the following day and the condition of the test sites was assessed 24 and 48 hr later. Two other groups of animals were similarly tested for dinitrochlorobenzene as positive control.

Results

The findings of the main study are presented in table B.6.2.6-1. Flutolanil did not induce dermal response, whereas the positive control, dinitrochlorobenzene, induced strong responses. Two animals in the flutolanil treated group found dead on days 17 and 18 were subjected to necropsy. There was no apparent association between these deaths and the treatment administered to either decedent.

Table B.6.2.6-1: Dermal responses of guinea pigs to flutolanil in Magnusson and Kligman Maximization test

GROUP	CHALLENGE TREATMENT	NUMBER OF ANIMALS	DERMAL RESPONSE			
			24 hr reading		48 hr reading	
			A	B	A	B
Control	Acetone	20	0	0	0	0
Flutolanil	Acetone	18	0	0	0	0
Control	60% Flutolanil in acetone	20	0	0	0	0
Flutolanil	60% Flutolanil in acetone	18	0	0	0	0
Control	Acetone	10	0	0	0	0
DNCB*	Acetone	9	0	0	0	0
Control	0.1% DNCB in acetone	10	0	0	0	0
DNCB	0.1% DNCB in acetone	9	0.55	0.83	0.78	1.22

A: Incidence = Number of dermal test sites showing a significant response / Number of treated sites

B: Severity = Total of all scores for dermal test sites / Number of treated sites
*DNCB: dinitrochlorobenzene

Conclusion

Under the conditions of this study, flutolanil did not show any sensitizing potential. The study is acceptable.

B.6.2.7 Phototoxicity

In the DAR (volume 3, Annex B, B2 physical and chemical properties; May 2005)) a study of Guesnet (2000) was evaluated. The threshold of ≥10 L/mol cm was exceeded for the molar absorption coefficient measured at ≥290 nm wavelength. This occurred at pH 7 (test concentrations 4.69 x 10^{-5} mol/L). Conduct of an *in vitro* phototoxicity test (Neutral Red uptake by 3T3 mouse fibroblast cells) was therefore triggered.

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study is considered acceptable.

reference	:	Valin M (2016)	vehicle	:	DMSO
Report number	:	43497 (T-3242)	doses	:	0.03 to 100 µg/mL
test substance		Flutalonil	GLP	:	yes
test system	:	Mouse fibroblasts (Balb/c 3T3, clone A31)	guideline	:	in accordance with OECD 432
			acceptability	:	acceptable

Executive summary

This assay assesses the cytotoxicity of chemicals applied to mouse fibroblasts (Balb/c 3T3, clone A31) in the presence or absence of exposure to a non-cytotoxic level of UVA light (5 J/cm^2). Also, to maximize exposure of the cells to residual UVB, the plates were irradiated without the lid. Cytotoxicity is measured as inhibition of the capacity to take up the vital dye Neutral Red (NR), 1 day after treatment.

According to results obtained in a preliminary test, the following concentrations were used for the main test: 6.73, 9.90, 14.56, 21.41, 31.48, 46.28, 68.03 and 100 µg/mL (serial dilution factor of 1.47). The following results were obtained:

- In the non-irradiated plate, a change in cell morphology (meaning potentially dying cells) was observed at 100 µg/mL, but no decrease in NR uptake was noted at any tested concentration.
- In the irradiated plate, a change in cell morphology (meaning potentially dying cells) was observed at concentrations ≥ 68.03 µg/mL, but no decrease in NR uptake was noted at any tested concentration.

An IC_{50} was not reached for either UVA or UVB, so a PIF (photo-irritation factor) could not formally be calculated and was concluded by default to be 1.0. The MPE (mean photo effect) was 0.017. It was concluded that Flutolanil technical was not phototoxic at up to 100 µg/mL in the test.

Materials and methods

Test item: Flutolanil technical

Batch No.: 9AE0011P

Purity:	98.7%
Expiry:	20 February 2021
Storage:	Ambient temperature, in the dark
Description:	Pale yellowish/greyish powder
Vehicle:	Dimethylsulfoxide (DMSO)
Positive control:	Chlopromazine hydrochloride (CPZ) in DMSO at 100 mg/mL
Media:	Hanks' Balanced Salt Solution (HBSS); Dulbecco's Modified Eagle's Medium (DMEM) buffered with sodium bicarbonate and supplemented with 10% fetal calf serum, 4 mM glutamine, 100 IU penicillin and 100 µg/mL streptomycin
Neutral Red (NR):	Final concentration in culture medium of 50 µg/mL
Desorption solution:	49% water, 1% glacial acetic acid, 50% ethanol
Cell line:	Balb/c 3T3, clone A31 (American Type Culture Collection No. CCL-163), from LGC Standards, UK. Batch 58215273, passages 97 and 74. Sensitivity to UVA checked every 6 months, last performed June 2015
Light source:	UVACUBE 400 (SOL-500 lamp) with H-1 (anti-UVB) filter and UVA meter (UV Technology, Germany). Distance from light source to bottom of the 96-well plate: ~60 cm, but to maximize exposure to residual UVB the plates were irradiated without the lid
Study start and completion:	23 November 2015 to 09 April 2016

Study design

A preliminary test was performed to determine the relevant concentration range at which cytotoxicity is obtained. During this assay, eight concentrations of the test item were tested using six replicates per concentration. These concentrations were 0.03 to 100 µg/mL, spaced using a decimal geometric dilution factor of 3.16. In parallel, eight concentrations of the positive control were also applied to cells in other plates. The procedure for performing the preliminary test was identical to the main test (see below). Following analysis of the results obtained in the preliminary test, an appropriate dilution scheme was used in the main test.

Main test:

Four 96-well plates were prepared: two were treated with the test item and the two others with the positive control (CPZ). Peripheral wells of each plate received 100 µL of HBSS, whilst the remaining wells received 1 x 10^4 3T3 cells in 100 µL culture medium. Plates were incubated for 24 ± 2 hours at 37°C, 5% CO_2, 90% humidity until the cells formed a semi-confluent monolayer.

After incubation, culture medium was removed and cells were washed with pre-warmed HBSS. Then, for each plate, eight concentrations of the test item and CPZ were applied to the cells (six replicates per concentration). The vehicle control and vehicle $control_{CPZ}$ were applied to the cells on the left and right sides of each test item-treated or positive control-treated plate (12 replicates/plate). HBSS was applied to the peripheral wells. The treatment volume for the test item, positive and vehicle controls

was 100 µL per well. The cells were exposed to the test item for 1 hour (± 5 minutes) with incubation at 37°C, 5% CO_2, 90% humidity.

At the end of the treatment period, one plate per test item or positive control was exposed to UV light (Irr+ plates) while the other plate remained in the dark (Irr- plates). For the Irr+ plates, the cells were irradiated with 5.2 and 5 J/cm2 in the preliminary and main tests, respectively. This irradiation was performed at room temperature in an UVACUBE 400 (Sol-500) equipped with an H-1 filter. As the lid of the 96-well plate has UVB-filtering effects, the lid of the plates was removed during the irradiation in order to maximize exposure to residual UVB. The Irr- plates were maintained in the dark at room temperature.

Fifty minutes after the start of light treatments, the solutions from each well of all plates (including Irr- plates) were removed and the cells were washed twice with pre-warmed HBSS. A volume of 150 µL of pre-warmed fresh culture medium was then added to each well containing cells and the plates were incubated at 37°C, 5% CO_2, 90% humidity for 18-22 h.

The cells were examined microscopically and any cytotoxic effects were recorded. The wells were washed with pre-warmed HBSS, which was then removed. Neutral Red (NR) culture medium (100 µL) was added to all wells and the plates incubated at 37°C, 5% CO_2, 90% humidity for 3 hours (± 6 minutes). After incubation, the NR medium was removed and the wells washed with HBSS. Desorption solution (150 µL) was added to each well. Plates were shaken on a plate shaker until NR was extracted and formed a homogenous solution. Absorption of the resulting coloured solution was measured at 540 nm using a plate reader and using the optical densities of the peripheral wells as blanks.

Data evaluation and analysis:

Following curve fitting of the data, IC_{50} values (concentration at which the cell viability is reduced by 50%) were calculated and reported for each curve. Additionally, a Photo Impact Factor (PIF) and a Mean Photo Effect (MPE) value were generated.

PIF is calculated using the formula: PIF = IC_{50}(Irr-)/IC50(Irr+). Where no IC_{50} values could be calculated, the PIF value was considered as 1.00 by default.

Where an IC_{50} was calculated for the irradiated plate but not for the non-irradiated plate, an accurate PIF cannot be calculated. However, an approximate PIF is calculated and used to evaluate the phototoxic potential. In this case (test item or CPZ), the maximum tested concentration in the non-irradiated plate is used as the IC_{50}(Irr-) to calculate the approximate PIF. This PIF value is referred to as > PIF in the study report and as "C PIF value" in the table. In that case, the formula is: > PIF = highest tested concentration (Irr-)/IC_{50}(Irr+).

MPE is based on comparison of the complete concentration response curves. This prediction model is useful in the data evaluation if no IC_{50} is achieved for the irradiated or non-irradiated plate. It is defined as the weighted average across a representative set of photo effect values.

$$MPE = \frac{\sum_{i=1}^{n} w_i \, PE_{c_i}}{\sum_{i=1}^{n} w_i}$$

The Photo Effect (PE_c) at any Concentration (C) is defined as the product of the Response Effect (RE_c) and the Dose Effect (DE_c) *i.e.* $PE_c = RE_c \times DE_c$. The Response Effect (RE_c) is the difference between the responses observed in the absence and presence of light, *i.e.* $RE_c = R_c$ (Irr-) - Rc (Irr+). The dose-effect is given by:

$$DE_c = \left| \frac{C/C^* - 1}{C/C^* + 1} \right|$$

Where C* represents the equivalence concentration, *i.e.* the concentration at which the Irr+ response equals the Irr- response at concentration C. If C* cannot be determined because the response values of the Irr+ curve are systematically higher or lower than RC (Irr-), the dose effect is set to 1. The weighting factors W_i are given by the highest response value, *i.e.* W_i = MAX {Ri (Irr+), Ri (Irr-)}. The concentration grid Ci is chosen such that the same number of points falls into each of the concentration intervals defined by the concentration values used in the experiment. The calculation of MPE is restricted to the maximum concentration value at which at least one of the two curves still exhibits a response value of at least 10%. If this maximum concentration is higher than the highest concentration used in the Irr+ experiment the residual part of the Irr+ curve is set to the response value "0".

Acceptance criteria:
The main test was considered valid if the following criteria were met:
For the vehicle control of the positive control plates:

- the mean $OD_{540\ nm}$ of the vehicle controls from each plate ≥0.4,
- the left and the right mean negative control of each plate should not differ by more than 15% from the mean of all controls of the plate (mean ± 15%),
- the mean viability of the irradiated vehicle controls should be ≥80% when compared with non-irradiated vehicle controls.

For the positive control (CPZ):

- the IC_{50} (Irr+) should be within the range 0.1 - 2.0 µg/mL,
- the IC_{50} (Irr-) should be within the range of 7.0 - 90.0 µg/mL, unless the concentration range used prohibits an IC_{50} to be calculated,
- the PIF value should be >6.

Evaluation criteria:
The following criteria are used to classify the test item:

- the test item is not phototoxic if PIF ≤ 2.0 or MPE ≤ 0.10,
- the test item is probably phototoxic if PIF > 2.0 but ≤ 5.0 or MPE > 0.10 but ≤ 0.15,
- the test item is phototoxic if PIF > 5.0 or MPE > 0.15.

Results

A solubility test showed that the test item was not soluble in HBSS (at 1 or 0.1 mg/mL) or in ethanol (at 100 mg/mL). It was soluble in DMSO at 100 mg/mL, but not after 100-fold dilution in HBSS; however,

at 10 mg/mL in DMSO it was then soluble after 100-fold dilution in HBSS. Therefore, the highest soluble concentration (100 µg/mL in HBSS containing 1% DMSO) was selected for use in the preliminary test.
The preliminary test used concentrations of 0.03, 0.10, 0.32, 1.00, 3.17, 10.02, 31.65 and 100 µg/mL in HBSS containing 1% DMSO (serial dilution factor 3.16).

For the non-irradiated plate:

- Change in cell morphology (meaning potentially dying cells) was noted at 100 µg/mL by microscopic observation,
- Slight decrease in cell viability at concentrations ≥ 31.65 µg/mL. However, no IC_{50} was calculated as the mean viabilities were >50%.

For the irradiated plate:

- Change in cell morphology (meaning potentially dying cells) at concentrations ≥ 31.65 µg/mL by microscopic observation,
- No decrease in NR uptake at any tested concentration.

Main test:
Considering that no meaningful cytotoxicity was observed in the preliminary test, a lower dilution factor (1.47) was employed in the main test, resulting in a concentration series of 6.73, 9.90, 14.56, 21.41, 31.48, 46.82, 68.03 and 100 µg/mL.

All acceptance criteria were fulfilled and the experiment was therefore considered valid.

The following results were obtained in the non-irradiated plate:

- a change in cell morphology (meaning potentially dying cells) was noted at 100 µg/mL by microscopic observation,
- no decrease in NR uptake was observed at any tested concentrations (Figure B.6.2.7-1; blue symbols).

The following results were obtained in the irradiated plate:

- change in cell morphology (meaning potentially dying cells) was noted at concentrations ≥ 68.03 µg/mL by microscopic observation,
- no decrease in NR uptake was observed at any tested concentrations (Figure B.6.2.7-2; yellow/white symbols).

Table B.6.2.7-1: vehicle control, positive control and test item main results

Date of treatment	Plates	Parameter	Value	Conclusion
17 December 2015	Positive control plates	Vehicle control$_{CPZ}$ (HBSS containing 1% DMSO) Irradiated plate[1]	OD_{540} = 0.682	Passed

	Vehicle control$_{CPZ}$ (HBSS containing 1% DMSO) Non-irradiated plate[1]	OD_{540} = 0.765	Passed
	% difference between left and right mean vehicle control$_{CPZ}$ Irradiated plate[2]	7.1%	Passed
	% difference between left and right mean vehicle control$_{CPZ}$ Non-irradiated plate[2]	3.3%	Passed
	% mean viability vehicle controlCPZ Irr+ *versus* mean viability Vehicle controlCPZ Irr-[3]	89%	Passed
	Positive control[4] CPZ	IC_{50} Irr+ = 0.6578 µg/mL IC_{50} Irr- = 33.72 µg/mL PIF = 51.352	Passed
Test item plates	Vehicle control (HBSS containing 1% DMSO) Irradiated plate[1]	OD_{540} = 0.629	Passed
	Vehicle control (HBSS containing 1% DMSO) Non-irradiated plate[1]	OD_{540} = 0.676	Passed
	Test item[5] Flutolanil Technical	IC_{50} Irr+ = not reached IC_{50} Irr- = not reached > PIF = 1.000 (by default) MPE = 0.017	Not phototoxic

[1]: OD_{540} for the vehicle controls wells ≥ 0.4.
[2]: the left and right mean vehicle controls$_{CPZ}$ should not differ by more than 15%.
[3]: the mean viability of the Irr+ vehicle control should be ≥ 80% when compared with non-irradiated vehicle controls.
[4]: for the positive control, IC_{50} Irr+ must be within 0.1-2.0 µg/mL; IC_{50} Irr- within 7.0-90.0 µg/mL and PIF > 6.
[5]: test item is not phototoxic if PIF ≤ 2.0 or MPE ≤ 0.10, probably phototoxic if PIF > 2.0 but ≤ 5.0 or MPE > 0.10 but ≤ 0.15 and phototoxic if PIF > 5.0 or MPE > 0.15.

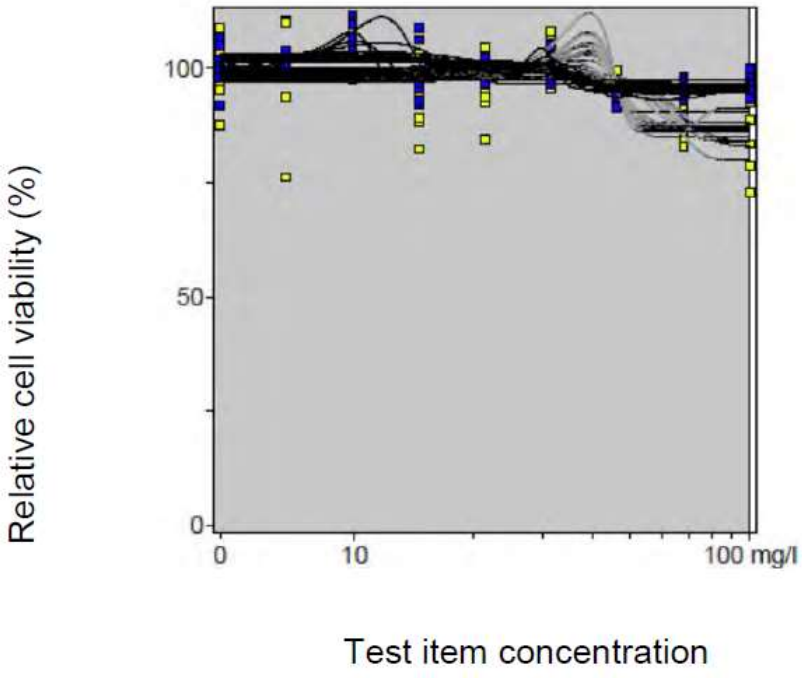


Figure B.6.2.7-1: NR uptake in 3T3 cells treated with flutolanil, main test

On this graph, the viability (%) of each replicate is represented for each tested concentration.

Blue symbols represent non-irradiated viability; and for each replicate the dose response is a black line.

Yellow symbols represent irradiated viability; dose response for each replicate is a grey line.

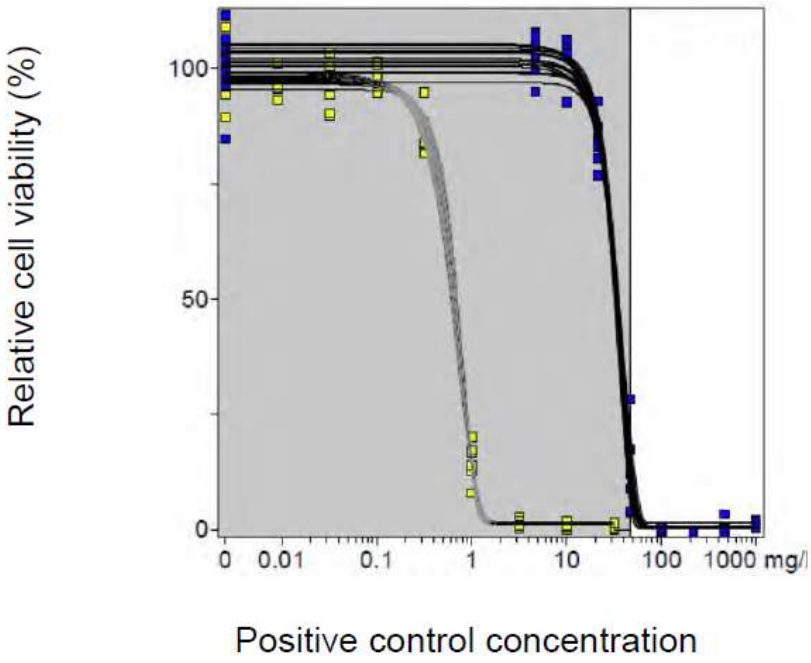


Figure B.6.2.7-2: NR uptake in 3T3 cells treated with CPZ (positive control), main test

On this graph, the viability (%) of each replicate is represented for each tested concentration.

In blue (or black) symbols, the non-irradiated viabilities are represented. For each replicate, the associated dose-response curve is represented in black.

In yellow (or white) symbols, the irradiated viabilities are represented. For each replicate, the associated dose-response curve is represented in grey.

Conclusion

Under the conditions of the study, flutolanil technical at up to 100 µg/mL was not phototoxic, according to the OECD guideline 432 criteria.

B.6.3 Short-term toxicity

B.6.3.1 Oral 28-day study

Previous evaluation	DAR (May 2005)
Evaluation of the RMS	No comments on the original evaluation. Some additional tabled results were added to the study summary. Some deviations from the current OECD 407 guideline (2008) exist, which include the following: - weights of epididymides, prostate and thymus were not measured - secondary sex organs, nerves, bone/bone marrow, lymph nodes and eyes not examined, although testes were examined (in response to a gross finding)

	- haematology: platelet count and blood clotting time/potential were not examined - clinical chemistry: urea and creatinine levels were not assessed - functional observations not described - data is generally presented as mean values instead of individual data.

reference	:	[redacted] (1977)	exposure	:	Via diet
Report number	:	T-3011	doses	:	400, 2000, 10000 and 50000 ppm
test substance	:	Flutolanil	GLP	:	no
species	:	Sprague Dawley rats	guideline	:	Generally complies with OECD 407
group size	:	20 animals (m/f) per group (control and treatment groups)	acceptability	:	acceptable

Test guideline and GLP

The study was not performed using any official guideline or test method, and it was not done under GLP. However, the study was performed in 1977 before GLP was required and in generally the method complies with OECD guideline 407 (Directive 67/548/EEC, Annex V, Methods B.7).

Study design

Groups of 10 Sprague-Dawley rats per sex received flutolanil (purity: >99%) in the feed at concentrations of 0, 400, 2000, 10000 and 50000 ppm for 31 days (male) or 32 days (female). According to the notifier, the treated rats ingested the following mean dosages of flutolanil: males: 0, 35.5, 180.2, 916.4 and 4875.1 mg/kg bw/day: females: 0, 41.2, 215.3, 1074.9 and 5435.0 mg/kg bw/day. The animals were observed for death and general condition daily. Haematological and blood chemistry analyses were done from samples taken at the end of the administration period. Urinalysis was done from urine collected on the day before the study termination. Organs were weighed and their ratios to body weight were calculated. In histopathological analysis, all the test animals were observed macroscopically. Sections of rats in 10 000 and 50 000 ppm groups were examined microscopically. Blood, liver, kidney, brain, and fat were sampled and gathered from each group for residue analysis. The test data was processed statistically.

Results

The compound did not cause death or signs of intoxication in any of the groups. Body weights were decreased in animals in high dose group. Absolute liver weights in 50 000 ppm groups and relative livers weight in groups receiving ≥10 000 ppm were increased significantly. In the liver, hypertrophy and vacuolar change of hepatocytes in centrilobular area were found in 7 out of 10 male rats and 4 out of 10 female rats in the 50 000 ppm group and in 4 out of 9 male rats and 3 out of 10 female rats in the 10 000 ppm group.

Table B.6.3.1-1: Body weight data for male and female rats treated with flutolanil

Male					
Group/dose (ppm)	**Control**	**400**	**2000**	**10000**	**50000**
Body weight gain (g)	186	188 (112%)	175 (94%)	179 (96%)	168 (90%)
Standard deviation	9	9	7	9	8
Female					
Group/dose (ppm)	**Control**	**400**	**2000**	**10000**	**50000**
Body weight gain (g)	95	85 (89%)	87 (92%)	87 (92%)	**78*** (82%)
Standard deviation	3	4	3	4	6

* statistically significant difference at the 95% (p,0.05) confidence level.
Data between brackets represent the bodyweight in percentage compared to the control

Table B.6.3.1-2: Haematological data for male and female rats treated with flutalonil

LEVEL	SEX	NO	RBC	WBC	HB	HCT
			(x10^4/mm^3)	(x10^2/mm^3)	(g/dl)	(%)
CONTROL	M	10	746 ± 7	105 ± 5	14.5 ± 0.1	46.0 ± 0.3
400 ppm	M	10	740 ± 7	100 ± 2	14.6 ± 0.1	**47.1 ± 0.3***
2000 ppm	M	10	**723 ± 5***	**91 ± 4***	13.9 ± 0.4	46.7 ± 0.5
10000 ppm	M	10	743 ± 11	**75 ± 5*****	14.4 ± 0.1	46.3 ± 0.5
50000 ppm	M	10	745 ± 7	99 ± 8	14.4 ± 0.2	47.0 ± 0.4
CONTROL	F	9	736 ± 10	82 ± 3	14.5 ± 0.2	47.4 ± 0.5
400 ppm	F	10	741 ± 7	**102 ± 8***	14.7 ± 0.1	47.1 ± 0.4
2000 ppm	F	10	743 ± 6	87 ± 5	14.3 ± 0.2	46.6 ± 0.6
10000 ppm	F	10	732 ± 13	81 ± 4	14.2 ± 0.2	46.1 ± 0.6
50000 ppm	F	10	736 ± 7	95 ± 7	14.1 ± 0.2	46.2 ± 0.6

*: Statistically significant difference at the 95% (p.<.05) confidence level
***: Statistically significant difference at the 99.9% (p.<.001) confidence level

Table B.6.3.1-3: Haematological data for male and female rats treated with flutalonil

LEVEL	SEX	NO	MCH	MCV	MCHC
			(pictogram)	(cubicgram)	(%)
CONTROL	M	10	19.4 ± 0.2	61.7 ± 0.7	31.4 ± 0.2
400 ppm	M	10	19.8 ± 0.2	**63.7 ± 0.5***	31.1 ± 0.2
2000 ppm	M	10	19.2 ± 0.6	**64.6 ± 0.7****	29.8 ± 1.0
10000 ppm	M	10	19.4 ± 0.3	62.4 ± 0.9	**31.2 ± 0.2***
50000 ppm	M	10	19.4 ± 0.2	63.1 ± 0.7	**30.7 ± 0.2***
CONTROL	F	10	19.8 ± 0.2	64.4 ± 0.6	30.7 ± 0.3
400 ppm	F	10	19.9 ± 0.2	63.6 ± 0.4	31.2 ± 0.3
2000 ppm	F	10	19.2 ± 0.2	62.7 ± 0.7	30.7 ± 0.3
10000 ppm	F	10	19.5 ± 0.1	63.0 ± 0.6	30.9 ± 0.1
50000 ppm	F	10	19.2 ± 0.3	62.8 ± 0.7	30.5 ± 0.4

*: Statistically significant difference at the 95% (p.<.05) confidence level
**: Statistically significant difference at the 99% (p.<.01) confidence level

Table B.6.3.1-4: Differential leucocyte counts for male and female rats treated with flutalonil

LEVEL	SEX	NO	NEUTRO	LYMPHO	MONO	EOSINO	BASO
			(%)	(%)	(%)	(%)	(%)
CONTROL	M	10	14.8 ± 1.8	82.9 ± 1.9	0.7 ± 0.2	1.6 ± 0.3	0.0 ± 0.0
400 ppm	M	10	11.3 ± 1.7	87.0 ± 1.6	0.6 ± 0.2	1.1 ± 0.3	0.0 ± 0.0
2000 ppm	M	10	11.2 ± 1.1	85.9 ± 1.4	0.9 ± 0.2	2.0 ± 0.5	0.0 ± 0.0
10000 ppm	M	10	14.0 ± 1.4	84.3 ± 1.6	0.7 ± 0.2	1.0 ± 0.3	0.0 ± 0.0
50000 ppm	M	10	15.0 ± 1.7	83.3 ± 1.9	0.6 ± 0.2	1.1 ± 0.3	0.0 ± 0.0
CONTROL	F	10	12.6 ± 2.4	85.6 ± 2.5	0.9 ± 0.1	1.0 ± 0.4	0.0 ± 0.0
400 ppm	F	10	17.9 ± 2.3	79.3 ± 2.4	0.7 ± 0.2	**2.1 ± 0.3***	0.0 ± 0.0
2000 ppm	F	10	14.3 ± 1.7	84.5 ± 1.7	**0.3 ± 0.2****	0.9 ± 0.3	0.0 ± 0.0
10000 ppm	F	10	15.6 ± 1.3	82.5 ± 1.2	**0.3 ± 0.2****	1.6 ± 0.3	0.0 ± 0.0
50000 ppm	F	10	**20.2 ± 2.3***	**77.9 ± 2.5***	0.6 ± 0.2	1.3 ± 0.3	0.0 ± 0.0

*: Statistically significant difference at the 95% (p.<.05) confidence level
***: Statistically significant difference at the 99.9% (p.<.001) confidence level

Table B.6.3.1-5: Clinical chemistry data for male and female rats treated with flutolanil

LEVEL	SEX	NO	GOT	GPT	LDH	CH-E
			(Karmen-U)	(Karmen-U)	(Wróblewski-U)	(SHµmoles/min/ml)
CONTROL	M	10	48 ± 2	16 ± 1	363 ± 40	0.310 ± 0.017
400 ppm	M	10	45 ± 2	15 ± 1	364 ± 36	0.337 ± 0.021
2000 ppm	M	10	**42 ± 2***	16 ± 1	411 ± 22	0.309 ± 0.013
10000 ppm	M	10	47 ± 2	19 ± 1	**493 ± 21***	0.320 ± 0.013
50000 ppm	M	10	**42 ± 1***	17 ± 1	449 ± 28	0.331 ± 0.012
CONTROL	F	10	43 ± 3	18 ± 0	444 ± 8	0.864 ± 0.051
400 ppm	F	10	46 ± 3	17 ± 1	465 ± 26	0.921 ± 0.044
2000 ppm	F	10	44 ± 2	18 ± 1	466 ± 8	0.843 ± 0.043
10000 ppm	F	10	42 ± 1	18 ± 1	449 ± 14	0.786 ± 0.077
50000 ppm	F	10	38 ± 2	17 ± 1	**503 ± 6*****	**0.695 ± 0.040***

*: Statistically significant difference at the 95% (p.<.05) confidence level
***: Statistically significant difference at the 99.9% (p.<.001) confidence level

TableB. 6.3.1-6: Clinical chemistry data for male and female rats treated with flutolanil

LEVEL	SEX	NO	AL-P	PROTEIN	BUN	GLUCOSE
			(Bessey-Lowry-U)	(g/dl)	(mg/dl)	(mg/dl)
CONTROL	M	10	7.1 ± 0.4	7.03 ± 0.06	21.5 ± 0.3	146 ± 4
400 ppm	M	10	8.2 ± 0.5 (n=9)	**7.42 ± 0.11***	21.0 ± 0.4	154 ± 9
2000 ppm	M	10	7.8 ± 0.4	**7.31 ± 0.10***	**19.3 ± 0.4*****	155 ± 4
10000 ppm	M	10	7.0 ± 0.6	**7.73 ± 0.15****	**18.4 ± 0.7****	157 ± 6
50000 ppm	M	10	6.8 ± 0.5	**7.88 ± 0.11*****	**18.9 ± 0.7****	**159 ± 3****
CONTROL	F	8	5.8 ± 0.6	5.68 ± 0.31	23.4 ± 1.5	142 ± 4
400 ppm	F	10	5.4 ± 0.4	6.12 ± 0.11	22.5 ± 0.6	**129 ± 3***
2000 ppm	F	10	5.9 ± 0.6	6.35 ± 0.10	22.8 ± 1.0	**129 ± 4***
10000 ppm	F	10	5.0 ± 0.3	**6.71 ± 0.13***	20.9 ± 0.8	**117 ± 2*****
50000 ppm	F	10	5.6 ± 0.5	**6.71 ± 0.21***	22.5 ± 1.0	**113 ± 3*****

*: Statistically significant difference at the 95% (p.<.05) confidence level
**: Statistically significant difference at the 99% (p.<.01) confidence level
***: Statistically significant difference at the 99.9% (p.<.001) confidence level

Table B.6.3.1-7a: Organ weight data for male and female rats treated with flutalonil

LEVEL	SEX	NO	BRAIN	HEART	LUNG	LIVER
			(%)	(%)	(%)	(%)
CONTROL	M	10	0.556 ± 0.019	0.322 ± 0.014	0.465 ± 0.032	4.365 ± 0.084
400 ppm	M	10	0.538 ± 0.016	0.323 ± 0.017	0.568 ± 0.049	**4.665 ± 0.105***
2000 ppm	M	10	0.560 ± 0.015	0.309 ± 0.006	**0.555 ± 0.029***	4.623 ± 0.091
10000 ppm	M	10	0.560 ± 0.014	0.319 ± 0.015	0.561 ± 0.040	**4.691 ± 0.108***

50000 ppm	M	10	0.567 ± 0.007	0.310 ± 0.007	**0.648 ± 0.069***	**5.321 ± 0.181*****
CONTROL	F	9	0.771 ± 0.034	0.344 ± 0.009	0.621 ± 0.027	4.284 ± 0.066
400 ppm	F	10	0.834 ± 0.017	1.321 ± 0.476	**0.801 ± 0.073***	4.377 ± 0.099
2000 ppm	F	10	0.832 ± 0.020	0.369 ± 0.012	0.739 ± 0.056	4.448 ± 0.144
10000 ppm	F	10	0.825 ± 0.022	0.358 ± 0.016	0.735 ± 0.057	**4.590 ± 0.098***
50000 ppm	F	10	0.844 ± 0.018	0.376 ± 0.015	0.810 ± 0.080	**5.396 ± 0.151*****

*: Statistically significant difference at the 95% (p.<.05) confidence level
***: Statistically significant difference at the 99.9% (p.<.001) confidence level

Table B.6.3.1-7b: Organ weight data for male and female rats treated with flutalonil

LEVEL	SEX	NO	KIDNEYS	SPLEEN	TESTES
			(%)	(%)	(%)
CONTROL	M	10	0.752 ± 0.023	0.207 ± 0.008	1.003 ± 0.043
400 ppm	M	10	0.773 ± 0.019	0.217 ± 0.011	1.013 ± 0.041
2000 ppm	M	10	0.705 ± 0.012	0.214 ± 0.010	0.987 ± 0.021
10000 ppm	M	10	0.721 ± 0.017	0.218 ± 0.005	0.940 ± 0.074
50000 ppm	M	10	0.737 ± 0.019	0.203 ± 0.09	0.992 ± 0.016
CONTROL	F	9	0.770 ± 0.027	0.222 ± 0.008	***** *****
400 ppm	F	10	0.787 ± 0.018	0.254 ± 0.013	***** *****
2000 ppm	F	10	0.828 ± 0.060	**0.249 ± 0.007***	***** *****
10000 ppm	F	10	0.747 ± 0.020	**0.254 ± 0.011***	***** *****
50000 ppm	F	10	0.789 ± 0.016	0.242 ± 0.010	***** *****

*: Statistically significant difference at the 95% (p.<.05) confidence level

Conclusion

The No observed adverse effect level (NOAEL) was 2000 ppm, which is equal to 180.2 mg/kg bw/day for males and 215.3 mg/kg bw/day for females. The NOAEL is based on increased liver weights and activated centrilobular hepatocytes at ≥10000 ppm dose. The study is acceptable.

B.6.3.2 Oral 90-day study

B.6.3.2.1 Oral 90-day study, study 1

Rat study

Previous evaluation	DAR (May 2005)
Evaluation of the RMS	No comments on the original evaluation. Some additional tabled results were added to the study summary. Some deviations from the current OECD 408 guideline exists, which include the following: - haematology: blood clotting time/potential were not examined - weight of epididymides not measured

reference	:	[redacted](1986a)	exposure	:	Via diet
Report number	:	85-2926 (T-3024)	doses	:	0, 500, 4000 and 2000 ppm
test substance	:	flutolanil	GLP	:	no
species	:	Sprague Dawley rats	guideline	:	Generally complies with OECD 408
group size	:	10/ sex/ group	acceptability	:	acceptable

Test guideline and GLP

The study was not performed using any official guideline or test method, and it was not done under GLP. However, the study was performed in 1986 in time before GLP was required and generally the

method complies with OECD guideline 408 (Directive 67/548/EEC, Annex V, Methods B.8). Deviations: no clotting parameters measured.

Study design

Groups of 10 male and 10 female Sprague-Dawley CD rats were administered flutolanil (purity: 97.5%) in the feed at concentrations of 0, 500, 4000 and 20000 ppm over a period of 13 weeks. Mean consumption of flutolanil was 0, 37, 299 and 1512 mg/kg bw/day for males and 0, 44, 339 and 1743 mg/kg bw/day for females in ascending order of dose. Body weight, food consumption and clinical signs were monitored routinely. Samples were taken for clinical chemistry and haematology (no clotting parameters measured) before sacrifice. Also, no samples for urine analysis were taken. Ophthalmoscopy was performed before treatment and at sacrifice. Extensive gross and microscopic examinations were performed on control and animals at the highest concentration; in animals at the two lower concentrations, microscopic examination was limited to lung, liver and kidneys. The test data was processed statistically.

Results

Mean terminal body weights for treated animals showed no significant differences from control in either males or females. By the end of the study, high dose males weighed slightly less than the controls, but this difference was less than –5%. In male rats, a linear dose-related trend for decreased brain weight was noted. This finding was not observed in female rats. When expressed as a percentage of the body weight, thyroid/parathyroid weight was significantly elevated by 23% and 20% in the mid- and high-dose males, respectively (Table B.6.3.2.1-1). Although rats are known to be sensitive with regard to thyroid effects, human relevance of the thyroid finding cannot be excluded based on the data available and the observed effects are therefore considered adverse. The most prominent treatment related effect occurred in the liver. High-dose males demonstrated a significant 14% increase in relative liver weight compared to controls. In female rats, both absolute and relative liver weights were elevated in both mid- and high-dose groups. This effect was linearly related to dose level. Serum enzyme markers of hepatotoxicity and TSH (thyroid stimulating hormone) levels were unaltered by treatment. The only consistent changes in clinical chemistry were small but statistically significant increases in albumin at the mid- (females) and high-dose (males and females) groups which was not considered adverse. Microscopic evaluation of tissues did not demonstrate any histomorphologic alterations.

Table B.6.3.2.1-1 Findings in rats given flutolanil in the diet for 13 weeks (Dewhurst I. Flutolanil, JMPR 2002)

Parameter	**Dietary concentration (ppm)**							
	0		**500**		**4000**		**20 000**	
	Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Intake (mg/kg bw per day)	0	0	34	40	230	340	1500	1700
Mean cell volume (μm^3)	51 ± 2	51 ± 2	49 ± 3	50 ± 1	50 ± 2	**49 ± 3***	50 ± 2	51 ± 2

Mean corpuscular haemoglobin (fg)	22 ± 1	23 ± 1	**21 ± 1***	22 ± 1	21 ± 1	**22 ± 1****	22 ± 1	22 ± 1
Platelet count (10^8/ml)	12 ± 2	11 ± 1	**10 ± 2***	11 ± 1	11 ± 1	11 ± 2	11 ± 1	12 ± 1
Albumin concentration (g/dl)	3.5 ± 0.2	3.6 ± 0.1	3.6 ± 0.1	3.8 ± 0.2	3.6 ± 0.1	**3.8 ± 0.1***	**3.7 ± 0.2***	**3.9 ± 0.2***
Bilirubin concentration (µg/dl)	28 ± 7	34 ± 0.1	28 ± 12	32 ± 4	28 ± 4	27 ± 5	24 ± 7	**26 ± 10***
Body weight (g)	490 ± 51	270 ± 26	500 ± 43 (102%)	270 ± 27 (100%)	510 ± 35 (104%)	270 ± 18 (100%)	470 ± 26 (96%)	260 ± 12 (96%)
Liver weight (absolute; g)	13 ± 2	6.8 ± 0.4	14 ± 2 (108%)	6.9 ± 0.5 (101%)	14 ± 2 (108%)	**7.5 ± 0.4**** (110%)	14 ± 1 (108%)	**8.1 ± 0.3**** (119%)
Liver weight (relative; %)	2.9 ± 0.2	2.8 ± 0.2	3.0 ± 0.3 (103%)	2.7 ± 0.2 (96%)	3.0 ± 0.3 (103%	**3.0 ± 0.2*** (107%)	**3.3 ± 0.1**** (114%)	**3.4 ± 0.1**** (121%)
Thyroid weight (relative; ± 10^5)	5.7 ± 0.9	6.6 ± 1.3	5.7 ± 0.8 (100%)	5.8 ± 1.2 (88%)	**7.0 ±1.1*** (123%)	6.6 ± 1.4 (100%)	**6.9 ± 0.8*** (121%)	7.5 ± 1.5 (114%)
Thyroid weight (absolute; range; mg)	22–31	12–20	22–34	11–21	26–40	11–22	27–34	14–25

***** p<0.05, ** p <0.01
Data between brackets represent the body- and organweight in percentage compared to the control

Conclusion

The NOAEL was 500 ppm, which is equal to 37 mg/kg bw/day in males and 44 mg/kg bw/day in females. It is based on increased weight of thyroid/parathyroid. The study is acceptable.

Note RMS: during the PRAPeR expert meeting 24 (June 2007), the experts agreed to set the NOAEL at 4000 ppm corresponding to 299 mg/kg bw/day in males and 339 mg/kg bw/day in females. The reason for this was that rats are known to be sensitive with regard to thyroid effects. Moreover, increased relative liver weight (<10%) without any histopathological findings was not considered adverse. The RMS agrees that the effect on liver weight at 4000 ppm (<10%) should not be regarded as adverse. However, no information on mode of action is available to exclude human relevance of the thyroid finding. Moreover, an effect on thyroid weight (>20%) was also observed in the 90-day dog study at the high dose albeit not statistically significant. The RMS therefore proposes to set the NOAEL at 500 ppm, the same as in the original DAR.

B.6.3.2.2 Oral 90-day study, study 2

Mice study

Previous evaluation	DAR (May 2005)
Evaluation of the RMS	No comments on the original evaluation. Some tabled results were added to the summary.

reference	:	[redacted] (1987)	exposure	:	Via the diet
Report number	:	86/NHH017/548 (T-3067)	doses	:	500, 5000 or 50000 ppm
test substance	:	flutolanil	GLP	:	Yes
species	:	CD-1(ICR)BR mice	guideline	:	Generally complies with OECD 408

group size	:	12 animals/ sex/ group	acceptability	:	Acceptable as supplementary study

Test guideline and GLP

The study was a range finding study for a mouse carcinogenicity study. It was not stated to be performed to conform with any official guideline or test method. The study was done under GLP and mainly complies with OECD guideline 408 (Directive 94/79/EC, Directive 92/69/EEC Method B.8.). No clinical chemistry, urine analysis or haematology was performed.

Study design

Groups of 12 male and 12 female CD-1 (ICR) BR mice were administered flutolanil (purity: 97.6%) in the feed at concentrations of 0, 500, 5000 and 50000 ppm over a period of 13 weeks. Mean consumption of flutolanil was 0, 69.24, 680.4 and 7510 mg/kg bw/day for males and 0, 80.19, 883.2 and 8825 mg/kg bw/day for females in ascending order of dose. Body weight, food consumption and clinical signs were monitored routinely. All animals were subjected to gross necropsy, and the adrenals, liver, bone marrow and spleen from controls and animals at the highest concentration, and also livers from two lower concentrations, were examined histologically. The findings were assessed statistically with the Dunnett or Student t tests.

Results

There were no clinical signs of toxicity. The only changes observed during the study were lower body weight gain among males receiving 50 000 ppm and females receiving 5000 ppm, and higher liver weights (absolute and relative) for males and females receiving 50 000 ppm (+23% for absolute liver weight and +30% for relative liver weight). There were no micro- or macropathological findings in the liver. The inferior bodyweight gain of females receiving 5000 ppm was considered anomalous as females receiving 50 000 ppm gained more weight and there was no corresponding response in the males.

Table B.6.3.2.2-1: Absolute organ weights of mice following flutolanil treatment for 90-days

SEX	MALE				FEMALE			
GROUP:	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
NUMBER:	**12**	**12**	**12**	**12**	**12**	**11**	**12**	**12**
	TERMINAL BODY WEIGHT (g)							
# IN GRP.:	12	12	12	12	12	11	12	12
MEAN:	39.1	38.3	39.3	36.7	32.0	32.0	**28.9***	30.4
STAND DEV:	3.4	3.7	2.3	3.1	2.7	3.4	2.0	2.2
	BRAIN							
# IN GRP.:	12	12	12	12	12	11	12	12
MEAN:	.487	.486	.492	.478	.500	.501	.494	.495
STAND DEV:	.025	.018	.025	.024	.023	.030	.024	.022
	HEART							
# IN GRP.:	12	12	12	12	12	11	12	12
MEAN:	.185	.185	.194	.177	.147	.145	.139	.153
STAND DEV:	.015	.013	.020	.014	.013	.019	.021	.021
	KIDNEYS (L & R)							
# IN GRP.:	12	12	12	12	12	11	12	12
MEAN:	.625	.669	.669	.595	.450	.441	.431	.421
STAND DEV:	.077	.062	.171	.083	.067	.041	.043	.055
	LIVER							
# IN GRP.:	12	12	12	12	12	11	12	12
MEAN:	2.07	1.98	2.15	**2.56****	1.78	1.71	1.61	**2.19****
STAND DEV:	.27	.32	.28	.34	.32	.25	.28	.35
	LUNGS							
# IN GRP.:	12	12	12	12	12	11	12	12
MEAN:	.211	.224	.249**	.214	.237	.206	.208	.269
STAND DEV:	.025	.032	.035	.023	.055	.036	.048	.067
	SPLEEN							
# IN GRP.:	12	12	12	12	12	11	12	12
MEAN:	.099	.096	.097	.086	.111	.099	.105	.127
STAND DEV:	.023	.014	.010	.010	.039	.017	.020	.053
	TESTES (L & R)							
# IN GRP.:	12	12	12	12	0	0	0	0
MEAN:	.245	.247	.248	.247				
STAND DEV:	.035	.037	.051	.031				

Statistical significance of differences from control (Dunnett's test): * p≤0.05, ** p≤0.01

Table B.6.3.2.2-2: Organ weights relative to terminal body weigth (%) following flutolanil treatment

SEX	MALE				FEMALE			
GROUP:	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
NUMBER:	**12**	**12**	**12**	**12**	**12**	**11**	**12**	**12**
	TERMINAL BODY WEIGHT (g)							
# IN GRP.:	12	12	12	12	12	11	12	12
MEAN:	39.1	38.3	39.3	36.7	32.0	32.0	**28.9***	30.4
STAND DEV:	3.4	3.7	2.3	3.1	2.7	3.4	2.0	2.2
	BRAIN							
# IN GRP.:	12	12	12	12	12	11	12	12
MEAN:	1.2535	1.2797	1.2587	1.3105	1.5728	1.5739	**1.7216***	1.6356
STAND DEV:	.1223	.1363	.1083	.1059	.1369	.1285	.1736	.1205
	HEART							
# IN GRP.:	12	12	12	12	12	11	12	12
MEAN:	.4746	.4839	.4964	.4833	.4645	.4538	.4842	.5049
STAND DEV:	.0385	.0387	.0576	.0371	.0616	.0543	.0834	.0733
	KIDNEYS (L & R)							
# IN GRP.:	12	12	12	12	12	11	12	12
MEAN:	1.6038	1.7545	1.7086	1.6185	1.4156	1.3839	1.5001	1.3808
STAND DEV:	.1865	.1691	.4536	.1542	.2141	.1196	.1629	.1191
	LIVER							
# IN GRP.:	12	12	12	12	12	11	12	12
MEAN:	5.298	5.140	5.462	**6.989****	5.540	5.339	5.589	**7.189****
STAND DEV:	.395	.502	.549	.751	.724	.532	.807	.848
	LUNGS							
# IN GRP.:	12	12	12	12	12	11	12	12
MEAN:	.5426	.5893	.**6376***	.5860	.7474	.6447	.7249	.8921
STAND DEV:	.0636	.0992	.0968	.0587	.1883	.1033	.1941	.2369
	SPLEEN							
# IN GRP.:	12	12	12	12	12	11	12	12
MEAN:	.2548	.2501	.2463	.2332	.3460	.3101	.3637	.4161
STAND DEV:	.0594	.0356	.0249	.0234	.1074	.0458	.0682	.1637
	TESTES (L & R)							
# IN GRP.:	12	12	12	12	0	0	0	0
MEAN:	.6296	.6508	.6362	.6773				
STAND DEV:	.0887	.1261	.1370	.1004				

Statistical significance of differences from control (Dunnett's test): * p≤0.05, ** p≤0.01

Conclusion

The NOAEL was 5000 ppm, which is equal to 680 mg/kg bw/day in males and 883.2 mg/kg bw/day in females. It is based on increased weight of the liver (absolute and relative) and lower body weight gain in males. The study is supportive.

B.6.3.2.3 Oral 90-day study, study 3

Dog study

Previous evaluation	DAR (May 2005) and addendum 1 to DAR (October 2006)
Evaluation of the RMS	No comments on the original evaluation. Some deviations with the current OECD 409 guideline exist, which include the following: - urinalaysis was not performed

	- epididymides were not weighed

reference	:	[redacted] (1986b)	exposure	:	Via gelatin capsules
Report number	:	85-2927 (T-3025)	doses	:	80, 400 and 2000 mg/kg bw/day
test substance	:	flutolanil	GLP	:	No
species	:	Beagle dogs	guideline	:	Generally complies with OECD 409
group size	:	4/ sex/ group	acceptability	:	acceptable

Test guideline and GLP

The study was not performed using any official guideline or test method, and it was not done under GLP. However, the study was performed in 1986 before GLP was required and generally the method complies with OECD guideline 409 (Directive 67/548/EEC, Annex V, Methods B.9).

Materials and methods

Groups of 4 male and 4 female Beagle dogs were administered flutolanil (purity: 97.5%) orally via gelatin capsules at doses of 0, 80, 400 and 2000 mg/kg bw/day for 3 months. Control groups received empty capsules. Clinical signs, deaths and food and water consumption were assessed routinely. Ophthalmologic examinations were performed before treatment and just before sacrifice. Samples for haematology and clinical chemistry were taken before treatment and at weeks 6 and 13. At sacrifice, all animals underwent gross and histopathological examination. The findings were assessed statistically with analysis of variance.

Results

There were no treatment-related deaths or clinical signs. The findings on ophthalmology were similar in treated and control animals. Treated females gained 1.3-1.5 kg (20-25%) more weight than controls (0.0 kg; 15%), even though their food consumption was slightly reduced (<5%). In the absence of a dose response relationship, this finding was considered not to be adverse. The only clinical chemistry finding of note was raised alkaline phosphatase activity in the males at the lowest and highest doses and females in highest dose (Table B.6.3.2.3-1). The absence of an effect in males at the intermediate dose and the high value before treatment in males at the lowest dose indicate that the finding at the lowest dose was probably not treatment-related. The absolute and relative weights of the liver were increased significantly in animals of each sex at 2000 mg/kg bw/day. The weight of the thyroid was increased >20% in animals at 2000 mg/kg bw/day and in females at 400 mg/kg bw/day, although the increase was not statistically significant. The only histopathological finding associated with the treatment was noted in the liver in the groups receiving 400 and 2000 mg/kg bw/day. The livers had an increased pallor and swelling of the hepatocytes. In these two groups, this change was compatible with an glycogen deposition (table B.6.3.2.3-1).

Table B.6.3.2.3-1: Findings in dogs given flutolanil in gelatine capsules for 13 weeks (Dewhurst I. Flutolanil, JMPR 2002)

Parameter	Time	Dose (mg/kg bw per day)			
		0	80	400	2000

		Males	Females	Males	Females	Males	Females	Males	Females
Erythrocyte count (10^9/ml)	Before test	6.5 ± 0.6	6.7 ± 0.6	6.0 ± 0.3	5.9 ± 0.5	6.2 ± 0.3	6.5 ± 0.5	6.2 ± 0.1	6.3 ± 0.5
	Week 6	6.7 ± 0.4	7.3 ± 0.3	6.2 ± 0.5	6.4 ± 0.3	6.8 ± 0.5	6.8 ± 0.7	**5.9 ± 0.4***	6.4 ± 0.4
	Week 13	6.6 ± 0.5	7.0 ± 0.4	6.7 ± 0.2	6.5 ± 0.1	6.8 ± 0.3	6.8 ± 0.6	5.8 ± 0.6	6.4 ± 0.6
Erythrocyte volume fraction (%)	Before test	45 ± 4	45 ± 4	42 ± 3	42 ± 3	43 ± 3	46 ± 4	43 ± 1	45 ± 3
	Week 13	46 ± 3	49 ± 2	47 ± 1	46 ± 1	48 ± 2	48 ± 4	42 ± 5	46 ± 3
Haemoglobin concentration (g/dl)	Before test	16 ± 1.3	16 ± 1.5	15 ± 1.2	15 ± 0.8	16 ± 1.0	17 ± 1.3	16 ± 0.5	16 ± 1.0
	Week 13	18 ± 1.3	19 ± 0.6	18 ± 0.6	18 ± 0.8	19 ± 0.7	19 ± 1.4	17 ± 2.0	18 ± 1.4
Alkaline phosphatase activity (IU/l)	Before test	72 ± 17	68 ± 19	100 ± 10	85 ± 35	75 ± 10	100 ± 16	79 ± 26	110 ± 12
	Week 13	52 ± 8	60 ± 17	**81 ± 17***	70 ± 25	58 ± 10	93 ± 17	**120 ± 16****	**110 ± 15***
Body weight (kg)		9.5 ± 1.4	7.4 ± 0.7	9.1 ± 0.9 (96%)	8.1 ± 0.6 (109%)	9.9 ± 0.7 (104%)	8.0 ± 0.8 (108%)	9.6 ± 0.7 (101%)	8.0 ± 1.1 (108%)
Liver weight (absolute) (g)		300 ± 24	240 ± 46	280 ± 29 (93%)	270 ± 31 (113%)	300 ± 10 (100%)	290 ± 19 (121%)	**380 ± 45**** (127%)	**320 ± 24*** (133%
Liver weight (relative) (g)		3.1 ± 0.4	3.3 ± 0.5	3.0 ± 0.4 (97%)	3.3 ± 0.5 (100%)	3.1 ± 0.3 (100%)	3.7 ± 0.3 (112%)	**3.9 ± 0.2**** (126%)	4.0 ± 0.5 (121%)
Thyroid weight (absolute) (g)		0.74 ±0.12	0.57 ±0.7	0.70 ±0.21 (95%)	0.58 ±0.24 (102%)	0.73 ±0.15 (99%)	0.69 ±0.15 (121%)	0.92 ±0.24 (124%)	0.70 ±0.18 (123%)
Thyroid weight (relative) (× 10^5)		7.7 ± 0.3	7.7 ± 0.3	7.8 ± 2.6 (101%)	7.2 ± 0.4 (94%)	7.4 ± 1.8 (96%)	8.6 ± 1.7 (112%)	9.5 ± 2.0 (123%)	8.8 ± 2.6 (114%)

***** *p<0.05, ** p* <0.01;analysis of variance

Data between brackets represent the bodyweight in percentage compared to the control

Table B.6.3.2.3-2: Glycogen deposition in the livers of dogs given flutolanil in gelatine capsules for 13 weeks

	Dose (mg/kg bw per day)			
	0	**80**	**400**	**2000**

Glycogen deposition	Males	Females	Males	Females	Males	Females	Males	Females
Minimal	3	1	4	0	0	0	1	1
Mild	1	3	0	4	4	4	1	1
Moderate	0	0	0	0	0	0	2	2

Conclusion

The NOAEL was 80 mg/kg bw/day. It was based on increased liver weights at 2000 mg/kg bw/day and histopathological alterations in the liver at doses of 400 and 2000 mg/kg bw/day. The study is acceptable.

Note RMS: ***The applicant derived a higher NOAEL of 400 mg/kg/day, which was based on liver weight increase observed at 2000 mg/kg/bw with hepatocyte swelling and pallor. However, this NOAEL is not accepted by the RMS since increased pallor and swelling of the hepatocytes was also observed in the livers of dogs administered 400 mg/kg/day. Therefore, the NOAEL of 80 mg/kg bw/day as defined in the DAR is considered acceptable.***

B.6.3.3 Other routes

There were no mortalities in the acute inhalation and dermal studies and the vapour pressure of flutolanil is very low (4.1 x 10-7 Pa at 20ºC). However, a short-term dermal study in rats has been conducted.

Previous evaluation	DAR, addendum 3 to DAR (May 2007)
Evaluation of the RMS	No comments on the original evaluation. Some tabled results were added to the summary.

reference	:	[redacted] (1990)	exposure	:	Dermal exposure
Report number	:	89-3497 (T-3070)	doses	:	1000 mg/kg/day
test substance	:	Flutolanil	GLP	:	yes
species	:	Sprague-Dawley rats	guideline	:	Complies generally with OECD 410
group size	:	5/ sex/ dose level	acceptability	:	Acceptable

Test guideline and GLP

The study was performed in compliance with GLP and the method used (FIFRA Pesticide assessment Guideline; US EPA 82-2 (1984)) generally complies with OECD guideline 410 (Directive 94/79/EC, Directive 92/69/EEC Method B.9.). The duration of the study was 21 days, only one dose level was used (limit-test) and test substance was administered only 5 times/week, but OECD guideline 410 regards these deviations acceptable.

Study design

The test substance (97,6% flutolanil) was administered to 10 Sprague-Dawley CD® rats (5/sex) at a dose level of 1000 mg/kg bw/day, 5 days per week for 21 days. The test substance was mixed with a small amount of distilled water prior to application to the clipped skin of the back. Control animals (5/sex) received distilled water.

Ophthalmoscopic examinations were performed pre-test and at study termination. Physical observations were performed pre-test and on each dose day. Dermal observations were scored pre-test and weekly during the study. Body weight and food consumption measurements were performed pre-test and weekly during the study. Haematology, clinical chemistry, and urinalyses parameters were evaluated for all survivors at study termination.

After 21 days of treatment, all survivors were sacrificed, selected organs were weighed and organ/body weight ratios calculated. Complete gross post-mortem examinations and histopathological evaluation of selected tissues were conducted on all animals.

Results

No evidence of dermal irritation or systemic toxicity was seen. Evaluations of clinical observations, dermal observations, ophthalmoscopic examinations, body weights, food consumption, clinical laboratory values (haematology, clinical chemistry and urinalysis) and results of gross and microscopic examinations revealed no apparent effects of flutolanil administration.

Organ weights showed no apparent effects of flutolanil administration. However, mean adrenal weights and adrenal/body weight ratios for treated males (638 mg and 1.63, respectively) were significantly lower than mean control values (794 mg and 1,98). In males, the weight of the adrenals was found to be decreased by 20% in animals treated with flutalonil at a dose of 1000 mg/kg bw. Similar differences were not present for treated females. According to the report, evaluation of historical control data reveals that adrenal weights for the control males (776-808 mg) were exceptionally high, while values for treated males (579-696 mg) were within expected ranges (350-748 mg). Therefore, the differences seen appear to reflect unusually high values for control animals, rather than an effect of flutolanil administration in treated males. To fully address this point the notifier is asked to provide the representative historical control data to assess their relevance for the current study.

Table B.6.3.3-1: Absolute organ weights of rats following dermal flutolanil exposure for 21-days

SEX	FEMALE		MALE	
DOSE:	**0**	**1000**	**0**	**1000**
	TERMINAL BODY WEIGHT (g)			
MEAN:	252.	259.	402.	394.
STAND DEV:	14.	17.	20.	34.
# IN GRP.:	5	5	5	4
	ADRENALS			
MEAN:	.0832	.0838	.0794	**.0638****
STAND DEV:	.0033	.0051	.0016	.0052
# IN GRP.:	5	5	5	4
	KIDNEYS			
MEAN:	2.014	2.167	3.131	3.053
STAND DEV:	.100	.164	.295	.208
# IN GRP.:	5	5	5	4
	LIVER			
MEAN:	7.426	8.038	11.247	11.568
STAND DEV:	.513	.767	1.457	1.060
# IN GRP.:	5	5	5	4
	SPLEEN			
MEAN:	.627	.583	.775	.688
STAND DEV:	.108	.113	.147	.104
# IN GRP.:	5	5	5	4
	TESTES/ EPIDEIDYMES			
MEAN:			4.7276	4.4024
STAND DEV:			.3615	.3768
# IN GRP.:			5	4

Statistical significance of differences from control: * p≤0.05, ** p≤0.01

Table B.6.3.3-2: Relative organ weights of rats following dermal flutolanil exposure for 21-days

SEX	FEMALE		MALE	
DOSE:	**0**	**1000**	**0**	**1000**
	ADRENALS			
MEAN:	3.30	3.25	1.98	**1.63***
STAND DEV:	.23	.23	.09	.21
# IN GRP.:	5	5	5	4
	KIDNEYS			
MEAN:	7.99	8.41	7.78	7.78
STAND DEV:	.40	.87	.66	.60
# IN GRP.:	5	5	5	4
	LIVER			
MEAN:	2.84	3.11	2.79	2.94
STAND DEV:	.13	.17	.25	.17
# IN GRP.:	5	5	5	4
	SPLEEN			
MEAN:	2.49	2.25	1.92	1.74
STAND DEV:	.48	.34	.32	.16
# IN GRP.:	5	5	5	4
	TESTES/ EPIDEIDYMES			
MEAN:			1.18	1.13
STAND DEV:			.10	.19
# IN GRP.:			5	4

Statistical significance of differences from control: * p≤0.05, ** p≤0.01

Table B.6.3.3-3: Male adrenal weight

Group	No. of Animals	Body Weights (g)	Adrenal Weights (mg)	Adrenal/Body Weight (%)
I – Control	5	383 – 431	776 – 808	0.019 – 0.021
II – FLUTOLANIL	4	352 – 435	570 – 696	0.013 – 0.018
Historical Controls	30	225 – 405	350 – 748	0.015 – 0.023

One treated male was found dead prior to dosing on Day 19 of the study. No unusual signs were evident in this animal prior to death. The only unusual observation seen at post-mortem examination was a moderately enlarged liver. However, no significant microscopic pathology was evident and no cause of death was established. In the absence of any other signs of local or systemic toxicity attributed to test substance administration, the death of this one animal does not appear to represent an effect of flutolanil. All other animals survived throughout the study.

Conclusion

Under conditions of this study, dermal administration of flutolanil to rats at a dose of 1000 mg/kg/day for 3 weeks produced no local effects (dermal irritation) and no apparent systemic toxicity. The death of one test animal on Day 19 of the study appeared to be unrelated to test substance administration. The NOAEL was greater than 1000 mg/kg bw/day. The study is acceptable.

B.6.4 Genotoxicity

B.6.4.1 *In vitro* studies

B.6.4.1.1 In vitro studies – Ames test

Previous evaluation	DAR (May 2005)
Evaluation of the RMS	No comments on the original evaluation. Some additional tabled results were added to the study summary.

reference	:	Moriya M (1981)	vehicle	:	DMSO
Report number	:	T-3016	doses	:	0, 10, 50, 100, 500, 1000, 5000, 10000, 25000 µg/ plate
test substance	:	NNF-136 (Flutolanil technical)	GLP	:	no
Test system	:	*Salmonella* typhimurium strains TA 98, TA 100, TA 1535, TA 1537 and TA 1538 and *Escherichia coli* WP2 *uvrA* Bacillus subtilis (H17, M45)	guideline acceptability	:	Generally complies with OECD 471 acceptable

Test guideline and GLP

The study was performed according to the Japanese guidelines. It was not done under GLP. The study was performed in 1981 before GLP was required. The method used in reverse mutation test complies generally with OECD guideline 471 (Directive 67/548/EEC, Annex V, Methods B.13 and B.14). However, plating was done only in duplicate.

Study design

Flutolanil (purity: 99.4%) was tested in reverse mutation tests using Salmonella typhimurium strains TA 98, TA 100, TA 1535, TA 1537 and TA 1538 and Escherichia coli WP2 uvrA at dose of 10, 50, 100, 500, 1000, 5000, 10000 and 25000 µg/plate in dimethylsulfoxide (DMSO). Studies were performed in the presence and absence of the metabolic activation system (S-9 mix). Plates were incubated at 37°C for 2 days. Appropriate compounds were used as positive control. Additionally, the DNA-damaging capability of flutolanil (purity: 99.4%) was studied with the rec-assay (DNA repair test) using Bacillus subtilis strains H17 (recombination-wild) and M45 (recombination-deficient) at doses of 20, 50, 100, 200, 500, 1000, 2000, 5000 and 10000 µg/plate. Plates were incubated overnight at 37°C. Kanamycin and mitomycin C were used as negative and positive control substances, respectively.

Results

In the reverse mutation tests, Flutolanil did not induce increases in the number of revertant colonies of any strain at any dose, compared with those of the corresponding control, whether S-9 mix was added or not. In the rec-assay, flutolanil did not cause any inhibitory zone in either strain even at the highest dose.

Table B.6.4.1.1-1 Reverse mutation tests with or without a liver metabolic system

Compound	µg/plate	S-9 Mix	No. of revertant colonies per plate					
			TA100	TA1535	WP2uvrA	TA98	TA1537	TA1538
Control		-	129	6	12	38	6	12
(DMSO)			117	12	24	49	12	11
NNF-136	10	-	111	7	15	33	14	21
			93	12	16	47	7	11
	50	-	100	11	17	48	8	15
			122	7	13	36	9	11
	100	-	105	8	21	47	6	12
			113	5	21	43	2	12
	500	-	96	9	13	50	4	21
			105	7	18	52	4	12
	1000	-	105	7	18	52	4	12
			77	10	11	41	6	14
	5000	-	98	9	16	39	6	13
			92	13	26	38	4	15
	10000	-	84	6	8	22	12	8
			74	5	13	21	6	11
	25000	-	90	5	14	28	8	8
			93	6	13	19	7	13
Control		+	150	6	17	53	9	25
(DMSO)			117	6	24	45	11	3
NNF-136	10	+	113	4	19	55	6	29
			127	10	17	41	5	28
	50	+	123	7	30	49	13	24
			99	8	25	34	10	21
	100	+	131	10	23	41	14	44
			127	11	14	56	12	31
	500	+	102	2	21	37	9	32
			117	10	24	46	11	29
	1000	+	118	8	17	61	6	22
			122	8	32	50	8	21
	5000	+	98	8	20	33	5	18
			100	8	16	35	3	9
	10000	+	106	7	15	25	3	18
			123	8	12	21	5	20
	25000	+	127	3	16	30	4	18
			129	4	10	38	3	24
Positive		-	393[a)]	2000[b)]	968[c)]	404[d)]	2000[e)]	336[f)]
Control			407	2000	910	358	2000	627
Positive		+	467[g)]	304[h)]	676[i)]	277[g)]	177[h)]	203[g)]
Control			492	239	656	255	134	188
(2-AA)		-	109	15	23	42	12	11
			104	12	21	39	8	21

a) AF-2,0.01 µg/plate. b) ENNG, 10 µg/plate. c) AF-2 0.04 µg/plate..
d) AF-2, 0.1 µg/plate. e) 9-AA, 80 µg/plate. f) 2-NF, 2 µg/plate.
g) 2-AA 0.5 µg/plate. h) 2-AA, 2 µg/plate. i) 2-AA, 40 µg/plate.

Table B.6.4.1-2 Rec-assay with *B. subtilis* M45 and H17

Compound	µg/disk	Inhibitory zone (mm)		Difference
		M45	H17	(mm)
Control (DMSO)		0	0	0
NNF-136	20	0	0	0
	50	0	0	0
	100	0	0	0
	200	0	0	0
	500	0	0	0
	1000	0	0	0
	5000	0	0	0
	10000	0	0	0
Kanamycin	10	8.5	6.5	2
Mitomycin C	0.1	9.5	1	8.5

Conclusion

No indication of mutagenic activity of flutolanil was found under the conditions of the test. The study is acceptable.

B.6.4.1.2 In vitro studies – mammalian chromosomal aberration test, study 1

Previous evaluation	DAR (May 2005)
Evaluation of the RMS	No comments on the original evaluation.

reference	:	Tokiwa T (1986)	vehicle	:	DMSO
Report number	:	T-3044	doses	:	12.1, 24.2 and 48.5 µg/ml
test substance	:	Flutolanil	GLP	:	No
Test system	:	Cultured Chinese hamster lung cells	guideline	:	Generally complies with OECD 473
			acceptability	:	Acceptable

Test guideline and GLP

The study was not done under GLP and it was not performed using any official guideline or test method. However, the study was performed in 1986 before GLP was required and Quality assurance unit audited it. The test method used generally complies with OECD guideline 473 (Directive 67/548/EEC, Annex V, Methods B.10). However, only 100 cells per treatment were examined.

Study design

Flutolanil (purity 97.5%), dissolved in dimethyl-sulfoxide (DMSO) as a vehicle, was applied to cultured Chinese hamster lung cells obtained from the National Institute of Genetics (Japan). The tests were performed at the dosages of 37.5, 75.0 and 150 µM (corresponding to 12.1, 24.2 and 48.5 µg/ml) in the presence or absence of metabolic activation system (S-9 mix). After treating the cell culture with the compound for 6 hr, the cells were washed and re-cultured for further 12 or 24 hr. 100 metaphases per treatment were analysed.

Results

At the highest dose, slight but significant increase in the frequency of chromosomal aberration, mainly gaps and breaks, was observed in the presence of metabolic activation after 12 hr but not after 24 hr re-culture (Table B.6.4.1.2-1). The reason for this may be that cells with chromosomal aberrations

could not survive and were eliminated from the culture during the extended expression period. No increment of numerical anomalies such as endoreduplications or polyploid cells were observed in the flutolanil-treated groups 12 or 24 hrs after the treatment. Positive control chemicals induced marked increases in the frequency of chromosomal damage.

Table B.6.4.1.2-1: Number of cells with chromosomal aberrations in Chinese Hamster Don cells treated with flutolanil

DOSE LEVEL (µM)	S-9 MIX	% ABERRATION			
		12 hr culture		24 hr culture	
		Total	Total - Gaps	Total	Total - Gaps
0 (DMSO)	-	4	2	4	3
37.5	-	2	2	0	0
75.0	-	1	1	2	1
150	-	4	2	1	0
Positive control[a)]	-	15**	11**	34**	33**
0 (DMSO)	+	6	0	0	0
37.5	+	13	5*	2	0
75.0	+	10	3	0	0
150	+	15*	8**	4	3
Positive control[b)]	+	57**	48**	34**	26**

Positive controls: a) Mitomycin C, b) Dimethylnitrosamine

* : Significantly different from control (P<0.05), ** : Significantly different from control (P<0.01)

Conclusion

Flutolanil induced a weak increase in number of cells with chromosomal aberrations 12 hrs after the treatment in the presence of S-9 mix. The study is acceptable.

B.6.4.1.3 In vitro studies – mammalian chromosomal aberration test, study 2

Previous evaluation	DAR (May 2005)
Evaluation of the RMS	No comments on the original evaluation.

reference	:	Jenkinson PC (1990)	vehicle	:	DMSO
Report number	:	G261-199/40 (T-3057)	doses	:	125 to 1000 µg/ml
test substance	:	Flutolanil	GLP	:	yes
Test system	:	Human lymphocytes	guideline	:	In accordance with OECD 473
			acceptability	:	Acceptable

Test guideline and GLP

The study was performed according GLP and method mainly complies with OECD guideline 473 (Directive 67/548/EEC, Annex V, Methods B.10). However, the cells were exposed to flutolanil only for 2 hours, instead of 3-6 hours required by the guideline.

Study design

Flutolanil (purity 97.6%), dissolved in dimethyl-sulfoxide (DMSO) as a vehicle, was applied to cultured human lymphocytes from a volunteer who had been previously screened for suitability. The tests were

performed at the doses of 125, 250, 500 and 1000 µg/ml in the presence or absence of metabolic activation (S-9 mix). After treating the cell culture with the compound for 2 hr, the cells were washed and re-cultured for a further 22 h. Duplicate cultures were established for the tests and 200 cells/treatment were scored for chromosomal aberrations.

Results

Flutolanil produced no significant increases in the frequency of chromosome aberrations either in the presence or absence of a liver enzyme metabolising system (Table B.6.4.1.3-1). Positive control chemicals induced significant increases in the frequency of chromosomal damage and all negative (solvent) controls gave frequencies of aberrations within the expected range for normal human lymphocytes.

Table B.6.4.1.3-1: Chromosomal aberrations in human lymphocytes treated with flutolanil

DOSE LEVEL (µG/ML)	S-9 MIX	MITOTIC INDEX	% ABERRATION	
		(% of Control)	Total	Total - Gaps
0 (DMSO)	-	100	0.5	0
125	-	79	1.0	1.0
250	-	96	2.5	2.0
500	-	45	1.0	0
1000	-	58	0.5	0.5
Positive control[a)]	-	66	39.2	25.6
0 (DMSO)	+	100	1.0	1.0
125	+	52	1.5	0.5
250	+	147	2.0	0.5
500	+	45	2.0	1.0
1000	+	75	0.5	0.5
Positive control[b)]	+	44	76.0	46.7

Positive control, a) : Ethyl methanesulphonate, b) : Cyclophosphamide

Conclusion

Flutolanil is considered to be non-clastogenic to human lymphocytes *in vitro*. The study is acceptable.

B.6.4.1.4 In vitro studies – mammalian cell gene mutation test

Previous evaluation	DAR (May 2005) and addendum 1 to DAR (October 2006)
Evaluation of the RMS	No comments on the original evaluation.

reference	:	Heidemann A (1989)	vehicle	:	acetone
Report number	:	145607 (T-3077)	doses	:	6.0, 15.0, 30.0, 60.0, 80.0 and 100 µg/ml
test substance	:	Flutolanil	GLP	:	yes
Test system	:	L5178Y cell line	guideline	:	In accordance to OECD 476
			acceptability	:	Acceptable

Test guideline and GLP

The study was performed according to GLP and the method complies with OECD guideline 476 (Directive 67/548/EEC, Annex V, Methods B.17).

Study design

Flutolanil (purity: 97.6%), dissolved in acetone, was applied to cultures of L5178Y TK+/- mouse lymphoma cells at doses of 6.0, 15.0, 30.0, 60.0, 80.0 and 100 µg/ml in the presence or absence of metabolic activation (S-9 mix). In the preliminary study, higher concentrations than 100 µg/ml precipitated in the culture medium. The highest concentration produced a decrease of cell culture growth and the cell growth observed at the lowest concentration was approximately in the range of the negative control. The cells were exposed to the compound for 4 hr. After washing, the cells were further incubated for 2 days and then plated in selective or non-selective medium. Mutant cell colonies were scored on selective plates containing 5-bromodeoxyuridine and cell survival was estimated from the non-selective plates. The assay was performed in two independent experiments and included positive controls and corresponding vehicle controls.

Results

In both experiments, the highest concentration of 100 µg/mL reduced growth of the cells, while higher concentrations precipitated in the culture medium. No increases in the number of mutant colonies were seen by the treatment with flutolanil either in the presence or absence of metabolic activation (table B.6.4.1.4-1). However, in experiment I 100 µg/mL with S9 the mutant frequency was increased by a factor 2. This effect was not observed in the absence of S9 and in experiment II in the presence of S9. In addition, the increase was not found to be concentration-related. The apparent increase in experiment I was therefore considered not biologically relevant. Moreover, the test item values were according to the study authors also in the range of the historical control data (0 to 32.0 mutants per 10^6 cells). Note that in the study report details on the historical data were not provided hampering a conclusion on the relevance of the historical control data. Positive control chemicals induced significant increases of mutant colonies.

Table B.6.4.1.4-1: Mutations detected in the mouse lymphoma thymidine kinase locus assay, in the absence of metabolic activation

DOSE LEVEL (µG/ML)	EXPERIMENT I		EXPERIMENT II	
	Rate of cell survival*	Mutant colonies/ 10^6 cells	Rate of cell survival	Mutant colonies/ 10^6 cells
Control	0.59	5.1	0.86	24.4
Control (acetone)	0.64	23.4	0.91	4.4
6	0.69	10.1	0.63	4.8
15	0.70	15.7		
30	0.70	8.6	0.59	5.1
60	0.76	0.0	0.72	20.8
80	0.74	9.5		
100	0.72	1.4	0.51	21.6
Positive control[a)]	0.10	520.0	0.61	141.0

Positive control, a) : Ethyl methanesulphonate dissolved in culture medium
* : Colonies counted in the non-selective plate/Number of cells seeded

Table 6.4.1.4-02: Mutations detected in the mouse lymphoma thymidine kinase locus assay, in the presence of metabolic activation

DOSE LEVEL (µG/ML)	EXPERIMENT I		EXPERIMENT II	
	Rate of cell survival*	Mutant colonies/ 10^6 cells	Rate of cell survival	Mutant colonies/ 10^6 cells

Control	0.76	7.9	0.89	21.3
Control (acetone)	0.71	14.1	0.88	0.0
Control (DMSO)	0.68	17.6	0.92	21.7
6	0.72	13.9	0.84	9.5
15	0.71	23.9		
30	0.64	14.1	0.73	30.1
60	0.68	13.2	0.88	28.4
80	0.72	15.3		
100	0.69	30.4	0.72	2.8
Positive control[a)]	0.68	88.2	0.58	155.2

Positive control, a) : 3-Methylcholanthrene dissolved in dimethylsulfoxide (DMSO)
* : Colonies counted in the non-selective plate/Number of cells seeded

Conclusion

Flutolanil is considered to be non-mutagenic in this mouse lymphoma thymidine kinase locus assay. The study is acceptable.

B.6.4.1.5 In vitro studies – unscheduled DNA synthesis assay

Previous evaluation	DAR (May 2005)
Evaluation of the RMS	No comments on the original evaluation.

reference	:	Fautz R (1989)	vehicle	:	acetone
Report number	:	145708 (T-3076)	doses	:	2.67, 8.00, 26,67, 53.33 and 80 µg/ml
test substance	:	Flutolanil	GLP	:	Yes
Test system	:	Freshly isolated rat hepatocytes (male Wistar)	guideline	:	In accordance with OECD 472
			acceptability	:	Acceptable

Test guideline and GLP

The study was performed according to GLP and the method complies with OECD guideline 482 (Directive 67/548/EEC, Annex V, Methods B.18).

Study design

Flutolanil (purity: 97.6%), dissolved in acetone, was given to cultures of freshly isolated hepatocytes obtained from male Wistar (CF HB) rats for 18 hr in the presence of radiolabelled thymidine (3H-TdR). The concentrations to be applied were chosen according to the results from the pre-experiments. Doses of flutolanil were 2.67, 8.00, 26,67, 53.33 and 80 µg/ml, and were tested in the two independent experiments. 2-Acetylaminofluorene (2-AAF) was used as positive control. For each concentration, including the controls, 100 cells were evaluated. The uptake of radioactivity to hepatocytes was determined by autoradiography.

Results

No dose-dependent increase in nuclear grain counts and net grain counts was observed up to the highest dose level of flutolanil (table B.6.4.1.5-1). In experiment I, cells treated with 53.33 and 80.00 µg/ml flutolanil showed slight toxic effects as determined by neutral red absorption assay. In experiment II, however, no toxicity was observed up to the highest concentration at which slight

precipitation of the test substance occurred during the incubation period. The positive control chemical induced distinct increases in both nuclear and net grain counts.

Table B.6.4.1.5-1: Unscheduled DNA synthesis in primary hepatocytes of male rats treated with flutolanil

DOSE LEVEL (µG/ML)	EXPERIMENT I			EXPERIMENT II		
	Grains per Nucleus	Grains per Cytoplasm Area	Net Grains per Nucleus	Grains per Nucleus	Grains per Cytoplasm Area	Net Grains per Nucleus
Control	29.78	26.73	3.05	13.26	12.87	0.39
Control (Acetone)	22.70	21.36	1.34	20.07	19.39	0.68
2.67	23.49	23.24	0.25	14.87	15.98	-1.11
8.00	30.84	24.54	6.30	19.78	19.52	0.26
26.67	21.11	25.57	-4.46	15.04	13.76	1.28
53.33	23.97	22.95	1.02	14.10	13.08	1.02
80.00	20.51	21.12	-0.61	16.20	16.36	-0.16
Positive control	69.39	16.19	53.20	54.16	7.61	46.55

Positive control: 2-Acetylaminofluorene dissolved in culture medium

Conclusion

Under the experimental conditions reported, flutolanil did not induce DNA repair synthesis in rat hepatocytes. The study is acceptable.

B.6.4.2 *In vivo* studies in somatic cells

B.6.4.2.1 In vivo studies – micronucleus test, study 1

Previous evaluation	DAR (May 2005)
Evaluation of the RMS	No comments on the original evaluation. Deviations from the current OECD 474 guideline (2016): - Only 1000 polychromatic erythrocytes were scored for the incidence of micronuclei. - Relatively high dose levels were used exceeding the 2000 mg/kg bw/day to be used for periods shorter than 14 days Since the study generally complies with the guideline adopted in 1983 which was in place when the study was performed. Therefore, this deviation is considered acceptable. Based on the study report it does not become clear if the target tissue (*i.e.* bone marrow) was reached. However, the ADME study of [redacted] 2012 has demonstrated exposure of the bone marrow, while the earlier [redacted] (1992) study also showed this by inference from the amounts demonstrated in bone (the femur).

reference	:	[redacted] (1983)	exposure	:	Single treatment or four consecutive treatments by gastric intubation
Report number	:	T-3019	doses	:	6400, 8000 or 10000 mg/ kg/ day
test substance	:	Flutolanil	GLP	:	No
Species	:	BDF1 mice	guideline	:	Generally complies with OECD 474
Group size	:	6 animals/ group	acceptability	:	Acceptable

Test guideline and GLP

The study was not done under GLP and it was not performed using any official guideline or test method. However, the study was performed in 1983 before GLP was required and Quality assurance unit audited it. Moreover, the method generally complies with OECD guideline 474 (Directive 67/548/EEC, Annex V, Methods B.12). Additionally, one group of animals were treated with flutolanil for 4 consecutive days.

Study design

Groups of six male and six female BDF1 mice were orally administered, by gastric intubation, flutolanil (purity 99.8%) in 2% Tween 80 solution. In the test with single treatment, flutolanil was given at 6400, 8000 and 10 000 mg/kg bw. Additionally, in the test with consecutive treatments, animals were treated with flutolanil for 4 consecutive days at a dose of 10 000 mg/kg bw. Bone marrow smears were prepared 12, 24, 48 and 72 hours after the last administration in the high dose groups. In the other groups, the smear was made 24 hours after dosage. Bone marrow smears on glass slides were observed by evaluating a total of 1000 polychromatic erythrocytes (PCE) per animal, by which incidence of PCE with micronucleus (MN-PCE) was calculated. The ratio of PCE to total erythrocytes: PCE+NCE (normochromatic erythrocytes) was also determined by counting a total of 1000 erythrocytes.

Results

In the experiments with single dosage, no significant increase in the incidence of MN-PCE was observed in either sex, at any sampling time, or at any dose, compared with the values of the corresponding controls (table B.6.4.2.1-1). Mitomycin C used as positive control induced marked increase in the incidence of MN-PCE. In addition, no significant increase was observed by consecutive administration. Also, the ratio of PCE to total erythrocytes (PCE+NCE) did not decrease even by the consecutive administration of the highest and technically limiting dose at 10000 mg/kg.

Table B.6.4.2.1-1: Micronucleus test of flutolanil in mice with single or consecutive administration, single administration

DOSE (MG/KG)	SAMPLING TIME (HR)	MALE		FEMALE	
		PCE/(PCE+NCE) (%)	MN-PCE/PCE (%)	PCE/(PCE+NCE) (%)	MN-PCE/PCE (%)
0	12	58.5	0.05	57.2	0.10
10000	12	65.6	0.13	63.2	0.02
0	24	64.8	0.13	55.9	0.05
10000	24	63.9	0.15	65.8	0.05
0	48	56.5	0.13	54.0	0.03
10000	48	60.7	0.08	64.9	0.10
0	72	57.2	0.03	54.5	0.05
10000	72	54.6	0.13	55.9	0.13
0	24	59.8	0.07	57.5	0.08
6400	24	56.1	0.05	61.2	0.10
8000	24	60.1	0.08	62.9	0.12
Positive control	24	49.7	4.48*	52.2	3.92*

PCE=polychromatic erythrocytes, NCE= normochromatic erythrocytes, MN-PCE= PCE with micronucleus,

Positive control: Mitomycin C, 2 mg/kg,

*: Significantly different from control at $p<0.001$

Table B.6.4.2.1-2: Micronucleus test of flutolanil in mice with single or consecutive administration, four consecutive administrations

DOSE (MG/KG)	SAMPLING TIME (HR)	MALE		FEMALE	
		PCE/(PCE+NCE) (%)	MN-PCE/PCE (%)	PCE/(PCE+NCE) (%)	MN-PCE/PCE (%)
0x4	12	61.4	0.12	65.9	0.13
10000x4	12	59.1	0.17	63.8	0.10
0x4	24	60.9	0.03	61.4	0.20
10000x4	24	65.1	0.07	62.2	0.07
0x4	48	63.3	0.15	60.5	0.12
10000x4	48	57.4	0.07	61.5	0.02
0x4	72	67.4	0.13	60.5	0.15
10000x4	72	66.2	0.13	60.1	0.05

Conclusion

Flutolanil did not induce any significant increase in the frequency of the erythrocytes with micronuclei, compared with the values of the corresponding control, in any groups of single or consecutive treatment in either sex. The study is acceptable.

B.6.4.2.2 In vivo studies – micronucleus test, study 2

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	A GLP statement was included in the report. The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study. Deviations from the current guideline (adopted 2016): - 2000 inmature erythrocytes were scored for the incidence of micronucleated immature erythrocytes and 200 erythrocytes were observed to determine the proportion of erythrocytes among the total erythrocyte count instead of 4000 and 5000 erythorcytes, respectively. However, the amount of erythrocytes scored is in line with the guideline adopted in 1997 which was in place when the study was performed (2011 - 2012). Therefore, this deviation is considered acceptable. Based on the study report it does not become clear if the target tissue (*i.e.* bone marrow) was reached. However, there was a mild decrease in immature versus total erythrocyte ratio which, although not statistically significant, suggest that the bone marrow was exposed. Moreover, the ADME study of [redacted] 2012 has demonstrated exposure of the bone marrow, while the earlier [redacted] (1992) study also showed this by inference from the amounts demonstrated in bone (the femur).

test substance	:	Flutolanil	GLP	:	yes
Species	:	Slc/ICR mice	guideline	:	In accordance with OECD 474 (1997)
Group size	:	5/ group	acceptability	:	yes

Executive summary

Flutolanil (98.7% purity) was suspended in 0.5% (w/v) methylcellulose solution and given by oral gavage to groups of 5 male Slc/ICR mice per group at dose levels of 0 (vehicle control), 500, 1000 or 2000 mg/kg/day (limit dose for this type of study), once daily for consecutive two days. A positive control group received a single intra-peritoneal injection of Mitomycin C. Bone marrow smears were obtained from each animal 24 hours after the last dose. Smear slides were stained with acridine orange and 2,000 immature erythrocytes were examined for each mouse by fluorescence microscopy for micronucleated immature erythrocytes (MNIE). The proportion of immature erythrocytes was assessed by examination of 200 total erythrocytes for each animal.

At every dose level of flutolanil, the frequency of MNIE in immature erythrocytes was comparable with the negative control group. Meanwhile, the frequency of MNIE was significantly increased with mitomycin C. The ratio of immature erythrocytes to total (immature + mature) erythrocytes was not affected by treatment of flutolanil, while it was slightly reduced by mitomycin C.

In conclusion, flutolanil did not show any clastogenic effect on immature erythrocytes in the bone marrow of mice, up to the limit dose level of 2000 mg/kg/day.

Materials and Methods

Test item:	Flutolanil
Batch No.:	9AE0011P
Purity:	98.7%
Expiry:	11 July 2013
Storage:	Room temperature, in the dark
Solubility:	8.01 µg/mL in water, at 20ºC
Vehicle:	0.5% w/v methylcellulose solution
Positive control:	Mitomycin C at 2.0 mg/kg (Lot 538AHI, expiry September 2012:Kyowa Hakko Kogyo Co., Ltd) by i.p. injection
Animals:	S1c/ICR mice ([redacted]), 8 weeks of age
Study start and completion:	18 November 2011 to 09 March 2012

In an acute oral toxicity study of flutolanil in the same mouse strain, death and abnormal clinical signs were not observed in either sex at up to 10000 mg/kg. In the previous micronucleus test, no animals died after administration at 10000 mg/kg/day for up to 4 days. Therefore, the limit dose at 2000 mg/kg was selected as the highest dose level in the current test. Additional dose levels of 1000 and 500 mg/kg were employed for middle and low dose groups.

Dosing suspensions were prepared just before the first dose and divided into two aliquots, one for immediate use, while the other was stored refrigerated for use on the next day. The required weight of flutolanil was ground in a mortar and then suspended in the vehicle, then topped up to the volume required at 200 mg/mL. This preparation was serially diluted with vehicle to produce concentrations of 100 and 50 mg/mL for dosing. This method was shown in earlier studies to produce homogeneous

suspensions, and storage stability at 0.8 and 240 mg/mL was shown to be satisfactory for at least 8 days. Mitomycin C was prepared just before dosing, diluting one vial of 2 mg with 6.6 mL of distilled water to produce the dosing formulation of 0.3 mg/mL, for immediate use.

Flutolanil was administered at 0 (vehicle only), 500, 1000 or 2000 mg/kg at a dose volume 10 mL/kg body weight, once daily for two consecutive days (24 h dosing interval), by oral gavage. Mitomycin C was administered by single intraperitoneal injection of 3 mg/kg at a dose volume of 10 mL/kg body weight. Each treatment group comprised 5 male mice.

The mice were observed for any clinical signs of toxicity just before and after each dose, and at 1, 3 and 6 h after each dose, then just before sacrifice. Body weight was recorded just before each dose and at sacrifice. At sacrifice the femurs were extracted and fetal bovine serum (FBS) was used to flush out the bone marrow erythrocytes, which were centrifuged and then resuspended in a small volume of FBS. Four smear slides were prepared from each animal, air dried, fixed in methanol, then coded and stored frozen. One slide from each animal was later stained with 0.04% acridine orange solution, rinsed with 1/15 M sodium phosphate buffer (pH 6.8), and coverslipped for examination, the remaining slides being stored as reserve (these reserves were discarded at the end of the study, after it was established that further examinations were not necessary).

Micronuclei in immature erythrocytes emit yellowish green fluorescence, same as nuclei, under fluorescent lighting using acridine orange excitation and emission wavelengths (band pass filter 515-560 nm, long-pass filter 590 nm). In contrast, cytoplasm of immature erythrocyte emits red fluorescence by acridine orange staining. Micronuclei were counted for one slide per animal. For the incidence of micronucleated immature erythrocytes (MNIE), 2000 immature erythrocytes were scored for each animal. For the evaluation of bone marrow toxicity, 200 erythrocytes were observed for each animal to measure the proportion of immature erythrocyte (IE) among total erythrocytes (TE: including immature and mature erythrocytes).

After decoding, the proportion (%) of immature erythrocytes among total erythrocytes (IE/TE) was calculated individually. The frequency (%) of micronucleated immature erythrocytes (MNIE) among 1000 immature erythrocytes was also calculated. These calculations, including the calculation of group mean and standard deviation, were performed for following statistical analysis: For frequency of MNIE in the flutolanil treated groups, statistical analysis was performed first by non-parametric Dunnett's type test (Steel's test) for pair-wise multiple comparison, considering $p \leq (0.14/\text{no. of treated groups} = 0.0467)$ as recommended by Kim *et al* (Mut Res 469, 233-241, 2000). For the ratio of IE/TE in the flutolanil treated groups, a non-parametric Dunnett's type test was used to compare with vehicle control group considering $p \leq 0.05$ as statistically significant. In addition, for frequency of MNIE, a Cochran-Armitage (C-A) trend test was also performed to detect a dose dependent increasing trend in flutolanil treated groups, considering $p \leq (0.0467)$ as statistically significant. For MNIE, such statistical significant levels for pair-wise comparison and C-A trend tests were chosen, as recommended by Kim *et al,* in order to maintain the experimentalwise type I error rate of 0.05. For the positive control group, the frequency of MNIE and ratio of IE/TE were compared with the vehicle control by Wilcoxon's rank sum test, considering $p \leq 0.05$ as statistically significant.

Validity of the test was accepted if:

a) In both vehicle and positive control groups, the proportion of IE/TE and the frequency of MNIE were within the historical control range.

b) In treated groups, the proportion of IE/TE was more than 20% of the control value.

Evaluation of the study results used the 3-step procedure of Kim *et al* (2000), which modified the original procedure proposed by Hayashi *et al* (Environ Mol Mutagen 13, 347-356, 1989):

1. Study is acceptable (acceptance criteria above);
2. Statistically significant increase of MNIE in treated groups compared to vehicle control;
3. Statistically significant dose-dependent increase of MNIE in treated groups.

Evaluation of ploidy was not necessary in this study, considering the negative results.

Results

The frequency of MNIE in the vehicle control group and mitomycin C group was within the corresponding historical control ranges. No deleterious or mutagenic effects were observed in the vehicle control group. Statistically and also biologically significant increase of the frequency of MNIE was clearly demonstrated by mitomycin C treatment, and while a slight but statistically significant reduction of IE/TE was observed, the proportion of IE/TE still remained at 85% level of the vehicle control group.

In each flutolanil-treated group, the proportion of IE/TE was comparable with vehicle control, being more than 20% of vehicle control value. For this parameter, there were no statistically significant changes except at 1,000 mg/kg. However, no treatment relationship was interpreted for this difference, owing to the lack of dose dependency.

These results established validity of the study.

At each dose level of flutolanil, the frequency of MNIE was comparable with vehicle control and no statistically significant increase was observed. The Cochran-Armitage trend test revealed also no increasing trend of MNIE for flutolanil treated groups.

Table B.6.4.2.2-1: MNIE/IE and IE/TE frequencies following flutalonil treatment

Test or control substance	Dose (mg/kg)	No. of animal	MNIE/IE (%)						IE/TE (%)			
			Mean	±	SD	Stat.			Mean	±	SD	Stat.
Vehicle control (0.5 % (w/v) MC)	-	5	0.09	±	0.08	-			51.0	±	4.6	-
Flutolanil	500	5	0.10	±	0.06	NS			51.7	±	3.6	NS
	1000	5	0.04	±	0.07	NS			58.0	±	0.7	#

	2000	5	0.11	±	0.12	NS			46.2	±	7.4	NS
Positive control (Mitomycin C)	3	5	3.76	±	2.48	*			43.5	±	4.0	*

MNIE: Micronucleated immature erythrocytes.
IE: Immature erythrocytes. TE: Total erythrocytes.
SD: Standard deviation. Stat.: Results of statistical analysis.
MC: Methylcellulose. -: Not applicable.
NS: Not significant (No statistically significant difference).
#: p≤0.05 vs vehicle control by Dunnett's type multiple comparison test.
*: p≤0.05 vs vehicle control by Wilcoxon's rank sum test.

Cochran-Armitage trend test revealed no significant trend of increase of MNIE for Flutolanil-treated groups.

Table B.6.4.2.2-2: Historical data

Item		Vehicle control	Positive control (Mitomycin C, 24hrs after single i.p. at 3 mg/kg)
MNIE (%)	N	18	16
	Mean	0.11	4.80
	SD	0.04	2.87
	Control range	0.00[a)]-0.22	1.00[b)]-13.40
IE/TE (%)	N	18	16
	Mean	49.9	42.0
	SD	3.5	2.9
	Control range	39.3-60.5	33.4-50.6

Above figures represent for the data obtained for male ICR mice at 8 weeks old at sampling for years through 1999-2011.
MNIE: Micronucleated immature erythrocytes.
IE: Immature erythrocytes. TE: Total erythrocytes
N: Number of studies collected. SD: Standard deviation
i.p.: Intra-peritoneal injection.
Control range was defined as the mean value ± 3 x SD.
a) Minimum acceptable level defined as 0.00%, as the mean value minus 3 x SD was below zero.
b) Minimum acceptable level defined as 1.00%, as the mean value minus 3 x SD was below zero.

There were no mortalities during the study, and no clinical signs of toxicity. Body weight was also unaffected by treatment, and there were no abnormal findings at necropsy.

Conclusion

Flutolanil had no clastogenic effect on immature erythrocytes in the bone marrow of mice at dosages up to the limit-dose of 2000 mg/kg/day for two consecutive days.

B.6.4.3 *In vivo* studies in germ cells

Specific studies in germ cells were considered not to be necessary.

B.6.5 Long-term toxicity and carcinogenicity

Rodent studies

B.6.5.1.1 Oral studies in rodent, study 1 (study in rats)

Previous evaluation	DAR (2005) and addendum 1 to DAR (October 2006)
Evaluation of the RMS	The study report does not contain a GLP-certificate. The performing lab is known to be GLP certified but the study was performed before GLP was in place. The study was generally performed according to OECD 453 (1981) which was in place when performing the study. The study slightly deviates from the current OECD 453 guideline (2009): - No ophthalmic or blood clotting examinations. - Haematology/blood chemistry at 3 months was conducted on 6 rats/sex only. - Residues in liver, kidney and fat were determined. The study is considered acceptable.

reference	:	[redacted] (1982)	exposure	:	Via the feed
Report number	:	54-039 (T-3013)	doses	:	0, 40, 200, 2000 and 10000 ppm
test substance	:	NNF-136 (flutolanil)	GLP	:	No (no applicable yet)
species	:	Sprague-Dawley CD rats	guideline	:	OECD 453 (1981)
group size	:	50/ sex	acceptability	:	acceptable

Test guideline and GLP

The study was not done under GLP and it was not performed using any official guideline or test method. However, the study was performed in 1980-1982 before GLP was required and Quality assurance unit audited it. In generally, the method complies with OECD guideline 453 (Directive 67/548/EEC, Annex V, Methods B.30). However, no ophthalmic examinations and blood clotting potential was examined, and haematological examination after 3 months was performed on 6 animals per group only. Additionally to the guideline, concentration of flutolanil residues in liver, kidney and fat were determined.

Materials and methods

Groups of 50 male and 50 female Sprague-Dawley CD rats were administered flutolanil (purity: 97.5 to 99.2 %) in the feed at concentrations of 0, 40, 200, 2000 and 10000 ppm over a period of 104 weeks. Additional groups for interim sacrifice were also used. Six animals of each group were killed at interim sacrifice at 3 months and 10 of each sex per group at 12 months. Mean consumption of flutolanil was 0, 1.8, 8.7, 86.9 and 460.5 mg/kg bw/day for males, and 0, 2.1, 10.0, 103.1 and 535.8 mg/kg bw/day for females.

Body weight, clinical signs and food consumption were recorded routinely, but no ophthalmic examinations were performed. Urine analysis and clinical chemical and haematological examinations were performed at 6 and 18 months on 10 animals of each sex per group and at interim and terminal

sacrifices. At sacrifice, a gross examination was performed, 11 organs were weighted, and 29 tissues (including eyes) and any abnormalities found were examined microscopically. The concentration of flutolanil residues in liver, kidney and fat were determined in 10 animals at each group at the terminal sacrifice.

Results

No clinical signs were found. The body-weight gain of males at the highest dose was reduced during the first 3 months but no thereafter (Table B.6.5.1.1-1). In contrast, the body-weights of males at the mid dose group and all treated females were frequently higher than those of controls, possibly due to increased food consumption at the beginning of the study.

Altered erythrocyte parameters (reduced haemoglobin concentration, haematocrit and mean corpuscular haemoglobin) were seen in females receiving 2000 or 10 000 ppm at 18 months only; the erythrocyte count was unaffected. Interpretation of these findings is difficult, as there was no clear dose–response relationship and the values for haemoglobin concentration and erythrocyte volume fraction in all groups at 18 months were notably higher than those at 12 or 24 months. The authors considered the pattern of effects in females 10 000 ppm to be treatment-related and adverse. Other variations in haematological parameters were not consistent with dose or duration and were within normal physiological ranges. They were thus considered not to be adverse effects of treatment.

Variations in a number of clinical chemical parameters were seen during the study, but most showed no consistency over time or dose, and many of these parameters, such as decreased bilirubin in older animals, were considered not to be adverse. Some of the alterations in animals at the highest concentration indicated a link with treatment: increased blood urea nitrogen, calcium and phosphate in males, decreased aspartate aminotransferase activity in males and females throughout the study and reduced cholesterol in females at 24 months. All the values except those for cholesterol were reported to be within the normal range. The results of urine analysis were generally similar in treated and control groups, with no indication of treatment-related nephropathy. Variations in the results of clinical chemistry and urine analysis within groups and over time (especially at 18 months) were greater than those usually reported (e.g. for sodium, potassium and chloride), possibly masking any small changes.

Table B.6.5.1.1-1: Findings in Crj:CD rats given diets containing flutolanil for up to 2 (adapted from Dewhurst I, Flutolanil JMPR 2002)

PARAMETER	DIETARY CONCENTRATION (PPM)									
	0		40		200		2000		10 000	
	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Body weight, week 10 (g)	480	290	480	300	490	300	470	300	**450***	300
Body weight, week 71 (g)	790	520	820	570	810	570	840	550	810	540

Food intake, week 10 (g/day)	25	19	**28 ****	**23 ****	**29 ****	**21 ****	**29 ****	**22 ****	**29 ****	**23 ****
Erythrocyte volume fraction, 18 months (%)	24	22	22 4	20	22	21	23	**20 ***	22	**20 ****
Haemoglobin concentration, 18 months (g/dl)	7.7	7.7	7.2 5	7.2	7.2	7.4	7.7	**6.8****	7.7	**7.0 ****
Mean corpuscular haemoglobin, 18 months (pg)	18	19	18	19	17	19	19	**19 ***	**18 ***	**18 ***
Total cholesterol, 24 months (mg/dl)	160	130	150	130	150	110	130	120	160	**96 ***
Calcium, 24 months (mg/dl)	9.9	10	10	10	10	11	10	11	10 *	10
Phosphate, 24 months (mg/dl)	5.2	3.8	5.5	3.8	5.2	3.6	5.6	3.8	**6.5 ***	3.5
Aspartate aminotransferase activity, 24 months (mIU/ml)	110	140	110	180	130	130	120	140	**93 ***	120
Blood urea nitrogen, 24 months (mg/dl)	17	18	20	20	18	17	18	**14 ***	33	16

* = $p < 0.05$; ** = $p < 0.01$; *t* test

At the 3-month interim sacrifice, a number of organs from flutolanil-treated animals showed statistically significant ($p < 0.05$) changes in weight relative to those of controls, including reduced absolute weights of the thymus and pituitary in all treated males, increased weights of the liver in animals of each sex at the highest concentration and increased kidney weights in males at this concentration (Tables B.6.5.1.1-2 and B.6.5.1.1-3). The changes in the thymus and pituitary did not show a clear dose–response relationship and diminished when corrected for body weight (data not shown). Females at the highest concentration had increased relative pituitary weights. At the 12-month interim sacrifice, the only notable effect on organ weights was an increase in liver weight. At terminal sacrifice, increased liver and kidney weights were seen at the highest concentration. Wide variations within groups (standard deviations as large as the mean) hindered evaluation of changes in the weights of many other organs.

Table 6.5.1.1-2: organ weights (mean g and Standard Deviation), males 3-month interim sacrifice

DOSE		Brain	Pituitary	Thyroid	Thymus	Lung	Heart	Liver	Kidney	Adrenals	Spleen	Reproductive organs
Control	MEAN	2.255	0.01520	0.02998	0.3630	1.490	1.690	18.017	3.603	0.06528	0.8902	3.352
	S.D.	0.060	0.00177	0.00613	0.0888	0.118	0.082	2.985	0.478	0.00865	0.0864	0.306
40 mg/kg	MEAN	**2.142***	**0.01243***	**0.02188***	0.2918	1.403	1.580	16.960	3.512	**0.05545***	**0.7865***	3.297
	S.D.	**0.098**	**0.00143**	**0.00167**	0.0776	0.091	0.089	1.995	0.315	**0.00597**	**0.0559**	0.236
200 mg/kg	MEAN	**2.157***	**0.01275***	**0.02198***	0.2727	1.338	1.532	16.093	3.322	0.06140	**0.7342***	3.218
	S.D.	**0.058**	**0.00158**	**0.00211**	0.0999	0.132	0.197	2.850	0.429	0.00863	**0.1159**	0.210
2000 mg/kg	MEAN	2.190	**0.01275***	0.02587	**0.2602***	**1.333***	**1.423*****	16.075	3.192	0.06115	**0.6742*****	3.360
	S.D.	0.045	**0.00203**	0.00350	**0.0543**	**0.070**	**0.063**	1.491	0.179	0.00938	**0.0568**	0.271
10000 mg/kg	MEAN	**2.130****	**0.01207****	0.02357	**0.2633***	1.413	1.572	17.558	3.382	0.05998	**0.6625*****	3.360
	S.D.	**0.068**	**0.00068**	0.00514	**0.0364**	0.202	0.133	1.071	0.169	0.00729	**0.0381**	0.271

* : Significantly different from control (p<0.05)
** : Significantly different from control (p<0.01)

Table 6.5.1.1-3: organ weights (mean g and Standard Deviation), females 3 month interim sacrifice

DOSE		Brain	Pituitary	Thyroid	Thymus	Lung	Heart	Liver	Kidney	Adrenals	Spleen	Reproductive organs
Control	MEAN	1.972	0.01374	0.01678	0.3142	1.058	0.970	9.870	1.978	0.07590	0.4708	0.10138
	S.D.	0.079	0.00180	0.00302	0.0477	0.095	0.067	0.485	0.195	0.00799	0.0118	0.01147
40 mg/kg	MEAN	2.020	0.01533	0.01812	0.3513	1.050	1.050	9.322	2.053	0.07035	0.4862	0.09070
	S.D.	0.054	0.00441	0.00197	0.0892	0.040	0.086	0.954	0.190	0.00618	0.0440	0.01336
200 mg/kg	MEAN	2.025	0.01992	0.01760	0.3370	1.067	0.993	9.838	2.070	0.06892	0.5223	0.09285
	S.D.	0.050	0.01000	0.00402	0.0823	0.039	0.074	1.162	0.148	0.00387	0.1030	0.01600
2000 mg/kg	MEAN	2.023	0.01595	0.01805	0.3442	1.013	1.045	10.180	2.203	0.07712	0.5348	0.10537
	S.D.	0.061	0.00387	0.00370	0.0736	0.162	0.120	1.156	0.372	0.00564	0.1013	0.01906
10000 mg/kg	MEAN	2.028	**0.01830***	0.02128	0.3112	1.092	**1.096***	**11.634****	2.158	0.07916	0.5180	0.10154
	S.D.	0.054	**0.00387**	0.00421	0.0456	0.073	**0.076**	**0.812**	0.148	0.00805	0.0701	0.00727

* : Significantly different from control (p<0.05)
** : Significantly different from control (p<0.01)

Gross pathological investigations did not show any treatment-related effects. A minimal increase in 'red change' of the lung in males at the highest concentration was not observed in females and was not correlated with any microscopic findings.

A number of the lesions occurred at higher incidence in some treated groups than in controls; however, many showed no dose–response relationship and/or no pattern consistent with other findings. Lesions associated with administration of flutolanil included vacuolar degeneration of the liver, atrophy and proliferation of splenic reticular cells in males at concentrations ≥ 2000 ppm, and bone marrow ('dyshaematopoiesis') in females at the highest concentration (table B.6.5.1.1-2). The patterns of nephrosis and prostate duct dilatation changed during the study, with apparent increases in treated groups at 12 months but with no dose–response relationship over a 250-fold range. These findings were not reproduced at termination. There were no adverse effects of treatment on the thyroid.

Table B.6.5.1.1-2: Non-neoplastic lesions in rats given diets containing flutolanil for up to 2 years (adapted from Dewhurst I, Flutolanil JMPR 2002)

Lesion	**Dietary concentration (ppm)**									
	0		**40**		**200**		**2000**		**10 000**	
	Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Terminal sacrifice										
Lung, atelectasia	8/21	19/26	**23/26***	20/26	15/19	16/20	15/19	19/22	**19/22***	19/24
Spleen, decreased cellular elements	1/21	4/26	4/26	5/26	3/19	1/20	**6/19***	3/22	**6/22***	6/24
Spleen, reticulocyte proliferation	0/21	0/26	0/26	2/26	0/19	2/20	0/19	4/22	1/22	**6/24***
Prostate duct, dilatation (≥ 3), 12 months	1/10		5/10		5/10		5/10		6/10*	
Prostate duct, dilatation (≥ 3), 24 months	11/21		8/26		**4/19***		**4/19***		**2/22***	
Kidney, nephrosis (> 2), 12 months	6/10	1/10	4/10	3/10	7/10	4/10	4/10	4/10	5/10	5/10
Kidney, nephrosis (> 2), 24 months	16/21	6/26	21/26	5/26	11/19	2/20	12/19	5/22	18/22	4/24
Liver, vacuolar degeneration (> 2), 12 months	0/10	2/10	1/9	3/10	0/10	2/10	1/10	2/10	2/9	5/10
Liver, vacuolar degeneration (> 2), 24 months	2/21	9/26	4/26	11/26	2/19	10/20	4/19	8/22	1/22	**16/24***
Liver, granulation	0/21	6/26	1/26	3/26	0/19	2/20	0/19	3/22	1/22	10/24
Bone marrow, dyshaematopoiesis	7/21	7/26	8/26	10/26	8/19	8/20	7/19	11/22	8/22	**13/24***
Died before study termination										

Liver, vacuolar degeneration (> 2)	7/28	11/24	6/25	12/24	4/30	15/30	8/30	16/28	11/29	18/26
Spleen, reticulocyte proliferation	0/28	2/24	0/25	7/24	0/30	5/30	0/30	3/28	1/29	1/26
Spleen, decreased cellular elements	6/28	2/24	6/25	5/24	7/30	6/30	2/30	8/28	6/29	**12/26***
Prostate duct dilatation (≥ 3)	7/28		12/25		18/30		11/30		13/29	

The overall incidences of tumours were similar in treated and control groups (Table B.6.5.1.1-3). A number of tumours were present at greater incidences in treated groups than in concurrent controls. In general, these were sporadic, since their incidence showed no dose–response, was not statistically significant, and the values were within the range of generic data for the strain of rat (although data for the test facility were reported to be no longer available). The pattern of pituitary tumours varied considerably between groups, with an increased incidence of chromophobe adenomas but a decrease in chromophobe adenocarcinomas in treated females. The overall incidences of pituitary tumours were considered to indicate that flutolanil is not tumorigenic in the pituitary gland.

Table 6.5.1.1-3: Total incidence of the tumours found by site, type and number in rats treated with flutolanil for 24 months

DOSAGES (PPM)	**0**		**40**		**200**		**2000**		**10000**	
Sex	**male**	**female**	**male**	**female**	**male**	**female**	**male**	**female**	**male**	**female**
Adrenals										
neuroblastoma	1	0	1	0	2	0	3	0	2	0
pheochromoblastoma	1	1	1	2	0	1	2	0	0	0
pheochromocytoma	0	1	3	0	4	2	2	2	4	0
cortical adenocarcinoma	0	1	1	0	0	0	0	0	1	0
Brain										
mixed glioma	0	0	1	1	2	0	0	0	0	0
Lymph nodes (inguinal)										
malignant lymphoma	0	0	0	0	0	0	0	1	0	0
Lymph nodes (mesenteric)										
histiocytic sarcoma	0	0	0	0	1	0	0	0	0	0
lymphocytic sarcoma	0	0	0	1	0	0	0	2	0	0
malignant lymphoma	0	0	0	0	0	0	0	1	0	0
lymph nodes (submaxl.)										
lymphocytic sarcoma	0	2	0	0	2	0	0	1	0	0
Liver										
cholangioma	0	0	0	0	0	0	0	1	1	1

Lungs									
adenoma	0	0	0	0	0	0	1	0	0 1
adenocarcinoma	2	0	1	0	1	0	0	0	0 0
malignant lymphoma	0	0	0	0	0	0	0	1	0 0
Mammary glands									
adenoma	1	1	0	0	0	0	0	2	0 1
adenocarcinoma	0	19	0	23	0	13	0	15	0 12
fibroadenoma	1	9	0	2	1	9	1	7	0 5
fibroadenocarcinoma	0	3	0	6	0	4	0	4	0 1
papillary adenocarcinoma	0	0	0	1	0	0	0	0	0 0
Pancreas									
islet cell adenoma	6	0	2	3	5	1	3	1	2 2
Pituitary									
chromophobe adenoma	8	10	4	18	11	17	11	15	8 16
chromophobe adenocarcinoma	12	24	7	15	12	16	1 0	13	9 11
chromophobe and eosinophilic adenoma	0	0	0	1	1	0	0	0	0 0
chromophobe and eosinophilic adenocarcinoma eosinophilic adenoma	0	1	0	0	0	3	0	1	3 0
	0	1	0	0	0	0	0	0	0 0
Prostate gland									
adenocarcinoma, metastasis	0	-	0	-	0	-	0	-	1 -
Salivary gland (submaxl)									
myxoma	1	0	0	0	0	0	0	0	0 0
Seminal vesicle									
adenocarcinoma	0	-	1	-	0	-	0	-	0 -
Small intestine									
papillary adenocarcinoma	0	0	0	1	0	0	0	0	0 0
Testis									
Leydig cell tumour	1	-	2	-	1	-	0	-	0 -
Thymus									
lymphocytic sarcoma	1	0	0	1	1	0	0	1	0 0
reticulum cell sarcoma	0	1	0	0	0	0	0	0	0 0
thymoma (m)	0	3	0	3	0	1	0	0	0 1
Thyroids									
C-cell adenoma	2	2	0	1	0	1	0	3	3 2
follicular adenoma	2	0	0	0	0	1	2	0	0 2
follicular adenocarcinoma	0	0	1	0	0	0	0	0	0 0
papillary adenocarcinoma	0	0	0	1	0	0	1	0	0 0
Urinary bladder									
papilloma	0	0	0	0	0	0	0	1	1 2
Uterus									
adenocarcinoma	-	0	-	1	-	0	-	0	- 0

Blood lymphocytic leukaemia	0 0	0 0	1 0	0 0	1 0

At the two higher concentration groups, two rare tumours were seen at increased incidences: liver cholangioma and urinary bladder papilloma. Although the incidences of these two tumour types were not statistically significant, none occurred in the groups at 0, 40 and 200 ppm. Furthermore, the liver is a target organ for flutolanil. The overall incidence of cholangioma was 3/200 (1.5%) (1/50 in males and 1/50 in females at 10 000 ppm and 1/50 in females at 2000 ppm group) in the two groups combined, which is not significantly greater than the control incidence. In the study report it is noted that '*according to reports on the spontaneous incidence of cholangioma in the liver of SD strain rats, two out of 24 rats developed the lesions*'. However, details on the respective studies are not provided hampering the determination of the relevance of those findings for the present study.
The incidence of urinary bladder papillomas was 1/50 in females at 2000 ppm, 1/50 in males at 10 000 ppm, 2/50 in females at 10 000 ppm. There was no evidence of a hyperplastic response in the bladder of flutolanil-treated animals. In general, the cholangioma and urinary bladder pappilomas observed were thus considered sporadic, since their incidence showed no dose–response, was not statistically significant, and the values were within the range of generic data for the strain of rat (although data for the test facility were reported to be no longer available). To fully address these points notifier is asked to provide representative historical control data for the liver cholangioma and urinary bladder paillomas findings if such information is available.

The residual concentration of flutolanil in organs revealed detectable residues in the fatty tissue of males and females in the two higher groups. The mean values of 0.18 ppm (males) and 0.24 ppm (females) were detected in the 2000 ppm group, and 0.58 ppm (males) and 0.66 ppm (females) in the 10000 ppm group.

Conclusion

The NOAEL was 200 ppm, which is equal to 8.7 mg/kg bw/day in males and 10.0 mg/kg bw/day in females, and is based on slight anaemia in females and histopathological splenic changes observed in males at 2000 ppm. Although many of the effects showed no or minimal dose–response relationships, the overall pattern of findings indicated that 2000 ppm was an effect level. Treatment with flutolanil at doses up to 10000 ppm for 104 weeks did not produce any neoplastic changes in rats. The study is acceptable.

B.6.5.1.2 Oral studies in rodent, study 2 (study in mice)

Previous evaluation	DAR (2005) and addendum 1 to DAR (October 2006)
Evaluation of the RMS	The study was generally performed according to OECD 453 (1981) which was in place when performing the study. The study slightly deviates from the current OECD 453 guideline (2009):

	- No blood chemistry or urinalysis The study is considered acceptable.

reference	:	[redacted] (1990)	exposure	:	Via the feed
Report number	:	89/NHH018/0043 (T-3041)	doses	:	0, 300, 1500, 7000 or 30000 ppm
test substance	:	flutalonil	GLP	:	yes
species	:	CD-1 (ICR) BR mice	guideline	:	Generally complies with OECD 453 (1981)
group size	:	52/ sex	acceptability	:	acceptable

Test guideline and GLP

The study was done under GLP and the method used complies generally with OECD guideline 453 (Directive 67/548/EEC, Annex V, Methods B.30).

Materials and methods

Groups of 52 male and 52 female CD-1 (ICR) BR mice were fed flutolanil (purity: 97.6%) in the feed at concentrations of 0, 300, 1500, 7000 and 30000 ppm over a period of 78 weeks. Additional groups of 10 males and 10 females for interim sacrifice after treatment for 13 weeks were allocated at each dose. Mean consumption of flutolanil was 0, 32, 162, 735 and 3333 mg/kg bw/day in males, and 0, 34, 168, 839 and 3676 mg/kg bw/day in females in ascending order of dose. All animals underwent a detailed gross necropsy. Extensive microscopic examinations were performed on animals that died during the study, all controls and animals at the highest concentration. Anomalies, liver, kidney and lungs from mice at the lowest and intermediate concentrations were also examined microscopically. The results were analysed by a range of statistical tests (Student's t-test, Dunnett's test, Cox's test, Fisher's exact probability test and Cochran-Armitage test).

Results

There was no increased mortality associated with the administration of flutolanil. Rather, a significant trend towards longer lifespan by the treatment was observed in females. Body weight gain was reduced in both sexes at the highest dose and with females receiving 7000 ppm (Table B.6.5.1.2-1).

Haematological investigations revealed no changes that were considered to be related to exposure to flutolanil. Analysis of organ weights revealed higher absolute and relative liver weights than those of controls for females in high dose group (20% and 34% increase, respectively). Also, increased absolute and relative spleen weights in females at the highest concentration were detected (15% and 23% increase, respectively).

Gross examination revealed no other signs of a treatment-related effect. The non-neoplastic findings were typical of those in aged mice, the general pattern indicating that treated animals had fewer degenerative changes than controls. Treatment-related findings were confined primarily to the liver, with increased panacinar and centriacinar fatty vacuolation at the highest concentration in females. Also at 7000 ppm, increased, but not significantly, centriacinar and periacinar fatty vacuolation was detected. In males, the increased periacinar fatty vacuolation detected at $\geq$ 1500 ppm was probably related to treatment, as the liver is a target organ. The only tumours for which the incidences were increased were hepatocellular adenoma and carcinoma in males. However, the increases were not

statistically significant (Fisher exact test), individually or combined, and, for animals at the highest concentration, were within the range for male CD-1 mice in the testing facility and in generic data from the supplier.

Table 6.5.1.2-1 Findings in CD-1 mice given diets containing flutolanil for up to 79 weeks (adapted from Dewhurst I, Flutolanil JMPR 2002)

Parameter	Dietary concentration (ppm)									
	0		300		1500		7000		30 000	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Body-weight gain, week 24 (g)	24	16	24 (100%)	16 (100%)	23 (96%)	16 (100%)	23 (96%)	14 * (88%)	20 * (83%)	14 ** (88%)
Terminal body weight (g)	50	44	49 (98%)	45 (102%)	48 (96%)	43 (98%)	52 (104%)	41 (93%)	47 (94%)	39 * (89%)
Liver weight (absolute, g)	3.2	2.0	3.3	2.0	3.0	1.9	3.3	2.1	3.5	2.4
Liver weight (relative, %)	6.5	4.7	6.9	4.6	6.3	4.4	6.3	5.1	7.3	6.3**
Hepatocellular carcinoma	3	0	9	0	3	1	3	0	5	0
Hepatocellular adenoma	3	0	1	0	0	0	0	0	7	1
Peri-acinar fatty vacuolation (n = 52)	4	1	6	2	9	0	14*[a]	0	9	2
Pan-acinar fatty vacuolation (n = 52)	0	0	1	1	0	2	0	0	2	5*[a]
Centri-acinar fatty vacuolation (n = 52)	0	2	0	0	0	0	0	2	0	7

* = p < 0.05 ** p < 0.01, *t* test, *[a] = *p* < 0.05, Fisher exact test
Numbers in brackets indicate percentage compared to control group

Conclusion

The NOAEL was 300 ppm for males, which is equal to 32 mg/kg bw/day, and is based on an increased incidence of periacinar hepatocytic fatty vacuolation at 1500 ppm and higher doses. For females, the NOAEL was 1500 ppm, equal to 168 mg/kg bw/day, on the basis of a significant

decrease in body-weight gain at 7000 ppm. Treatment with flutolanil at doses up to 30000 ppm for 79 weeks did not produce any neoplastic changes in the mice. The study is acceptable.

Dog studies

B.6.5.1.3 Oral studies in dog, study 1

Previous evaluation	DAR (2005) and addendum 1 to DAR (October 2006)
Evaluation of the RMS	The study was performed before GLP was in place and generally complies with OECD 452. The study is considered acceptable.

reference	:	[redacted] (1982)	exposure	:	Orally via gelatin capsules
Report number	:	T-3014	doses	:	0, 50, 250 or 1250 mg/kg body weight per day
test substance	:	flutolanil	GLP	:	No
species	:	Beagle dogs	guideline	:	Generally complies with OECD 452 (1981)
group size	:	6/ sex	acceptability	:	yes

Test guideline and GLP

The study was not done under GLP and it was not performed using any official guideline or test method. However, the study was performed in 1979-1981 before GLP was required and Quality assurance unit audited it. In generally the method used complies with OECD guideline 452 (Directive 67/548/EEC, Annex V, Methods B.30).

Materials and methods

Groups of 6 male and 6 female beagle dogs were administered flutolanil (purity 97.5-99.4%) orally via gelatin capsule at doses of 0, 50, 250 and 1250 mg/kg bw/day over a period of 104 weeks. In addition to routine observations for this type of study, body temperature and pulse were measured weekly 6 h after dosing, electrocardiography and ophthalmoscopy were performed before treatment and at termination, sperm parameters were measured at termination and the oestrus cycle regularly. Urine, faeces and blood samples were obtained before treatment and at 1.5, 3, 6, 12, 18 and 24 months. Haematological examinations included measurements of prothrombin time. Excretion of bromosulfonaphthalein (liver function test) and phenolsulfonphthalein (kidney function test) was investigated at 24 months. Extensive gross and histopathological examinations were performed on all animals. The results were analysed by analysis of variance or Student t test as appropriate.

Results

Emesis, salivation and excretion of soft faeces were found in the ≥250 mg/kg bw/day group animals after 15 month of treatment (tables B.6.5.1.3-1 and B.6.5.1.3-2). The incidence and extent were rather severe at the highest dose. The food consumption and body weight of animals at the highest dose were decreased after about 18 months. The reduction in body weight of these animals during the final 6 months was about 0.8 kg (7%), whereas all other groups showed increases of 0.1–0.2 kg (1–2%) during the same period. The absolute weights of organs were similar in the treated and control groups. Some organ weights were increased relative to body weight in animals at the highest dose, the

increase for the brain and heart reaching statistical significance ($p < 0.01$) in both sexes. The relative liver weight was increased by <10%.

Table 6.5.1.3-1 Occurrence of clinical signs in dogs treated with flutolanil for 104 weeks

	Dietary concentration (mg/kg bw/day)							
	0		50		250		1250	
Clinical sign (no of animals/group)	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Emesis	0/6	0/6	0/6	0/6	5/6	4/6	6/6	6/6
Salivation	0/6	0/6	0/6	0/6	2/6	4/6	2/6	3/6
Soft faeces	0/6	0/6	0/6	0/6	4/6	6/6	6/6	6/6

Table 6.5.1.3-2: Findings in dogs treated with flutolanil for 104 weeks

DOSAGES	**0, 50, 250 and 1250 (mg/kg bw/day)**	
Mortality	Nad	
Clinical signs	≥250 mg/kg:	emesis, salivation, excretion of soft faeces
Body weight gains	1250 mg/kg:	decreased
Food consumption	1250 mg/kg:	decreased
Water consumption	Nad	
Body temperature	Nad	
Electrocardiogram	Nad	
Ophthalmoscopy	Nad	
Haematology	Nad	
Clinical chemistry	Nad	
Urinalysis	Nad	
Gross pathology	1250 mg/kg:	intestinal hyperaemia
Organ weights	1250 mg/kg:	brain and heart (relative)
	Nad	
Sperm parameters	Nad	
Oestrus cycle	Nad	

Nad : no abnormalities detected

Tables 6.5.1.3-3 and 6.5.1.3.4 provide an overview of the bodyweight relative organ weights in males and females, repectively.

Table 6.5.1.3-3: Bodyweight-relative organ weights (mean % and Standard Deviation), males

DOSE		Brain	Pituitary	Thyroid	Heart	Lung	Liver	Adrenal	Kidney	Spleen	Testis
Control	MEAN	7.1333	0.0055	0.0732	8.2500	7.9333	28.6667	0.1210	4.5817	2.5750	1.7483
	S.D.	0.3670	0.0005	0.0109	0.5541	0.7967	2.7031	0.0070	0.4113	0.4706	0.1671
50 mg/kg	MEAN	7.2500	0.0055	0.0712	0.4167	7.9033	29.1167	0.1222	4.6217	2.7483	1.7317
	S.D.	0.3332	0.0005	0.0109	0.4665	0.7757	2.0673	0.0084	0.3455	0.3084	0.1489
250 mg/kg	MEAN	7.100	0.0053	0.0753	0.1667	7.8500	28.9833	0.1180	4.6567	2.5667	1.7733
	S.D.	0.0894	0.0005	0.0093	0.4320	0.8550	2.5996	0.0073	0.2438	0.5267	0.1174
1250 mg/kg	MEAN	7.7500*	0.0057	0.0797	9.0667*	8.5167	30.8500	0.1300	4.9867	2.8433	1.9117
	S.D.	0.3619	0.0005	0.0087	0.4590	0.6735	2.1989	0.0090	0.2386	0.4342	0.1509

* : Significantly different from control (p<0.05)
** : Significantly different from control (p<0.01)

Table 6.5.1.3-4: Bodyweight-relative organ weights (mean % and Standard Deviation), females

DOSE		Brain	Pituitary	Thyroid	Heart	Lung	Liver	Adrenal	Kidney	Spleen	Ovary
Control	MEAN	7.3833	0.0055	0.0743	8.1833	8.1500	29.100	0.1252	4.4967	2.7183	0.1337
	S.D.	0.3061	0.0005	0.0104	0.4792	0.9072	2.4438	0.0067	0.3523	0.5938	0.0310
50 mg/kg	MEAN	7.3167	0.0055	0.0762	8.2833	8.4333	30.2833	0.1230	4.9400	2.8033	0.1378
	S.D.	0.2401	0.0055	0.0111	0.4070	0.8189	2.3592	0.0064	0.3625	0.5156	0.0197
250 mg/kg	MEAN	7.5333	0.0057	0.0712	8.1167	8.3500	28.3667	0.1247	5.0017	2.7600	0.1332
	S.D.	0.2582	0.0005	0.0102	0.3251	0.6473	2.5057	0.0059	0.3197	0.4565	0.0197
1250 mg/kg	MEAN	8.0333**	0.0060	0.0817	8.9667*	8.9833	31.8667	0.1373**	5.3900	2.9483	0.1455
	S.D.	0.2422	0.0000	0.0082	0.3830	0.5419	2.1491	0.0041	0.3383	0.4652	0.0290

* : Significantly different from control (p<0.05)
** : Significantly different from control (p<0.01)

The number of viable sperm observed was 2.7 to 3.6 x 10^8, with 85 to 96% viability and 6 to 7% abnormal sperm, which were all within the normal range. The estrous cycles were normal, with maximal estrus maintained for 3-4 days.

Hyperaemia of the intestinal tract was observed in both male and female dogs in high dose groups. In males the hyperaemia was observed in duodenum (2), jejunum (2), and ileum (1). In females it appeared in duodenum (1), duodenum + jejunum (2) and jejunum (1). Any other abnormalities in gross observations were not seen. Histological examination showed no clear differences in the pattern of findings in treated and control animals. The gastrointestinal effects may have been a local effect due to administration of the capsules, but it is unusual for such effects to become more prevalent towards the end of a study. The authors did not propose a mechanism for the gastrointestinal lesions but noted that they were consistent with irritation.

Conclusion

The NOAEL was 50 mg/kg bw/day based on clinical signs (emesis, salivation, excretion of soft faeces) arised during the latter part of the study. The study is acceptable.

B.6.6 Reproductive toxicity

B.6.6.1 Reproductive toxicity, study 1

Previous evaluation	DAR (2005)
Evaluation of the RMS	No comments on the original evaluation. However, some deviations from the current OECD 416 guideline can be identified *i.e.* no pre-mating evaluation of estrous cycles and no sperm evaluations (although estrous cycles and some sperm parameters were evaluated in the long-term dog study, with negative results). No functional investigations (neurotoxicity endpoints) or evaluation of pubertal development. However, since the study complies with the OECD guideline in place when the study is performed the study is considered acceptable.

reference	:	[redacted] (1991)	exposure	:	Via diet
Report number	:	89-3417 (T-3069)	doses	:	0, 200, 2000 or 20000 ppm
test substance	:	flutalonil	GLP	:	
species	:	Sprague-Dawley (Crl:CD®(SD)BR) rats	guideline	:	In accordance with OECD 416 (1983)
group size	:	30/ sex/ dose	acceptability	:	Acceptable

Test guideline and GLP

The study was performed in compliance with GLP and according to OECD test guideline 416 (1983)/Directive 87/302/EEC, Part B, ”two generation reproduction test”, and US EPA Guideline Subdivision F (1982), and Japanese MAFF No. 4200 (1985).

Deviations: None

Material and methods

A two-generation reproduction study was conducted with flutolanil (purity: 97.6 - 98.3%) administered in the diet at dose levels of 0 (vehicle control), 200, 2 000, and 20 000 ppm to 30 male and 30 female Sprague-Dawley (Crl:CD® (SD) BR) rats (42 days old) per dose level. F0-generation animals (30 males and 30 females) were bred within their treatment groups to produce F1 litters after a 13-weeks pre-mating treatment period. Females were allowed to three 7-day mating periods with different males until sperm in the vaginal smear or copulation plug were observed. From a pool of two F1 pups/sex/group at weaning, animals for F1 adult generation were selected. These 30 males and 30 females of F1 generation were bred to produce F2 pups after a 14-week premating period. Litters were culled by random selection to a total of eight pups (four/sex) on day 4 postpartum. Mortality, behaviour, clinical observations, body weight development, food consumption, mating performance, gestation period, reproduction and litter data were examined. At sacrifice, each adult generation animal was given a gross postmortem examination and spleen, liver, reproductive tissues and gross lesions were taken and preserved. Histopathological evaluations of these tissues were performed for

control and high dose animals for both generations. Gross lesions were evaluated histopathologically also for all low and mid-dose adults for both generations. Liver and spleen were weighed for all parental generation animals.

F1 and F2 pups were examined externally and internally for gross abnormalities and only abnormal tissues were saved. F1 pups were sacrificed either at weaning or at time of selection of the F1 adults. F2 pups were sacrificed at weaning.

The average intakes of flutolanil at 200, 2 000 and 20 000 ppm were approximately 0, 16, 161 and 1 636 mg/kg bw/day for F0 males; 0, 16, 157 and 1614 mg/kg bw/day for F1 males; 0, 19, 188 and 1 917 mg/kg bw/day for F0 females; and 0, 20, 191 and 1 955 mg/kg bw/day for F1 females.

Results

No adverse effects were observed on survival, body weight data, food consumption, reproductive performance or physical observation data for the parental animals or on any litter parameter or on post-mortem examination. The only treatment-related effect found was increase in liver weight (up to approximately 20%) in the parental animals at the highest dietary level of flutolanil (20 000 ppm). This increase in liver weights, both absolute and relative to body weights, was most apparent in the F0 adult animals (males and females) and the F1 parental females.

The NOAEL was 2 000 ppm, no reproductive effects were observed at a dietary level up to and including 20 000 ppm.

Table B.6.6.1-1: Main observations in two-generation reproduction study

Parameter	Control	200 ppm	2 000 ppm	20 000 ppm
F0 generation				
-Mean body weights (g)	512/300	524/294	524/297	508/288
-terminal (m/f)[a]				
-bw gain during gestation	128	120	123	129
-bw gain during lactation	13	12	15	23
-Gestation length (days)	22.0	22.1	21.9	22.0
-Organ weights (g or %)				
-liver, absolute, g (m/f)- liver, absoluteg % (m/f)	18.3/9.7	18.9/9.6 103/99	19.3/9.8 105/101	19.9/10.7** 109/110
-liver, relative, g (m/f)	3.6/3.2	3.6/3.3	3.7/3.3	3.9**/3.7**
- liver, relative, % (m/f)		100/103	103/103	108/116
-spleen, absolute (m/f)	0.74/0.52	0.73/0.53	0.74/0.52	0.73/0.49
-spleen, relative (m/f)[b]	1.44/1.72	1.40/1.80	1.42/1.75	1.44/1.69
F1 generation, adults				
-Mean body weights (g)				
-terminal (m/f)	568/316	570/316	582/318	556/317
-bw gain dring gestation	125	119	113	122
-bw gain during lactation	6	6	13	11
-Gestation length (days)	22.0	22.0	22.3	22.1

-Organ weights (g) -liver, absolute, g (m/f) - liver, absolute, % (m/f) -liver, relative, g (m/f) - liver, relative, % (m/f) -spleen, absolute (m/f) -spleen, relative (m/f) **F1 generation, pups** -Body weights, day 21 (g)	 19.5/10.2 3.4/3.2 0.77/0.51 1.36/1.61 46.3	 19.6/9.8 101/96 3.5/3.1 103/97 0.79/0.50 1.39/1.59 46.3	 19.7/10.3 101/101 3.4/3.3 100/103 0.78/0.53 1.34/1.67 47.3	 19.6/11.1 101/109 3.5/3.5* 102/109 0.73/0.49 1.31/1.54 45.5
F2 generation, pups -Body weights, day 21 (g)	 42.9	 45.8	 46.3	 43.4

[a] males/females

[b] organ weight/body weight x 1000

* p <0.05, ** p<0.01

Conclusions

The NOAEL for reproduction and pup toxicity was ≥ 20 000 ppm (corresponding 1 636-1 614 mg/kg/day for males and 1 917-1 955 mg/kg/day for females) and the NOAEL for parental toxicity (increased liver weight up to almost 20%) was 2 000 ppm (corresponding to 157-161 mg/kg bw/day in males and 188-191 mg/kg/day in females). It should be noted that according to the OECD guideline the highest dose level should be chosen with the aim to induce toxicity but not death or severe suffering. In the present study effects on liver weight were observed indicating parental toxicity. Moreover, the top dose was equivalent to 1600/1900 mg/kg bw/day which is relatively high also considering that the rat is the most sensitive species for flutalonil treatment. Therefore, the current study is considered to be acceptable to study reprotoxic effects of flutalonil. The study is acceptable.

B.6.6.2

B.6.6.3 Developmental toxicity studies

B.6.6.3.1 Developmental toxicity, study 1 (rat)

Previous evaluation	DAR (2005)
Evaluation of the RMS	The study slightly deviated from the current OECD 414 guideline since the test substance was administered from gestation day 6 through day 15 instead of administrering daily from implantation to the day prior to scheduled caesarean section. However, since the study complies with the OECD guideline in place when the study is performed the study is considered acceptable.

reference	:	[redacted] (1987, as amended 1992)	exposure	Via gavage
Report number	:	87/NHH023/554 (T-3043)	doses	0, 40, 200 or 1000 mg/kg/day
test substance	:	Flutolanil	GLP	Yes
species	:	Sprague-Dawley CD rats	guideline	In accordance with OECD 414
group size	:	22/ dose	acceptability	Acceptable

Test guideline and GLP

The study was performed in compliance with GLP and according to OECD guideline 414 (Directive 87/302/EEC, Part B, ”teratogenicity study - rodent and nonrodent”), and also USA EPA guidelines (1982) and Japanese MAFF (1985).

Deviations: None

Material and Methods

Groups of 22 pregnant female Sprague-Dawley CD rats received daily oral doses of flutolanil (purity: 97.5%) at levels of 0, 40, 200 and 1 000 mg/kg bw/day administered by gavage in aqueous 0.5% (w/v) methylcellulose mucilage from gestation day 6 through 15 (day 0 = sperm positive day or copulation plugs). Individual daily doses were determined from the individual daily body weights on dosing days. All animals were killed on day 20 of gestation for examination of their uterine contents.

Individual maternal body weights were recorded on gestation days 0, 3, from 6 to 16, 18 and 20. Food consumption was recorded on days 0, 3, from 6 to 18 daily, and 20 of gestation. Water consumption was recorded daily throughout gestation. On gestation day 20, the foetuses were removed from dams by caesarean section. The number and distribution of live and dead foetuses, number of early and late resorptions and number of implantation sites and corpora lutea were recorded. Dams were examined macroscopically for evidence of disease or adverse reaction to treatment and specimens of abnormal tissues were retained. All foetuses were weighed, sexed and examined for external malformations. Placental weights and abnormalities were recorded. The neck, thoracic and abdominal cavities of approximately one-half of each litter were dissected and examined, and foetuses were fixed in alcohol, stained with Alizarin-red and examined for skeletal malformations and variations. The remaining half of the foetuses in each litter were preserved in Bouin´s fixative and examined for visceral malformations and variations.

Results

There were no treatment-related effects on any of the parameters examined. Maternal body weights, food consumption and water consumption were similar in all groups.

There was no change in foetal viability, weights, placental weights or any other reported parameters. Numbers of foetuses with increasing or decreasing ossification depending of bone observed were increased mainly at 1 000 mg/kg bw/day. However, these changes indicate no teratogenic potential of the substance since the changes were all within the historic control data as presented in Table B.6.6.2.1-1 and in the case of incomplete ossification thoriacic vertebral centra did not show a clear dose-response. Note that in the study report details on the historical data were not provided hampering a conclusion on the relevance of the historical control data. The NOAEL for maternotoxic and embryonic/foetal developmental effects was ≥ 1 000 mg/kg bw/day. To fully address this point the notifier is asked to provide the representative historical control data to assess their relevance for the current study.

Table B.6.6.2.1-1: Main results of the rat developmental toxicity study

Parameter	Control	40 mg/kg bw/day	200 mg/kg bw/day	1 000 mg/kg bw/day	HCD Mean (ranges)
Maternal body weights (g) -day 6 -day 20	 249 373	 244 363	 246 368	 247 369	 248 (236-258) 363 (346-383)[1]
Food consumption (g/rat/day) -day 6 -day 18-19	 26 26	 25 25	 25 25	 24 26	 26(25-27)[2] No data
Water intake (ml/rat/day) - day 6 - day 19	 47 52	 51 50	 45 53	 52 57	No data
Number of maternal observations	0	0	2	6	
Foetal body weights (g) Placental weights (g)	3.23 0.51	3.33 0.50	**3.37*** 0.51	3.34 0.53	3.32 (3.00-3.55)
Number of foetuses (litters) with					
-external malformations	0.3(1)[a]	0.3(1)[b]	0.3(1)[c]	0.3(1)	0.01 (0.0-0.4)[b]
-skeletal malformations	0.6(1)[e]	0.6 (1)[f]	0	4(4)[g]	0.01(0.0-0.9)[e] 0.04 (0.0-1.4)[f]
-visceral malformation	2(1)[h]	0	0	1[i]	
-skeletal variations (%), e.g., - Ossification 1st cervical centra [j]	16.5%	7.7%	**2.6%***	**2.0%***	6.82 (0.0-22.2)[3]
- In complete ossification thoracic vertebral centra [k]	22.2%	**36.8%***	26.3%	**32.7%***	26.24 (8.6-58.3)[4]
metacarpals/ metatarsals (4/4)	10.1(10)	19.4(10)	19.7(11)	**22.9*(14)**	0.94 (6.2-71.4)[4]
- % small foetuses	5.7% (11)	2.3% (5)	**0.7% (2)****	2.3 (5)	3.61 (0.0-16.9)[5]
Number of viable foetuses Number of resorptions	14.4 1.0	14.0 0.9	13.7 0.7	13.9 1.2	13.6 (11.1-15.3)[6] 0.9 (0.3-1.8)

*p<0.05, ** p<0.01

[a]craniorrhachischisis, left eyelids absent, neck and rib cage reduced, thyroids absent

[b]one foetus with a slight right hindlimb flexure, other with tail absent

[c]one foetus with agnathia, left eye absent, proboscis, right eye reduced

[d]one foetus with lower jaw protruding, left eye reduced, other with abnormal tail – proximal section threadlike, distal section swollen and haemorrhagic

[e]one foetus with anomalous vertebral column and rib cage

[f]one foetus with agenesis of vertebrae caudally from lumbar region

[g]one foetus with agenesis of vertebrae from the proximal caudal region and associated anomalous sacral vertebrae and tail, one foetus with limb long bones reduced in length and generalized reduction of ossification, two foetuses with abnormal forelimb flexure

[h]one foetus with a small diaphragmatic hernia, the other with a cardiovascular abnormality, severe generalized subcutaneous oedema, reduction in musculature of abdominal wall, reduced kidneys and subcutaneous vesicle, nasal region

[i]one foetus with retro-oesophageal right subclavian artery

[j]vetral arch ossification

[k]incomplete ossification

[1] data from 10 studies

[2] data from 3 studies

[3] data from 116 studies

[4] data from 119 studies

[5] data from 119 studies

[6] data from 146 studies

Conclusions

The NOAEL for maternal and foetal toxicity and developmental toxicity was ≥ 1 000 mg/kg bw/day. The study is acceptable.

B.6.6.3.2 Developmental toxicity, study 2 (rabbit)

Previous evaluation	DAR (2005)
Evaluation of the RMS	The study slightly deviated from the current OECD 414 guideline since the test substance was administered from gestation day 6 through day 18 instead of administrering daily from implantation to the day prior to scheduled caesarean section. However, since the study complies with the OECD guideline in place when the study is performed the study is considered acceptable.

reference	:	[redacted] (1987)	exposure	Via gavage
Report number	:	T-3042	doses	0, 40, 200 and 1 000 mg/kg bw/day
test substance	:	flutalonil	GLP	Yes
species	:	New Zealand White rabbit	guideline	In accordance with OECD 414
group size	:	13 to 16/ group	acceptability	Acceptable

Test guideline and GLP

The study was performed in compliance with GLP and OECD guideline 414 (Directive 87/302/EEC "teratogenicity study – rodent - nonrodent"; and according to guidelines of the USA EPA (1982) and Japanese MAFF (1985).

Deviations: none

Material and Methods

Groups of 13-16 pregnant New Zealand White rabbits were administered daily oral doses of flutolanil (purity: 97.5%) at levels of 0, 40, 200 and 1 000 mg/kg bw/day by a stomach tube in 2% (w/v) arabic gum aqueous solution from day 6 to day 18 of gestation.

Individual doses were determined from the individual body weights recorded on gestation day 6. The control groups received the vehicle only at a volume of 10 ml/kg. All animals were weighed on day 0, 6, 9, 12, 15, 18, 23 and 28 of gestation. Food intakes were measured individually and daily during the gestation. On gestation day 28, the foetuses were removed from dams by caesarean section. The number of viable foetuses, dead foetuses, resorptions and the number of total implantations and corpora luteae were recorded. The abdominal, thoracic and pelvic cavities and organs of the dams were examined for gross changes and the following organs were weighed: liver, kidneys, spleen and adrenals. All viable foetuses were weighed, sexed and examined for external abnormalities. Each foetus was examined for visceral abnormalities. After visceral examination, skeletal abnormalities and degree of ossification were examined after staining the skeleton with alizarin red S.

Results

There were no mortalities, clinical signs or adverse body weight changes during the study (Table B.6.6.2.2-1). Maternal organ weights and the mean foetal body weight were not reduced in groups. Incidence of resorptions and deaths were slightly increased at 200 and 1 000 mg/kg bw/days. The resportions and deaths occurring at the top dose tested occurred in 5 different litters (out of 13 litters). However, the number of live fetuses remained unaffected. There were no other signs of toxicity and no increased incidence of malformations or variations in treated groups. One foetus had multiple malformations at 1000 mg/kg bw/day and one foetus had acrania at 200 mg/kg/bw/day. To fully address this point the notifier is asked to provide the representative historical control data to assess their relevance for the current study.

Table 6.6.2.2-1: Main observations in the rabbit developmental study

Parameters	0 mg/kg bw/day	40 mg/kg bw/day	200 mg/kg bw/day	1000 mg/kg bw/day
Number of mated females	16	16	16	16
Dams without implants	1	0	3	3
Dams excluded	1[a]	1[b]	0	0
Number of dams with litters	14	15	13	13
Mean body weights (kg)				
- gestation day 0	3.04	2.96	3.00	2.97
- gestation day 6	3.51	3.38	3.40	3.39
- gestation day 18	3.71	3.54	3.57	3.56
- gestation day 28	3.87	3.72	3.80	3.70
Maternal organs weights (g) /relative weight %)				
- liver	118.5/3.1	124.3/3.4	119.0/3.1	118.9/3.2
- kidney	18.8/0.49	19.1/0.52	18.3/0.48	19.1/0.52
- spleen	1.4/0.04	1.6/0.04	1.4/0.04	1.4/0.04
- adrenals	0.34/0.009	0.32/0.009	0.33/0.009	0.30/0.008
Mean number of live foetuses	7.4	6.6	7.8	7.3
Number of resorptions and deaths (litters)	1	0	4 (3)	7 (5)
Foetal mortality (%)[f]	1.0	0	3.8	6.9
Sex ratio (male/female)	1.36	1.20	1.02	0.86
Mean foetal body weights (g) male/female	43.4/42.9	44.6/45.0	45.3/43.8	45.7/42.5
Foetuses/litters with malformations	0	0	1[c]	1[d]
Foetuses with skeletal or visceral abnormalities	0	0	1[e]	0
Foetuses with skeletal or visceral variations	0	0	0	0

[a] swallowed down a stomach tube
[b] found dead
[c] acrania
[d] multiple malformation (shortening of crown-rump length, hydrocephaly, gastroschisis, myeloschisis)
[e] fusion of 2nd, 3rd and 4th sternebrae
[f] (total number of resorptions and deaths/ total number of implantations) x 100

Conclusions

In the DAR it was concluded that the NOAEL-level was ≥1 000 mg/kg/day for dams and for intrauterine development. However, the RMS notes that the positive trend of resportions and deaths occurring in 5 different litters (out of 13 litters) should not be neglected even a statistical significant difference was not observed. Therefore, the LOAEL for intrauterine development wat set at 200 mg/kg bw/day. The study is acceptable.

B.6.6.3.3 Developmental toxicity, study 3

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study.

reference	:	[redacted] (2012)	exposure	Via gavage
Report number	:	R-1093 (T-3229)	doses	0, 100, 300 or 1000 mg/kg/day
test substance	:	flutolanil	GLP	Yes
species	:	New Zealand White rabbit	guideline	In accordance to OECD 414
group size	:	19 to 22/ group	acceptability	Acceptable

Executive summary

Flutolanil (99.1% purity) was administered orally to groups of New Zealand White female rabbits, 19 to 22 pregnant animals per group, at dose levels of 0 (control), 100, 300 or 1000 mg/kg/day (limit dose) at a standard dose volume of 5 mL/kg, once daily from gestational day (GD) 6 to 27 (from implantation to one day prior to the day of scheduled kill). The test item was suspended in 0.5% aqueous methylcellulose solution.

The animals were observed twice daily for clinical signs of reaction to treatment and weighed on GD 0, 3 and 6-28. Food consumption was recorded daily from GD 1-28. At terminal sacrifice on GD 28 the animals were subjected to gross necropsy and the reproductive tract and contents were examined in detail. Corpora lutea, implantations and live and dead implants were counted and recorded. Live fetuses were examined externally and internally for abnormalities, the brain and heart being removed and examined separately, then the carcasses were prepared for skeletal examination.

There were no mortalities and no treatment related effects on body weight or food consumption. There were no abortions of pregnancy and no effects on the numbers of implantations or embryofetal deaths. Fetal weight was also unaffected. There was no treatment related incidence of any external, soft tissue or skeletal abnormalities in the fetuses.

It was concluded that a NOAEL for flutolanil administered orally to rabbits by gavage from gestation day 6 to 27 inclusive was 1000 mg/kg/day (the limit dose) in terms of effects on the dams and on embryofetal development.

Materials and Methods

Test item:	Flutolanil
Batch Nos.:	101141
Purity:	99.1%
Expiry:	22 July 2015
Appearance:	Pale yellowish/greyish powder
Storage:	Room temperature in the dark
Vehicle:	0.5% (w/v) aqueous methylcellulose (Metolose SM-400, Shin-Etsu Chemical Co., Ltd), Lots 8105606, 1065396
Animals:	New Zealand White nulliparous female rabbits, Kbl:NZW strain ([redacted]), 17-19 weeks of age at start of mating
Study start and completion:	05 December 2011 to 29 June 2012

The females were mated one-to-one with stock males of the same strain at the laboratory, then allocated to the four study groups by block randomization. The day of observed mating was designated gestation day (GD) 0. Each group consisted of 24 mated females, to receive flutolanil at a dosage of 0 (vehicle control), 100, 300 or 1000 mg/kg/day (limit dose) once daily by gavage on GD 6-27 inclusive.
Dosing formulations were prepared by pulverizing weighed flutolanil in a mortar with small amounts of vehicle, washing the resulting suspension into a measuring cylinder and then making up to volume with more vehicle. Mixing was then by inversion, with subsequent maintenance on a magnetic stirrer. The suspensions were prepared at least once weekly and divided into aliquots for daily dosing, which were stored refrigerated and used within 7 days. Achieved concentration, homogeneity and stability were verified by analysis at the laboratory. The formulations were administered to the animals at a standard dose volume of 5 mL/kg body weight (weight as measured at the time of adminstration) orally using a Nelaton catheter as a gavage, once daily in the morning.
All animals were observed for general condition such as external appearance, appearance of excrement, nutritional condition, posture and behaviour before dosing and approximately 1 and 4 hours after dosing every day during the administration period and once (GD 28) or twice a day (in the morning and/or afternoon) during the other periods. They were weighed on GD 0, 3 and 6-28 inclusive. Food consumption was measured from GD 1 to 28.
Terminal sacrifice was on GD 28, when the organs and tissues of the thoracic and abdominal cavities were examined macroscopically. The intact uterus was removed and weighed, and the number of corpora lutea in each ovary was recorded. The uterus was opened and the numbers of live fetuses and embryofetal deaths were recorded. The placentae were examined for any abnormalities. Apparently non-pregnant uteri were immersed in 10% ammonium sulphide to detect implantation sites. Live fetuses were weighed and examined for external abnormalities, then the thoracic and abdominal cavities were dissected and examined macroscopically. The brain and heart were extracted and fixed in 10% formalin, the brain for freehand sectioning and the heart for micro-dissection. The fetuses were then processed and stained with Alizarin red S for examination of the skeleton for abnormalities and ossification status.
The statistical significance of any intergroup differences among the results was assessed using appropriate statistical tests.

Results

Formulation analyses verified satisfactory achieved concentration and homogeneity.

Group sizes in terms of pregnant animals were 19, 21, 21 and 22 in the respective groups (0, 100, 300 and 1000 mg/kg/day). There were no mortalities or abortions of pregnancy.
The incidence of dams showing reduced fecal output was slightly higher in the treated groups than in control. Body weight and food consumption, however, remained unaffected.

At necropsy, one female in each of the 300 and 1000 mg/kg/day groups had aplasia of the gall bladder, regarded as an incidental finding, not related to treatment.

There was no treatment related effect on the numbers of corpora lutea, implantations or pre-implantation losses.

Table B.6.6.6-1: caeserian section data following flutolanil treatment

Dose mg/kg/ day	No. of dam s		No. of corpora lutea	No. of implant-tations	Implan-tation index %[a]	Pre-implan-tation loss[b]	No. of embryo-fetal deaths			No. of live fetuses	Gross evaluati on of placent a
							Total (%)[c]	Early[d]	Late[e]		
0	19	Total	182	154			6	2	4	148	No abnormal findings
		Mean	9.6	8.1	83.5	16.5	(3.7)			7.8	
		S.D.	1.4	2.9	23.9	23.9	5.7)			2.8	
100	21	Total	216	191			6	3	3	185	No abnormal findings
		Mean	10.3	9.1	88.0	12.0	(3.1)			8.8	
		S.D.	2.0	2.6	17.4	17.4	(6.5)			2.7	
300	21	Total	205	186			16	12	4	170	No abnormal findings
		Mean	9.8	8.9	90.9	9.1	(11.5)			8.1	
		S.D.	2.1	2.3	13.4	13.4	(22.3)			3.0	
1000	22	Total	221	194			19	10	9	175	No abnorm findings
		Mean	10.0	8.8	86.9	13.1	(12.5)			8.0	
		S.D.	2.0	2.7	18.7	18.7	(21.9)			2.7	

a): (No. of implantations in each dam / No. of corpora lutea in each dam) X 100

b): [(No. of corpora lutea - No. of implantations in each dam) / No. of corpora lutea in each dam] X 100

c): (No. of embryo-fetal deaths in each dam / No. of implantations in each dam) X 100

d): Implantation trace, resorbed embryo and placental remnant

e): Early macerated fetus, late macerated fetus and dead fetus

No significant difference from control group in any treated group

Table B.6.6.6-2: External fetal examination and fetal weight following flutolanil treatment

Dose mg/kg/day	No. of dams		No. of males	No. of females	Sex ratio[a]	Fetal body weight (g)		No. of fetuses with external abnormalities (%)
						male	female	
0	19	Total	68	80				0
		Mean	3.6	4.2	0.44	35.29	33.54	(0.0)
		S.D.	2.1	1.9		0.22	4.05	4.57 (0.0)
100	21	Total	98	87				0
		Mean	4.7	4.1	0.53	35.33	35.03	(0.0)
		S.D.	1.8	2.1	0.16	5.69	4.43	(0.0)
300	21	Total	81	89				1[c]
		Mean	3.9	4.2	0.47	34.78	33.90	(0.7)
		S.D.	1.9	1.9	0.15	4.11	4.81	(3.2)
1000	22	Total	85	90				0
		Mean	3.9	4.1	0.49	35.29	34.64	(0.0)
		S.D.	2.0	2.0	0.17	5.21	4.61	(0.0)

a): No. of live male fetuses in each dam / No. of live fetuses in each dam

b): (No. of live fetuses with external abnormalities in each dam / No. of live fetuses observed in each dam) X 100

c): Umbilical hernia

No significant difference from control group in any treated group

There were no statistically significant differences between the control group and any treated group in the number or type of embryo-fetal deaths, the number of live fetuses, the index of external abnormalities in fetuses, live fetal weight or sex ratio, although the number of embryo-fetal deaths at 300 mg/kg/day or more was slightly high compared with the control group. Total numbers of embryo-fetal deaths at 0 (control), 100, 300 and 1000 mg/kg/day were 6, 6, 16 and 19, respectively. In the study report it is noted that the findings occurring at the highest dose levels are considered to be unrelated to flutolanil treatment. The study authors relate this finding to one exceptional dam (No 3110 at 300 mg/kg bw/day and No. 4110 at 1000 mg/kg bw/day) bearing no live fetuses. Recalculating the

numbers of embryo-fetal deaths at the two highest doses results in 7.1% and 8.3% for dams treated with 300 mg/kg bw/day and 1000 mg/kg bw/day, respectively, when excluding the two respective dams. The study authors also note that the actual values remained within background data. To fully address this point the notifier is asked to provide the representative historical control data to assess their relevance for the current study. At 300 mg/kg/day, 1 fetus was observed with umbilical hernia. No other abnormalities were observed in the external-gross examinations.

The incidences of soft tissue abnormalities in the treated groups was comparable with control. Similarly, the incidences of skeletal abnormalities showed no treatment effect. Ossification of the sacral and caudal vertebrae were marginally accelerated at 100 mg/kg/day, an isolated difference that in the absence of a dosage relationship across the groups was regarded as fortuitous.

Table B.6.6.6-3: soft tissue findings

Dose mg/kg/day	0	100	300	1000
No. of dams examined	19	21	20	21
No. of fetuses examined	148	185	170	175
No. of fetuses with abnormalities (%:Mean±S.D.)	1 (0.5± 2.1)	2 (1.0± 3.1)	1 (0.6± 2.5)	0 (0.0± 0.0)
Small left atrioventricular ostium	0 (0.0± 0.0)	1 (0.4± 2.0)	0 (0.0± 0.0)	0 (0.0± 0.0)
Small right ventricular chamber	0 (0.0± 0.0)	0 (0.0± 0.0)	1 (0.6± 2.5)	0 (0.0± 0.0)
Persistent truncus arteriosus	1 (0.5± 2.1)	0 (0.0± 0.0)	0 (0.0± 0.0)	0 (0.0± 0.0)
Coronary artery fistula	0 (0.0± 0.0)	1 (0.5± 2.4)	0 (0.0± 0.0)	0 (0.0± 0.0)
No. of fetuses with variations (%:Mean±S.D.)	5 (3.7± 8.2)	4 (2.7± 6.5)	2 (1.1± 3.4)	5 (2.9± 5.5)
Abnormal lung lobation (absent accessory lobe)	1 (1.3± 5.7)	1 (0.4± 2.0)	2 (1.1± 3.4)	4 (2.3± 5.0)
Abnormal origin of right subclavian artery	2 (1.3± 5.7)	1 (0.4± 2.0)	0 (0.0± 0.0)	1 (0.6± 2.7)
Retrocaval ureter (right)	2 (1.1± 3.2)	2 (1.9± 6.1)	0 (0.0± 0.0)	0 (0.0± 0.0)

No significant difference from control group in any treated group

Table B.6.6.6-4: Skeletal findings

Dose mg/kg/day	0	100	300	1000
No. of dams examined	19	21	20	21
No. of fetuses examined	148	185	170	175
No. of fetuses with abnormalities (%:Mean+S.D.)	1 (0.5+ 2.1)	4 (1.5+ 5.3)	2 (1.3+ 3.8)	1 (0.6+ 2.7)
Branched rib	0 (0.0+ 0.0)	1 (0.4+ 1.7)	0 (0.0+ 0.0)	0 (0.0+ 0.0)
Fused thoracic centrum	1 (0.5+ 2.1)	3 (1.1+ 5.0)	0 (0.0+ 0.0)	0 (0.0+ 0.0)
Thoracic hemivertebra	1 (0.5+ 2.1)	1 (0.4+ 1.7)	1 (0.6+ 2.8)	0 (0.0+ 0.0)
Fused sternebra	0 (0.0+ 0.0)	1 (0.4+ 2.0)	1 (0.6+ 2.8)	1 (0.6+ 2.7)
No. of fetuses with variations (%:Mean+S.D.)	54 (34.2+26.0)	55 (30.7+18.4)	54 (33.0+31.1)	60 (4.2+31.3)
Splitting of sternebra	0 (0.0+ 0.0)	3 (1.5+ 3.9)	4 (1.9+ 4.8)	2 (1.0+ 3.0)
Splitting of lumbar arch	0 (0.0+ 0.0)	1 (0.6+ 2.7)	0 (0.0+ 0.0)	0 (0.0+ 0.0)
Unossified talus	9 (4.8+ 8.9)	3 (1.2+ 2.9)	4 (1.6+ 5.5)	6 (2.4+ 9.2)
25 presacral vertebrae	1 (1.8+ 7.6)	1 (0.6+ 2.7)	0 (0.0+ 0.0)	0 (0.0+ 0.0)
27 presacral vertebrae	48 (9.8+24.4)	50 (8.5+19.6)	50 (31.4+32.1)	54(31.7+31.6)
Progress of ossification				
Region of sternebrae (%:Mean+S.D.)				
1st	148(100.0+ 0.0)	185(100.0+ 0.0)	170(100.0+ 0.0)	175(100.0+0.0)
2nd	148(100.0+ 0.0)	185(100.0+ 0.0)	170(100.0+ 0.0)	175(100.0+0.0)
3rd	148(100.0+ 0.0)	185(100.0+ 0.0)	170(100.0+ 0.0)	175(100.0+0.0)
4th	148(100.0+ 0.0)	185(100.0+ 0.0)	170(100.0+ 0.0)	175(100.0+0.0)
5th	130(88.4+16.0)	173(92.3+ 8.7)	140(84.3+21.3)	152(88.5+18.7)

6th	122(85.4+25.0)	148(79.0+21.4)	138(82.8+24.1)	132(77.6+31.0)
Sternebrae	5.74+0.29	5.72+0.25	5.68+0.34	5.66+0.45
Forelimbs (right)				
Metacarpal (phalanges)	4.71+0.31	4.86+0.19	4.80+0.21	4.75+0.25
Proxima (phalanges)	4.97+0.07	4.98+0.06	4.98+0.06	4.99+0.07
Middle (phalanges)	3.66+0.36	3.84+0.27	3.78+0.46	3.86+0.30
Distal (phalanges)	5.00+0.00	5.00+0.02	5.00+0.00	5.00+0.00
Forelimbs (left)				
Metacarpal (phalanges)	4.73+0.27	4.87+0.17	4.81+0.20	4.80+0.25
Proxima (phalanges)	4.97+0.07	4.98+0.06	4.98+0.06	4.99+0.04
Middle (phalanges)	3.65+0.37	3.85+0.26	3.80+0.37	3.86+0.30
Distal (phalanges)	5.00+0.00	5.00+0.00	5.00+0.00	5.00+0.00
Hindlimbs (right)				
Metacarpal (phalanges)	4.00+0.00	4.00+0.00	4.00+0.00	4.00+0.00
Proxima (phalanges)	4.00+0.00	4.00+0.00	3.99+0.04	4.00+0.00
Middle (phalanges)	3.93+0.19	3.98+0.09	3.95+0.14	3.97+0.15
Distal (phalanges)	4.00+0.00	4.00+0.00	4.00+0.00	4.00+0.00
Hindlimbs (left)				
Metacarpal (phalanges)	4.00+0.00	4.00+0.00	4.00+0.00	4.00+0.00
Proxima (phalanges)	4.00+0.00	4.00+0.00	3.99+0.04	4.00+0.0
Middle (phalanges)	3.94+0.15	3.99+0.07	3.95+0.14	3.97+0.15
Distal (phalanges)	4.00+0.00	4.00+0.00	4.00+0.00	4.00+0.00
Sacral and caudal vertebrae	19.18+0.41	19.50+0.28*D	19.33+0.43	19.37+0.4

*: p<0.05 (Significant difference from control group)

D: Dunnett's test

No significant difference from control group in any treated group

Conclusions

The NOAEL for flutolanil administered orally to rabbits by gavage from gestation day 6 to 27 inclusive was 1000 mg/kg/day (the limit dose) in terms of effects on the dams and on embryofetal development.

B.6.7 Neurotoxicity

In the DAR the following was stated: *Flutolanil does not have chemical structures that are similar or related to those capable of inducing delayed neurotoxicity, such as organophosphates. There was no evidence of neurotoxicity in the toxicity studies that have been conducted with flutolanil. It is therefore considered that flutolanil does not have a potential to induce neurotoxicity in mammals and no studies are required.*

Subsequently, however, a programme of neurotoxicity studies in rodents has been conducted and therefore these studies are submitted for the renewal.

B.6.7.1 Neurotoxicity in rodents

B.6.7.1.1 Neurotoxicity in rodents, single dose study

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study. The study is considered acceptable.

reference	:	[redacted] (2011)	exposure		single oral gavage
Report number	:	T-3201	doses		0, 125, 500 or 2000 mg/kg bw
test substance	:	flutolanil	GLP		Yes
species	:	Wistar rats	guideline		In accordance with OECD 424 (1997)
group size	:	10/ sex/ group	acceptability		Acceptable

Executive summary

Four groups of Wistar rats (10/sex/group) received flutolanil (98.7% purity) as a single oral gavage dose at doses of 0 (vehicle control), 125, 500 or 2000 mg/kg body weight, followed by a 14-day observation period.

There were no treatment related effects on mortality, clinical signs, body weight and food consumption, functional observations, locomotor activity, organ weights, or on macroscopic/microscopic findings at necropsy.

It was concluded that flutolanil administered to rats as a single dose of up to 2000 mg/kg was well tolerated. No treatment effects were observed in the neurobehavioural observations made in-life, nor in subsequent neuropathological investigations. The NOAEL was considered to be 2000 mg/kg for both sexes.

Materials and Methods

Test item:	Flutolanil technical
Batch Nos.:	9AE0011P
Purity:	98.7%
Expiry:	11 July 2013
Appearance:	Off-white powder
Storage:	Room temperature in the dark
Vehicle:	1.0% aqueous methylcellulose
Animals:	Wistar rats, HsdHan:WIST strain, from [redacted], 8 weeks of age, 191-264 g (males) and 146-189 g (females) at start of dosing
Study start and completion:	28 April 2011 to 23 September 2011

Four groups of rats, 10/sex/group, were formed using a randomized allocation procedure, to receive a single dose of flutolanil at 0 (vehicle control), 125, 500 or 2000 mg/kg body weight, by oral gavage. The high dose was selected as being devoid of toxic effects, as shown in an earlier study.

Dosing formulations were prepared on the day prior to dosing as a suspension in 1.0% aqueous methylcellulose, first grinding the test item in a mortar with a little vehicle to form a paste, then transferring to a formulation bottle, making up to volume with more vehicle and mixing using a Silverson homogenizer. The resulting formulations were stored at room temperature in sealed containers. They were stirred continuously before and during the dosing procedures. Stability, homogeneity and achieved concentration were verified by analysis at the laboratory.

The animals received their respective formulation in a single dose on day 1, by oral gavage at a dose volume of 10 mL/kg body weight.

The rats were observed daily for signs of ill health or toxicity, plus a detailed physical examination pre-treatment, on day 1 and weekly thereafter. Post-dosing observations were conducted at 0.5, 1, 2 and 4 h after dosing. Individual body weights were recorded at days -7, 1, 7 and 14. Food consumption was determined for each day.

A functional observation battery (FOB) was applied as a series of behavioural tests and observations conducted on one occasion during week -2 and on days 1, 7 and 14. Where possible the observations were conducted at approximately the same time on each occasion (approximately 2 h post-dose, to coincide with an estimation of t_{max}). The observations were performed 'in blind' (observer unaware of each animal's treatment group).

Cage-side and in-hand observations

Observations for: Posture, activity, gait, tremor, convulsion, excessive vocalization, arousal upon opening the cage.

Each animal then removed from the cage and observed for: Ease of removal, ease of handling, excessive vocalization, tremor, convulsion, palpebral closure, exophthalmus, lacrimation, lacrimation type, salivation, abnormal respiration, piloerection, appearance of fur, and any other abnormality.

Open field observations

Placed in open field for 2 min and observed for: Latency to first step, posture, arousal, circling, gait type and severity, stereotypy, tremor, convulsion, and any other abnormality. The number of rears, faecal boli and urine pools, faecal consistency and any polyuria were also recorded.

Open field tests

Tests for: Approach response, touch response, tail pinch, air righting ability, pupillary response, corneal tactile response, auditory startle response, hindlimb foot splay, forelimb and hindlimb grip strength.

Locomotor activity

Spontaneous motor activity was assessed for each animal in automated photocell equipment for 30 min, recording counts at 2-min intervals (total activity and total mobile counts).

Positive control data were previously generated by the laboratory, demonstrating the validity of the testing methods using acrylamide and trimethyltin chloride over a 4-week dosing period. Inter-observer reliability for the functional observation battery is also maintained.

Terminal procedures

Necropsies were performed after overnight fasting. Five/sex/group were sacrificed by exsanguination under isofluorane anaesthesia, while the remaining 5/sex/group were given intraperitoneal sodium pentobarbitone, then flushed with heparinized sodium nitrate solution prior to perfusion with 50% Karnovsky's fluid. A full macroscopic examination was performed and a comprehensive list of tissues were preserved from the non-perfused animals and appropriately preserved:

Adrenals	Peyer's patch
Brain	Popliteal lymph nodes
Caecum	Prostate
Colon	Rectum
Duodenum	Sciatic nerves
Eyes (a)	Seminal vesicles
Femur with bone marrow and stifle joint	Cervical spinal cord
Gross lesions	Lumbar spinal cord

Head	Thoracic spinal cord
Heart	Spleen
Ileum	Sternum with bone marrow
Jejunum	Stomach
Kidneys	Testes and epididymides (b)
Liver	Thymus
Lungs with mainstem bronchi	Thyroids with parathyroids
Mandibular lymph nodes	Trachea, tracheal bifurcation
Mesenteric lymph nodes	Urinary bladder
Optic nerves	Uterus and cervix
Ovaries	Vagina

Fixatives: 10% neutral buffered formalin, except a = Davidson's fluid; b = Bouin's fluid

The above tissues were held in fixative and not processed further.

The following neural tissues were taken from the perfused animals and preserved in appropriate fixative for neuropathological examination:

Olfactory bulb
Forebrain (including hippocampus)
Caudate nucleus
Hypothalamus/thalamus
Midbrain
Pons
Cerebellum
Medulla
Entire spinal cord (to include enlargement of C4 level), thoracic
and lumbar (to include enlargement at L4/5 level) regions
Cervical dorsal root ganglia **†**
Lumbar dorsal root ganglia **†**
Trigeminal ganglia
Eye including retina
Optic nerve, Left and Right†
Proximal and distal sciatic nerve TS & LS, Left, Right†
Proximal and distal tibial nerve TS & LS, Left, Right†
Sural nerve TS & LS, Left, Right†
Anterior tibialis muscle (left side)
Gastrocnemius muscle (left side)
Any macroscopic lesions

The above were trimmed, processed and embedded in paraffin wax for staining with H & E, except **†**trimmed, processed and embedded in epoxy resin and then stained with 0.5% Alkaline Toluidine blue solution

The animals were weighed before necropsy, and the brain from the perfused animals was weighed at the time of sectioning, following an adequate fixation period.
All the tissues listed above for neuropathology were examined microscopically for the control and high dosage groups.

Data evaluation

Statistical analyses were applied where appropriate:
Body weight gains, necropsy body weights, locomotor activity data and functional observational battery data were analyzed 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≥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 analyzed 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.
Brain weights were analyzed using analysis of covariance (ANCOVA) and Dunnett's test, for each sex separately, using the necropsy body weight as the single covariate. Levene's test for equality of variances was applied across groups, and in all cases this showed no evidence of heterogeneity (P≥0.01).
For each macroscopic and microscopic finding, comparisons were made between the incidence in each of the treated groups and that in the control group, using Fisher's Exact Probability test (two-sided). There were no results of statistical significance (P≥0.05 for all tests).

Results

Homogeneity and stability of the dietary formulations, and achieved concentrations, were satisfactorily demonstrated. Test item was not detected in Group 1, control. There was no mortality and no treatment related clinical signs. There were no treatment effects on body weight or food consumption.

Males dosed at 2000 mg/kg were noted to have a relative increase in ambulation between 18 to 22 minutes of the observation period on Day 1, when compared to concurrent controls. This increase in ambulation was not a consistent phenomenon, was isolated to discrete intervals and the values observed were within the expected ranges for animals of this species, strain, sex and age. As such these observations were considered to be incidental to an effect of the test article.

There were considered to be no test article related effects on functional observations. A slightly higher incidence of rats being noted as hyperactive during the open field tests was noted in females dosed at 500 mg/kg when compared to concurrent controls (Table B.6.7.1.1-01). In addition, a slightly higher incidence of hyperactive rats (1 rat, males only) was also observed at a dose of 125 mg/kg. However,

this finding did not demonstrate a dose response relationship, was isolated to a single sex, was not corroborated by related observations and was within the expected realms of variance for animals of this species, strain and age, as such this finding was considered incidental to treatment.

Table B.6.7.1.1-1: summary of open field observations – arousal

	Week -2		Day 1		Day 7		Day 14	
	M	F	M	F	M	F	M	F
Control								
Number observed	10	10	10	10	10	10	10	10
Normal	10	9	10	10	10	9	10	9
Hypoactive	0	0	0	0	0	0	0	0
Hyperactive	0	1	0	0	0	1	0	1
125 mg/kg								
Number observed	10	10	10	10	10	10	10	10
Normal	10	9	9	10	9	8	9	7
Hypoactive	0	0	1	0	1	0	1	0
Hyperactive	0	1	0	0	0	2	0	3
500 mg/kg								
Number observed	10	10	10	10	10	10	10	10
Normal	10	10	10	8	10	7	10	8
Hypoactive	0	0	0	0	0	0	0	0
Hyperactive	0	0	0	2	0	3	0	2
2000 mg/kg								
Number observed	10	10	10	10	10	10	10	10
Normal	10	10	10	9	10	10	10	9
Hypoactive	0	0	0	0	0	0	0	0
Hyperactive	0	0	0	1	0	0	0	1

The RMS notes that in the open field observations a significant difference in the number of rears was observed. In fact, in males exposed to 125 mg/kg flutolanil a decrease in the number of rears was observed at day 7 only while in males a significant dose response test was determined after 14 days showing an increase in the number of rears in the high dose. Since both increases and decreases in rear numbers were observed which were not dose-response related, isolated to one sex and not substantiated by related (locomotor) observations this finding is not considered treatment related.

Table B.6.7.1.1-2: summary of open field observations – number of rears

	Week -2		Day 1		Day 7		Day 14	
	M	F	M	F	M	F	M	F
Control								
N	10	10	10	10	10	10	10	10
Mean	12	22	10	26	8	16	7	17
SD	6	10	4	17	2	8	2	9
125 mg/kg								
N	10	10	10	10	10	10	10	10
Mean	10	21	10	23	4*	19	5	23
SD	4	7	4	8	4	9	2	10
500 mg/kg								
N	10	10	10	10	10	10	10	10
Mean	10	18	10	21	6	19	5	22

SD	5	5	4	16	3	11	3	10
2000 mg/kg								
N	10	10	10	10	10	10	10	10
Mean	15	18	13	18	10	18	11	19
SD	7	6	6	9	4	5	6	9
							DR*	

N = number of non-missing values
* P<0.05
** P<0.01
*** P<0.001
DR = significant dose response test

There were no meaningful intergroup differences in brain weight. Similarly, there were no macroscopic or microscopic findings related to treatment.

Conclusions:

A single dose of flutolanil by gavage to rats at 125, 500 or 2000 mg/kg was well tolerated. There were no test item related neurobehavioural or neuropathological changes, and the NOAEL was concluded to be 2000 mg/kg flutolanil.

B.6.7.1.2 Neurotoxicity in rodents, repeated dose study

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study. The study is considered acceptable.

reference	:	[redacted] (2012)	exposure	Via the diet
Report number	:	T-3214	doses	0, 350, 2000 or 1000 ppm
test substance	:	flutolanil	GLP	Yes
species	:	Sprague-Dawley rats	guideline	In accordance with OECD 424 (1997)
group size	:	10/ sex/ group	acceptability	Acceptable

Executive summary

Groups of Sprague-Dawley rats (10/sex/group) received flutolanil (98.7% purity) by admixture in the diet, at concentrations of 0 (control), 350, 2000 or 10000 ppm for 13 consecutive weeks. Achieved concentrations were between 90-110% of nominal and no test item was detected in control.

Mortality, clinical signs, body weight and food consumption were monitored throughout. Ophthalmoscopy and a functional observation battery were applied, including open field testing and automated spontaneous motor activity monitoring. At terminal necropsy, 5/sex/group were subjected to perfusion fixation and a comprehensive set of neural tissues were taken for neuropathological examination. The remaining 5/sex/group were subjected to conventional necropsy and fixation of a comprehensive range of tissues for histopathological examination. Tissues were stained with haematoxylin and eosin or toluidine blue, as appropriate.

All dosages were well tolerated and achieved nominal dosages were 62.6, 250 and 1000 mg/kg/day. There were no treatment related effects in the study, and in particular there were no neurobehavioural effects, nor any neuropathological changes.

A NOAEL for flutolanil of a nominal 1000 mg/kg/day administered in the diet for 13 weeks was concluded for both sexes.

Materials and Methods

Test item:	Flutolanil technical
Batch Nos.:	9AE0011P
Purity:	98.7%
Expiry:	11 July 2013
Appearance:	Off-white powder
Storage:	Room temperature in the dark
Diet:	Rat and Mouse Maintenance Diet No. 1 (expanded, fine-ground) SQC, from Special Diet Services, UK
Animals:	Wistar rats, HsdHan:WIST strain, from [redacted], 6 weeks of age, 151-215 g (males) and 123-161 g (females) at start of dosing
Study start and completion:	05 July 2011 to 10 April 2012

Diet formulations were prepared weekly, the concentrations based on the expected mean body weight and food intake for each group. The formulations were stored at room temperature in sealed containers.

The diet formulations prepared at 350, 2000 and 10000 ppm were analyzed for homogeneity and for stability over periods of 24 h and 12 days. Achieved concentrations were assayed for test formulations prepared for use in weeks 1 and 13 of treatment.

Four groups, each of 10 males and 10 females, were formed by means of a randomization procedure, to receive target dosages of 0 (control), 62.6, 250 or 1000 mg/kg/day flutolanil (the limit dose), by means of admixture with the diet at concentrations that were adjusted at each (weekly) body-weighing interval on the basis of the predicted mid-interval group mean body weight and an estimate of food consumption. The diet and water were available to the animals *ad libitum*.

The animals were housed at up to 5 per cage. However, 24 h prior to functional observation tests, they were housed singly, then on completion of that testing they were returned to their home cages.

All animals were observed daily for signs of ill health or overt toxicity. A more detailed physical examination was performed at weekly intervals. Body weights were recorded before treatment on the first day, then weekly to coincide with functional observations and prior to necropsy. Food consumption was measured weekly. Ophthalmoscopy was performed for all animals pre-treatment, then again for the control and high dosage groups during week 12.

A functional observation battery was applied for all animals pre-treatment (week -1) and during weeks 2, 4, 8 and towards the end of week 13. The observations were performed ‘in blind’ (observer unaware of each animal’s treatment group), and as far as possible they were conducted at about the same time on each occasion.

Cage-side and in-hand observations, Weeks -1, 2, 4, 8, 13

Observations for: Posture, activity, gait, tremor, convulsion, excessive vocalization, arousal upon opening the cage.

Each animal then removed from the cage and observed for: Ease of removal, ease of handling, excessive vocalization, tremor, convulsion, palpebral closure, exophthalmus, lacrimation, lacrimation type, salivation, abnormal respiration, piloerection, appearance of fur, and any other abnormality.

Open field observations, Weeks -1, 2, 4, 8, 13

Placed in open field for 2 min and observed for: Latency to first step, posture, arousal, circling, gait type and severity, stereotypy, tremor, convulsion, and any other abnormality. The number of rears, faecal boli and urine pools, faecal consistency and any polyuria were also recorded.

Open field tests, Weeks -1, 2, 4, 8, 13

Tests for: Approach response, touch response, tail pinch, air righting ability, pupillary response, corneal tactile response, auditory startle response, hindlimb foot splay, forelimb and hindlimb grip strength, rectal body temperature. *Note: Rectal temperatures were omitted in error for weeks -1, 2 and 4. The data from the later occasions were considered adequate for the appraisal and it was considered that this deviation did not impact on study objectives.*

Locomotor activity, Weeks -1, 2, 4, 8, 13

Spontaneous motor activity was assessed for each animal in automated photocell equipment for 30 min, recording counts at 2-min intervals (total activity and total mobile counts).

Positive control data were previously generated by the laboratory, demonstrating the validity of the testing methods using acrylamide and trimethyltin chloride over a 4-week dosing period. Inter-observer reliability for the functional observation battery is also maintained.

Terminal procedures:

Necropsies were performed after overnight fasting. Five/sex/group were sacrificed by exsanguination under isofluorane anaesthesia, while the remaining 5/sex/group were given intraperitoneal sodium pentobarbitone, then flushed with heparinized sodium nitrate solution prior to perfusion with 50% Karnovsky's fluid. A full macroscopic examination was performed and a comprehensive list of tissues were preserved from the non-perfused animals and appropriately preserved:

Adrenals	Peyer's patch
Brain	Popliteal lymph nodes
Caecum	Prostate
Colon	Rectum
Duodenum	Sciatic nerves
Eyes (a)	Seminal vesicles
Femur with bone marrow and stifle joint	Cervical spinal cord
Gross lesions	Lumbar spinal cord
Head	Thoracic spinal cord
Heart	Spleen
Ileum	Sternum with bone marrow
Jejunum	Stomach
Kidneys	Testes and epididymides (b)
Liver	Thymus
Lungs with mainstem bronchi	Thyroids with parathyroids

Mandibular lymph nodes	Trachea, tracheal bifurcation
Mesenteric lymph nodes	Urinary bladder
Optic nerves	Uterus and cervix
Ovaries	Vagina

Fixatives: 10% neutral buffered formalin, except a = Davidson's fluid; b = Bouin's fluid

The following neural tissues were taken from the perfused animals and preserved in appropriate fixative for neuropathological examination:

Olfactory bulb
Forebrain (including hippocampus)
Caudate nucleus
Hypothalamus/thalamus
Midbrain
Pons
Cerebellum
Medulla
Entire spinal cord (to include enlargement of C4 level), thoracic
and lumbar (to include enlargement at L4/5 level) regions
Cervical dorsal root ganglia **†**
Lumbar dorsal root ganglia **†**
Trigeminal ganglia
Eye including retina
Optic nerve, Left and Right†
Proximal and distal sciatic nerve TS & LS, Left, Right†
Proximal and distal tibial nerve TS & LS, Left, Right†
Sural nerve TS & LS, Left, Right†
Anterior tibialis muscle (left side)
Gastrocnemius muscle (left side)
Any macroscopic lesions

The above tissues trimmed, processed and embedded in paraffin wax for staining with H & E, except **†**trimmed, processed and embedded in resin and stained with 0.5% Alkaline Toluidine blue solution

The animals were weighed before necropsy, and the brain from the perfused animals was weighed at the time of sectioning, following an adequate fixation period.

All the tissues listed above for neuropathology were examined microscopically for the control and high dosage groups.

Data evaluation:

Statistical analyses were applied where appropriate:

Body weight gains, locomotor activity data and functional observational battery data were analyzed 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≥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 analyzed either 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.
Necropsy body weights were analyzed 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≥0.01 for all 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 recognized alone only where none of the pairwise comparisons was significant.
For each macroscopic and microscopic finding, comparisons were made between the incidence in each of the treated groups and that in the control group, using Fisher's Exact Probability test (two-sided). There were no results of statistical significance (P≥0.05 for all tests).

Brain weights were analyzed using analysis of covariance (ANCOVA) and Dunnett's test, for each sex separately, using the necropsy body weight as the single covariate. Levene's test for equality of variances was applied across groups, and in all cases this showed no evidence of heterogeneity (P≥0.01).

Results

Homogeneity and stability of the dietary formulations were satisfactorily demonstrated. Achieved concentrations at weeks 1 and 13 were also satisfactory, and no test item was detected in the control diets. There were no mortalities, and no treatment related clinical signs of overt toxicity. Body weight and food consumption were considered to have been unaffected by treatment.
There were no treatment related ophthalmological findings.

It was considered that no treatment related effects occurred for any of the functional parameters.
Lower hindlimb grip strength was recorded for the high dose females at week 13, but the difference from control was quite small, the value remained within the historical control range and there were no changes in any related parameters. This intergroup difference was therefore considered fortuitous and unrelated to treatment. Some other statistically significant variations occurred in the high dose group, including the number of rears (Table B.6.7.1.2-01), hindlimb footsplay (Table B.6.7.1.2-02) and rectal temperature (Table B.6.7.1.2-03), but these were considered unrelated to treatment, owing to inconsistent/temporal occurrence and absence of changes in related parameters.

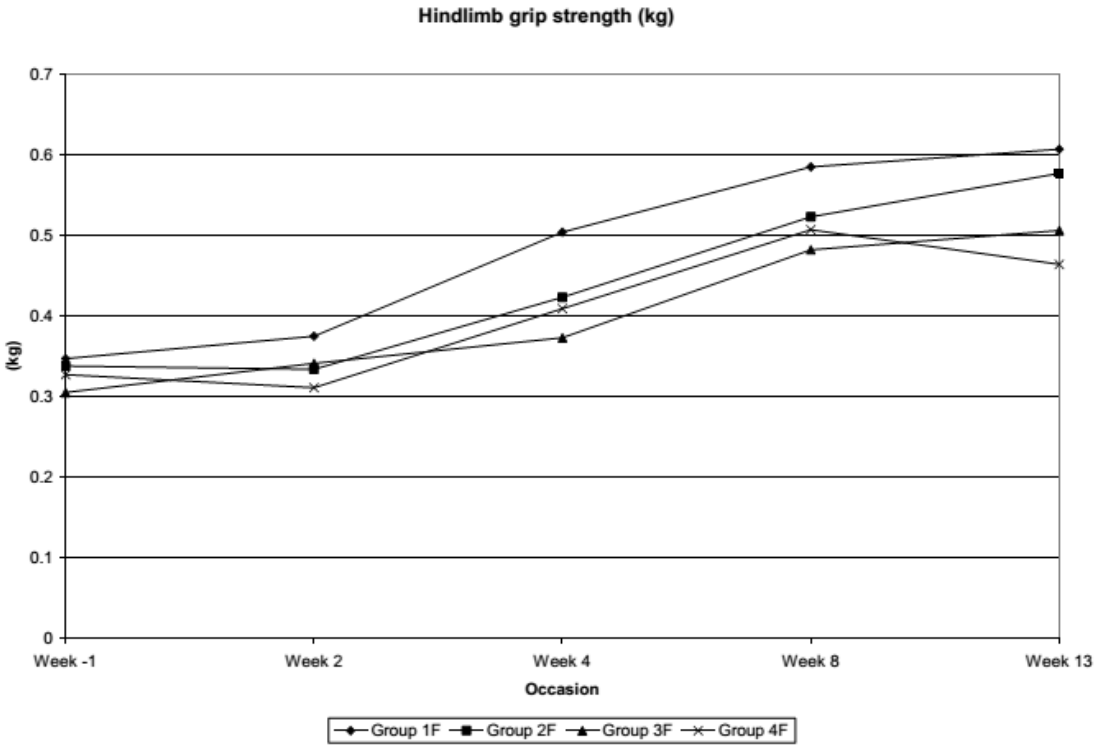


Figure B.6.7.1.2-1: group mean hindlimb grip strenght in females at all observation junctures

Table B.6.7.1.2-1: summary of open field observations – number of rears

	Week -1		Week 2		Week 4		Week 8		Week 13	
	M	F	M	F	M	F	M	F	M	F
Control										
N	10	10	10	10	10	10	10	10	10	10
Mean	12	18	10	17	8	13	10	16	8	19
SD	5	4	7	7	4	6	3	11	3	9
62.6 mg/kg										
N	10	10	10	10	10	10	10	10	10	10
Mean	10	18	6	19	3**	15	6*	19	3*	17
SD	4	6	3	12	3	10	4	11	3	9
250 mg/kg										
N	10	10	10	10	10	10	10	10	10	10
Mean	13	20	8	15	5	14	6*	18	7	14
SD	5	6	4	6	2	6	3	11	4	4
1000 mg/kg										
N	10	10	10	10	10	10	10	10	10	10
Mean	12	17	8	16	5*	14	6*	16	5	18
SD	8	6	5	9	3	7	3	10	2	11

* P<0.05

** P<0.01

Table B.6.7.1.2-02: summary of open field observations – hindlimb footsplay (mm)

	Week -1		Week 2		Week 4		Week 8		Week 13	
	M	F	M	F	M	F	M	F	M	F
Control										

N	10	10	10	10	10	10	10	10	10	10
Mean	39	56	38	70	48	66	47	69	52	61
SD	9	11	5	15	13	15	19	19	21	22
62.6 mg/kg										
N	10	10	10	10	10	10	10	10	10	10
Mean	43	65	37	67	55	68	48	73	52	70
SD	14	16	11	11	10	19	13	22	17	21
250 mg/kg										
N	10	10	10	10	10	10	10	10	10	10
Mean	44	61	47	61	51	63	47	60	55	59
SD	11	14	15	13	17	12	17	19	23	16
1000 mg/kg										
N	10	10	10	10	10	10	10	10	10	10
Mean	43	57	48	57	54	61	45	55	48	54
SD	8	13	11	12	13	22	10	12	14	10
			DR*	DR*				DR*		

* P<0.05

DR = significant dose response test

Table B.6.7.1.2-03: summary of open field observations – rectal temperature (deg C)

	Week -1		Week 2		Week 4		Week 8		Week 13	
	M	F	M	F	M	F	M	F	M	F
Control										
N	0	0	0	0	0	0	10	10	10	10
Mean							38.4	38.6	36.5	38.8
SD							0.6	0.4	1.8	0.6
62.6 mg/kg										
N	0	0	0	0	0	0	10	10	10	10
Mean							38.1	38.2	36.8	38.2
SD							0.8	1.0	0.8	0.7
250 mg/kg										
N	0	0	0	0	0	0	10	10	10	10
Mean							37.9	38.1	36.7	38.0*
SD							0.5	0.6	1.1	0.8
1000 mg/kg										
N	0	0	0	0	0	0	10	10	10	10
Mean							37.9	37.8*	37.0	38.1
SD							0.6	0.5	1.1	0.7

* P<0.05

There were no treatment related macroscopic findings. There were no meaningful intergroup differences in brain weight.

Microscopic findings were infrequent, of a minor nature and consistent with the normal pattern of findings in animals of this strain and age. None of the findings were related to treatment.

Conclusions

Dietary administration of flutolanil to rats at nominal dose levels of 62.6, 250 and 1000 mg/kg over a 13-week exposure period was well tolerated. There were no test article related neurobehavioural observations made in-life, nor any neuropathological changes noted at histopathological examination. The NOAEL was considered to be nominally 1000 mg/kg/day for both sexes.

B.6.7.2 Delayed polyneuropathy studies

Flutolanil does not have chemical structures that are similar or related to those capable of inducing delayed neurotoxicity, such as organophosphates, and there was no evidence of neurotoxicity in the studies that have been conducted with flutolanil, including specific subacute (4-week) neurotoxicity studies in rats. It was considered in this context that no studies of delayed polyneuropathy are required.

B.6.8 Other toxicological studies

B.6.8.1 Toxicity studies of metabolites and relevant impurities

The safety of two newly-identified metabolites of flutolanil requires investigation. These are 2 (trifluoromethyl)-benzamide (coded M-101) and 2-(trifluoromethyl)-benzoic acid (coded M-102), and arise from cleavage of the amide bridge in the flutolanil molecule. These compounds have been detected as minor metabolites in rats, appearing in urine at 0.05 to 0.08% of administered radioactivity at an oral dose of 20 mg/kg ([redacted] 2012, B.6.1.1.3 in this document). The metabolism was indicated in earlier studies not to be dependent on dose.

In support of re-approval of flutolanil, a Letter of Access has been obtained permitting access to relevant data from the dossier supporting approval of the product fluopyram. Although the fluopyram dossier does not contain studies specifically on M-101 or M-102 alone, the ADME studies indicate that fluopyram is metabolized such that the animals are exposed to considerably higher levels of M-101 and M-102 than is the case with flutolanil.

An ADME study by [redacted] (2008) with fluopyram (DAR B.6.1.1) used compound labelled in the appropriate phenyl ring, and showed that at single dosages of 5 or 250 mg/kg the urine contained 16 to 25% of M-101 (termed 'fluopyram-benzamide') and 4 to 7% of M-102 ('fluopyram-benzoic acid') in terms of percentage of administered radioactivity. Meanwhile, in the ADME study with flutolanil ([redacted] 2012, B.6.1.1.3 in this document), again using appropriately labelled material, a 20 mg/kg dose of flutolanil resulted in a urine content of 0.06% of M-101 and 0.05 to 0.08% of M-102 in terms of percentage of administered radioactivity. This indicates that in the fluopyram studies, rats would have been exposed to levels of these two metabolites greatly exceeding the levels obtained with flutolanil (by a factor of at least 250 for M 101 and at least 50 for M-102, assuming that the excreted amounts adequately reflect internal dosage).

The preceding indicates that the rat studies with fluopyram provide adequate toxicological cover for the metabolites M-101 and M-102, in support of the flutolanil re-approval. An overall NOAEL of 12.5 mg/kg/day was determined for the short-term toxicity of fluopyram, from a 90-day rat study, while the overall NOAEL for long-term toxicity/carcinogenicity was 1.2 mg/kg/day, again from a rat study. For flutolanil, the overall NOAEL (short-term toxicity) is 37 mg/kg/day, from a 90-day rat study, while the long-term NOAEL is 8.7 mg/kg/day, again from a rat study.

The formation of metabolite M-101 and M-102 in the metabolism of flutolanil were found to be below 10% and therefore it cannot be concluded that the rat studies with flutolanil provide adequate toxicological cover for metabolite M-101 and M-102. Therefore, in addition to the information available

from the fluopyram dossier, additional studies on these metabolites have been conducted, including an acute toxicity study, 28-day study and a genotoxicity test battery:

B.6.8.1.1 Studies with 2-(trifluoromethyl)-benzamide

Acute oral toxicity, 2-(trifluoromethyl)-benzamide

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study. The study is considered acceptable. Based on the outcome of the study, 2-(trifluoromethyl)-benzamide should be classified as harmful if swallowed (H302).

reference	:	[redacted] (2011)	exposure	:	single oral dose (gavage)
Report number	:	LSRC-T11-086A (T-3232)	doses	:	300 and 2000 mg/kg
test substance	:	2-(trifluoromethyl)-benzamide	GLP	:	yes
species	:	Sprague Dawley rat (Crl:CD)	guideline	:	In accordance with OECD 423 (2001)
group size	:	3/ group/ dose	acceptability	:	Acceptable

Executive summary

The acute oral toxicity to rats of 2-(trifluoromethyl)-benzamide (M-101, 100% purity) was examined by the acute toxic class method. Female Sprague-Dawley rats were administered a single oral dose (gavage) of test item suspended in 0.5% (w/v) carboxymethylcellulose sodium solution containing 0.2% (v/v) Tween 80. Daily observations for mortality and clinical signs were made for 14 days after treatment. Body weight was measured on days 1, 7 and 14. Necropsy was conducted on day 14 or when an animal was found dead.

Three rats were administered 300 mg/kg in a single dose by gavage, resulting in no mortality. A further 3 rats were administered 300 mg/kg in a single dose by gavage, which again resulted in no mortality. Another 3 rats were then administered 2000 mg/kg in a single dose by gavage, and all animals died within 24 h after treatment. Animals receiving 300 mg/kg showed abnormal gait and piloerection. Animals receiving 2000 mg/kg showed abnormal gait, lacrimation and moribund condition.

Body weight in the 300 mg/kg group, where all animals survived, was not affected by treatment and increased steadily.

At necropsy, out of all three decedents administered 2000 mg/kg, 2 animals showed urinary retention, considered possibly to be an effect of treatment.

The acute LD_{50} of 2-(trifluoromethyl)-benzamide in female rats was estimated to be greater than 300 mg/kg and less than 2000 mg/kg.

Materials and Methods

Test item:	2-(trifluoromethyl)-benzamide (M-101)
Batch No.:	336876
Purity:	100.0%
Expiry:	11 July 2011
Appearance:	Not stated
Storage:	Refrigerated, in the dark

Vehicle:	0.5% (w/v) aqueous carboxymethylcellulose sodium, containing 0.2% (v/v) Tween 80
Animals:	Sprague-Dawley female rats, Crl:CD strain ([redacted]), 8-9 weeks of age, 174-187 g at the first administration and 189-211 g at the third administration
Study start and completion:	16 May 2011 to 02 September 2011

The animals were assigned to 3 groups, each of 3 females, such that initial mean body weights were comparable across the groups. They were fasted for 16-18 h before administration and for approximately 3 h afterwards.

Dosing formulations were prepared just before administration. The test substance was weighed, pulverized in a mortar and pestle and suspended in 0.5 % (w/v) carboxymethylcellulose sodium aqueous solution containing 0.2 % (v/v) Tween 80.

The formulations were administered in the morning by oral gavage as a single dose at a volume of 10 mL/kg, based on the body weight measured immediately prior to administration. The initial dose level of 300 mg/kg was administered to the 3 rats in Group 1. The same dosage was administered to Group 2 two days later, then a dosage of 2000 mg/kg was administered to Group 3 after a further seven days.

The animals were observed for mortality and clinical signs immediately before dosing, immediately afterwards, at 15 and 30 min and 1, 3 and 6 h after administration, then once a day until day 14. Body weights were recorded at allocation to the test groups, at the start of fasting, on the day of administration (immediately before administration), and at approximately the same time of day on days 1, 7 and 14 after administration. On day 14, all surviving animals were sacrificed by exsanguination via the abdominal aorta under ether anaesthesia and subjected to necropsy. Animals found dead were subjected to immediate necropsy.

Results

Since there were no deaths among the 3 animals that received the first administration of 300 mg/kg (Group 1), another 3 animals (Group 2) were also administered 300 mg/kg, and again no deaths occurred.

Among the 3 animals administered 2000 mg/kg, one animal died within 6 h after administration and the remaining 2 animals died within 24 h. No further administration was therefore done.

In Group 1 (300 mg/kg), piloerection was observed in 1/3 animals at 1 h, and in all animals at 3 h after administration. Abnormal gait was also observed in 1 animal at 3 hours after administration. All findings were recovered and not detected on day 1 after administration. There were no abnormalities in any of these animals thereafter.

In group 2 (300 mg/kg), abnormal gait was observed in 1/3 animals and piloerection in 2/3 animals at 3 h after administration. These changes were recovered and not detected on day 1 of administration. There were no abnormalities in these animals thereafter.

In group 3 (2000 mg/kg), abnormal gait or decreased exploratory activity were observed at 15 min after administration in 1 or 2 animals. From 30 min to 1 h after administration, abnormal gait,

decreased locomotor activity and moribundity were observed in 1 or 2 animals. All animals became moribund and 2 animals showed lacrimation at 3 h after administration. From 6 hours to day 1 after administration all animals died.
There was no effect on body weight at 300 mg/kg, other than that of pre-treatment fasting.

Table B.6.8.1-1: Body weight changes in rats treated with 2-(trifluoromethyl)-benzamide

Dose (mg/kg)	Animal number	Body weight (g)					
		Pre fasting	Pre dosing	days after dosing			death
				1	7	14	
300	01F01	198.72	186.71	193.68	218.51	236.77	-
	01F02	190.69	174.54	189.37	214.59	228.08	-
	01F03	190.00	173.57	184.44	210.82	226.45	-
	Mean	193.14	178.27	189.16	214.64	230.43	
	S.D.	4.85	7.32	4.62	3.85	5.55	
	02F01	202.58	190.26	201.24	218.98	233.27	-
	02F02	199.09	187.58	206.66	226.36	243.80	-
	02F03	196.80	181.34	204.27	227.58	242.75	-
	Mean	199.49	186.39	204.06	224.31	239.94	
	S.D.	2.91	4.58	2.72	4.65	5.80	
2'000	03F01	228.00	211.27	-	-	-	211.28
	03F02	220.89	206.64	-	-	-	204.20
	03F03	204.72	189.48	-	-	-	181.86
	Mean	217.87	202.46				199.11
	S.D.	11.93	11.48				15.36

S.D.:Standard deviation

At necropsy there were no abnormal findings in the animals that received 300 mg/kg. Meanwhile, 2 of the animals that received 2000 mg/kg showed urine retention, which was thought possibly an effect of treatment.

Conclusion:

The acute oral LD_{50} of 2-(trifluoromethyl)-benzamide (M-101) in female rats was estimated to be greater than 300 mg/kg, but less than 2,000 mg/kg.

Oral 28-day study, 2-(trifluoromethyl)-benzamide

Previous evaluation	Newly submitted for the purpose of renewal
Evaluation of the RMS	The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study. The study deviates slightly from OECD 407:

	- The weight of the prostate + seminal vesicles with coagulating glands was not recorded during gross necropsy. However, histopathological analysis was performed. - In addition to the requirements laid down in OECD 407 immunohistochemical examinations were also performed. The study is considered acceptable.

reference	:	[redacted] (2012)	exposure	:	Via diet
Report number	:	LSRC-T11-124A (T-3231)	doses	:	50, 200 and 800ppm
test substance	:	2-(trifluoromethyl)-benzamide	GLP	:	Yes
species	:	Sprague Dawley rats (Crl:CD(SD))	guideline	:	In accordance with OECD 407 (2008)
group size	:	10/ sex/ dose	acceptability	:	Acceptable

Executive summary

M-101 (2-(trifluoromethyl)-benzamide, 100% purity) was mixed into powdered basal diet at concentrations of 0 (control), 50, 200 or 800 ppm and administered to test groups consisting of 10 male and 10 female Sprague Dawley rats, for 28 or 29 days. Achieved mean test item intake was 4.2-4.4, 17-18 and 68 mg/kg/day for the respective diet concentrations. During the treatment period, mortality and clinical signs were observed daily, and detailed clinical observations, body weights and food consumption were recorded weekly. Functional observations were conducted before treatment and in the 4th week of treatment for all animals. Ophthalmological examination was conducted before treatment for all animals and in the 4th week of treatment for all animals in the control and 800 ppm dose groups. Urinalysis, haematology, blood chemistry, necropsy and organ weight measurements were conducted at the 4th week of treatment for all animals. Histopathological examination was performed for all animals in the control and 800 ppm dose groups, while the liver from both sexes and kidneys and thyroids from males in the 50 and 200 ppm groups were also examined.

At 800 ppm the body weight gain of both sexes showed significant decreases or decreasing trend throughout the treatment period. In the 1st week of treatment, food consumption and feed efficacy showed a significant decrease. Haematological examination revealed a significant extension of activated partial thromboplastin time in males, small but statistically significant decreases in red blood cell count, haemoglobin concentration and haematocrit in females. In blood chemistry examination, males and females showed significant increases in total protein, albumin and total cholesterol. In addition, males showed significant decreases in triglyceride and glucose, while females showed a decrease of albumin/globulin ratio and an increase of triglyceride. At urinalysis, males showed significant increasing in protein, leukocytes and epidermal cells in sediment. At necropsy, males and females showed significant increases in the incidence of enlargement, dark discoloration and/or accentuated lobular pattern in the liver. In addition, males also showed significant increases in the incidence of pale discoloration in the kidney and enlargement in the thyroid. In organ weight measurement, males and females showed significant increases in absolute and relative weights of the liver, and males also showed significant increases in absolute and relative weights of the kidneys and thyroid glands. At histopathology, males and females showed significant increases in the incidence of centrilobular hepatocyte hypertrophy in the liver. The incidence of follicular cell hypertrophy in the thyroid glands, excessive hyaline droplets accumulation in tubular epithelial cells, renal tubular degeneration/necrosis, basophilic change of renal tubules, and cellular infiltration in the kidney were also significantly increased in males.

At 200 ppm the blood biochemistry examination, showed a significant increase in total cholesterol for both sexes. At urinalysis, males showed significant increases in leukocytes and epidermal cells in sediment. At necropsy, males and females showed a significantly increase in the incidence of accentuated lobular pattern in the liver. The incidence of pale discoloration in the kidney was also significantly increased in males. Among organ weights, males and females showed a significant increase in relative weight of the liver. In addition, males showed significant increases in absolute and relative weights of the kidneys and relative weight of the thyroid glands. At histopathology, the incidence of centrilobular hepatocyte hypertrophy in the liver increased significantly in both sexes. In males, the incidence of follicular epithelial cell hypertrophy in the thyroid gland, excessive hyaline droplet accumulation in tubular epithelial cells, renal tubular degeneration/necrosis, basophilic change of renal tubules, and cellular infiltration in the kidney, showed significant increases.
At 50 ppm, males showed a significant increase in relative weight of the kidneys. Histopathologically, males showed significant increases in the incidences of excessive hyaline droplet accumulation in tubular epithelial cells, basophilic change of renal tubules, and cellular infiltration in the kidney. One male showed renal tubular degeneration/necrosis in the kidney.
The hyaline droplets in tubular epithelial cells of the kidney at ≥50 ppm were identified as α_{2u}-globulin by immunohistochemical examination. Since excessive deposition of α_{2u}-globulin is a male rat specific finding, it is considered not to be relevant to human risk assessment.
A NOAEL of 50 ppm 2-(trifluoromethyl)-benzamide (M-101) in the diet was concluded, equivalent to an average of 4.2 mg/kg/day for males, 4.4 mg/kg/day for females. This excluded the α_{2u}-globulin deposition in male kidneys.

Materials and Methods

Test item:	2-(trifluoromethyl)-benzamide (M-101)
Batch No.:	336876
Purity:	100.0%
Expiry:	11 July 2011
Appearance:	Not stated
Storage:	Refrigerated, in the dark
Diet:	MF powdered diet (Oriental Yeast Co., Ltd, Japan)
Animals:	Sprague-Dawley rats, Crl:CD strain ([redacted]), 5 weeks of age, 127-148 g (males) and 115-133 g (females) at the start of administration
Study start and completion:	11 April 2011 to 19 July 2012

The rats were distributed into four groups, each of 10 males and 10 females, by means of a randomization procedure stratified by body weight, to receive diets containing test item at concentrations of 0 (control), 50, 200 or 800 ppm for 28 or 29 consecutive days (first day of administration = day 0). The dosages were selected on the basis of earlier studies, where after 14 days of administration at ≥300 ppm in males there were depressions of body weight gain and food consumption, with liver changes, while pharmacokinetic data indicated that at ≥100 ppm there was no

increase in effect associated with prolongation of the dosing period. Based on this, 800 ppm was selected for the present study, in the expectation of causing clear toxicity in both sexes.
Test diets were prepared by first grinding the test item in a mortar, adding some diet and grinding the mixture further, then adding this mixture to further diet in a mixing machine to produce the highest required concentration. Aliquots of this were diluted with further diet in the mixer to produce the lower concentrations. The formulated diets were stored at room temperature in closed containers and used within 2 weeks of preparation. Samples were taken from each preparation, and those from the first preparation and representative samples from the highest and lowest concentrations later in the study were analyzed for achieved concentration, homogeneity and stability (over 0, 3, 8 and 15 days of storage at room temperature in the dark).

The animals were observed daily for mortality and overt clinical signs. Detailed clinical observations were conducted once weekly:

Cage-side: Body position/posture, abnormal vocalization, tremor, convulsion, abnormal behavior, abnormal gait.

Hand-held: Ease of removal from cage, ease of handling, muscle tone, palpebral closure, lacrimation, exophthalmos, pupil diameter, salivation, secreta, piloerection, condition of fur, body temperature, abnormal respiratory sound, changes in the skin, and changes in the visible mucosa.

Open field: Rearing, abnormal vocalization, tremor, convulsion, spontaneous movement, abnormal behaviour, abnormal gait, grooming, breathing, defecation, urination.

Functional observations were conducted before the start of treatment and on day 27 or 28 of administration:

Sensory reactions: Visual response, touch response, auditory response, pain response, air-righting reflex, landing foot splay of hindlimb, grip strength (forelimb and hindlimb), and spontaneous locomotor activity (1 h in an automated system).

Body weights were recorded on days 0, 7, 14, 21 and 28 of administration, then terminally after fasting on day 29 or 30. Food consumption was determined for each week of administration, as g/rat/day.
Ophthalmology was conducted, including use of a slit lamp, for all animals at the start of administration and for the control and high dosage groups on day 28. As there were no treatment related effects, the lower dosage groups were not examined on day 28.
Urinalysis was conducted for all animals on day 28 or 29, examining pH, protein, glucose, ketones, bilirubin, urobilinogen, occult blood, specific gravity, leucocytes and sediment, urine colour and volume.
Just prior to necropsy the animals were anaesthetized and blood was collected from the posterior aorta for haematology and blood chemistry:

Haematology: White cell count, red cell count, haemoglobin, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count, differential white cell count, prothrombin time (PT), activated partial thromboplastin time (APTT) and reticulocyte count.

Blood chemistry: Total protein (TP), albumin (Alb), A/G ratio, glucose (Glu), total cholesterol (TC), triglyceride (TG), total bilirubin (T-Bil), urea nitrogen (UN), creatinine (CRE), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltranspeptidase (γ-GTP), alkaline phosphatase (ALP), sodium (Na), potassium (K), calcium (Ca), chlorides (Cl) and inorganic phosphorus (P).

Organ weights were recorded at necropsy for all animals:

Liver, kidneys, adrenal glands, thymus, spleen, heart, brain, thyroid including parathyroids (weighted post-fixation), testes with epididymides, ovaries.

The following organs were preserved:

Skin, lymph nodes (cervical lymph node and mesenteric lymph node), aorta, salivary glands, bone and bone marrow (sternum and femur), thymus, trachea, lungs and bronchi, heart, thyroid glands (including parathyroid glands), oesophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, liver, pancreas, spleen, kidneys, adrenal glands, urinary bladder, brain (including medulla oblongata, cortex of cerebellum and cortex of cerebrum, except rhinencephalon), pituitary, sciatic nerves, skeletal muscles (femoral), spinal cord (cervical, thoracic and lumbar), eyeballs, harderian gland, testes, epididymides, seminal vesicle /coagulation gland, prostate, ovaries, uterus, vagina, mammary gland, and any macroscopic lesions at necropsy.

Fixation was in 10% neutral buffered formalin. Testes were first fixed in buffered formalin and acetic acid, and the eyeballs in Davidson's fluid, overnight before transfer to 10% NBF.

H&E stained specimens were prepared after paraffin embedding and sectioning. For histopathological examination, all organs were examined for the control group and the high dose group, while only the organs that showed histopathological abnormalities in the high dose group were examined for the middle and low dose groups. Pathological findings were classified by severity (–: no effects, ±: minimal, +: slight, 2+: moderate, and 3+: severe).

Immunohistochemistry: Kidneys of the male rats were examined by staining with anti-alpha 2u globulin antibody. Two or five animals were randomly selected from 0ppm and 800ppm groups, and paraffin-embedded specimens prepared from the kidneys were examined. Serial sections were prepared and mounted on slides coated with antistripping agent. The specimens were treated with xylene/alcohol for deparaffinization and hydrophilization, and then boiled for 40 min in 1 mM tris-EDTA solution (pH 9). After a drying treatment (20 min at room temp.), they were treated with the protease to generate reactivity to the antibody, and further treated with methanol containing 3% (v/v) hydrogen peroxide to inactivate endogenous peroxidases. The specimens were then incubated with primary antibody (goat polyclonal anti-rat alpha 2u-globulin antibody) overnight at 4°C and then with peroxidase conjugated anti-rat IgG. The specimens were then stained with peroxidase reaction and the nuclei were further stained with haematoxylin and the specimens mounted. A specimen from the 0 ppm group was used as control, in which non-specific binding to the secondary antibody was excluded by skipping the primary antibody (anti-alpha 2u globulin) treatment. Eosinophil granulocyte accumulation at the alpha 2u globulin positive sites was examined using serial sections of the sites, by H&E staining.

Statistical analyses: For the body weight, food consumption, organ weight (including relative weight), results of haematological and blood biochemistry examinations, grip strength, spontaneous movement and landing foot splay of hindlimbs, group mean with standard deviations were calculated. They were firstly evaluated by Bartlett's test for homogeneity of variance. When group variances were homogeneous (p≤0.05), Dunnett's multiple comparison test was conducted to determine which group was significantly different from the control. When group variances were heterogeneous (p≤0.05), Steel's multiple comparison test was conducted between each test item group and the control group. Data from the detailed clinical observations, the results of visual response, touch response, auditory response, pain response and air-righting reflex, and the urinalysis data that were obtained from survivors at the end of the administration period were evaluated by Steel's multiple comparison test between the control group and each test item group. However, the dichotomous response data in detailed clinical observations, clinical signs, the results of ophthalmological examination, gross pathological findings and histopathological findings were evaluated by Fisher's exact probability test. A probability value of p≤0.05 was considered statistically significant.

Results

Test diets were formulated to within ±6.3% of nominal concentrations, with coefficients of variation (CV) for the means of approximately 3 to 5%. Storage stability was also satisfactory, with residual concentrations of 101 and 107% at room temperature.

There was mortality during the study, and no abnormalities in general condition of the animals. Among the more detailed clinical observations, including the various functional observations, there were also no treatment related findings.

There was slight depression of body weight gain in both sexes at 800 ppm. (See following tables).

Table B.6.8.1-2: Mean body weight of male rates treated with 2-(trifluoromethyl)-benzamide

Test substance	Dose (ppm)		Body weight (g)									
			Week									
			0		1		2		3		4	
2-(trifluoromethyl)benz-amide	0	Mean	137.39		197.60		254.45		310.11		357.07	
		S.D.	5.04		7.98		8.27		15.42		20.26	
		N	10		10		10		10		10	
	50	Mean	137.49		200.13		258.21		310.58		353.99	
		S.D.	4.43		7.59		10.53		14.71		18.63	
		N	10		10		10		10		10	
	200	Mean	137.50		197.29		250.19		303.69		345.29	
		S.D.	5.92		10.75		13.63		14.30		18.56	
		N	10		10		10		10		10	
	800	Mean	137.65		**181.92**	**	**237.45**	*	**290.00**	*	**332.34**	*
		S.D.	4.54		9.19		15.89		20.47		26.22	
		N	10		10		10		10		10	

S.D.:Standard deviation
N:Number of animals examined
*: p<0.05, **;p<0.01: Significantly different from the control (Dunnett's test).

Table B.6.8.1-3: Mean body weight of female rats treated with 2-9trifluoromethyl)-benzamide

Test substance	Dose (ppm)		Body weight (g)									
			Week									
			0		1		2		3		4	
2-(trifluoromethyl)benz-amide	0	Mean	123.61		156.76		176.75		197.43		215.01	
		S.D.	5.18		6.70		8.83		13.95		15.26	
		N	10		10		10		10		10	
	50	Mean	123.57		157.93		179.34		197.44		217.21	
		S.D.	4.97		7.73		8.84		8.91		12.95	
		N	10		10		10		10		10	
	200	Mean	123.63		153.93		174.69		196.85		216.09	
		S.D.	4.50		5.43		8.19		11.84		14.49	
		N	10		10		10		10		10	
	800	Mean	123.72		**145.01**	**	167.64		183.81		199.13	
		S.D.	4.99		7.46		12.12		14.31		18.52	
		N	10		10		10		10		10	

S.D.:Standard deviation
N:Number of animals examined
*: p<0.05, **;p<0.01: Significantly different from the control (Dunnett's test).

Food consumption was depressed in the first week of treatment at 800 ppm (following table).

Table B.6.8.1-4: Food consumption of male rats treated with 2-(trifluoromethyl)-benzamide

Test substance	Dose (ppm)		Food consumption (g/rat/day)							
			Week							
			1		2		3		4	
2-(trifluoromethyl)benz-amide	0	Mean	20.08		23.91		24.07		24.54	
		S.D.	0.95		1.15		0.68		1.47	
		N	5		5		5		5	
	50	Mean	20.12		24.10		23.23		23.71	
		S.D.	0.52		0.75		1.07		1.75	
		N	5		5		5		5	
	200	Mean	19.70		22.56		23.26		23.05	
		S.D.	1.05		1.40		0.98		1.47	
		N	5		5		5		5	
	800	Mean	**17.43**	**	22.77		23.62		22.79	
		S.D.	1.43		2.40		1.76		2.10	
		N	5		5		5		5	

S.D.: standard deviation
N: number of animals examined
**; p<0.01: significantly different from the control (Dunnett's test)

There were no treatment related ophthalmological findings.

For urinalysis, the 800 ppm males showed significant increases in protein, white blood cell count and epithelial cells. Females at this dosage showed only a minor increase in volume, which was most probably sporadic. At 200 ppm the males showed increased white cell count and epithelial cells in sediment, and also an incidental reduction in urinary casts that was not dose related.

Table B.6.8.1-5: Urinalysis, week 4 of administration with 2-(trifluoromethyl)-benzamide

Item	Sex and Dose level (ppm)	
	Males	Females

		50	200	800	50	200	800
Urine volume		118	134	122	122	165	↑178
Protein		-	-	↑	-	-	-
White blood cell		-	↑	↑↑	-	-	-
Urinary sediment	Cast	-	↓	-	-	-	-
	Epithelial cells	-	↑↑	↑↑	-	-	-

The values for urinary volume are percentage of the control group.
"–" indicates that there was no statistically significant difference.
↑↓: $p < 0.05$, ↑↑: $p < 0.01$ (Dunnett's test or Steel's test)

Haematology: Females in the 800ppm group showed small but statistically significant decreases in red blood cell count, haemoglobin concentration and haematocrit, and a marginal increase in MCHC, with a slight compensatory upward trend in reticulocyte number. Males in this group showed a decrease in MCV and prolongation of APTT, both of which were considered incidental. In the 200 and 50 ppm groups, there were no changes that were considered to be effects of the test item. Other than the above, females in the 50 ppm group showed a statistically significant increase in MCHC, but this was judged to be incidental and not an effect of treatment, considering its very small magnitude and the lack of a meaningful dosage relationship.

Table B.6.8.1-6: Haematology changes following treatment with 2-(trifluoromethyl)-benzamide, expresssed as % of control

Item	Sex and Dose level (ppm)					
	Males			Females		
	50	200	800	50	200	800
Red blood cell count	101	102	103	97	97	↓ **94**
Hemoglobin concentration	103	100	99	98	97	↓↓ **94**
Hematocrit	102	100	99	97	96	↓↓ **92**
MCV	101	98	↓96	100	99	98
MCHC	101	100	100	↑**101**	101	↑↑**102**
APTT	97	111	↑**122**	100	97	96

↓↑: $p < 0.05$,↑↑ ↓↓: $p < 0.01$ (Dunnett's test)

Blood chemistry: In the 800ppm group, males showed significant decreases in AST, ALP (alkaline phosphatase), TG (triglyceride), Glucose and Cl and significant increases in TC (total cholesterol), UN (urea nitrogen), TP (total protein), Albumin and Ca, while females showed significant decreases in AST, ALP, CRE (creatinine), A/G (albumin/globulin ratio) and Cl, and significant increases in TG, TC, TP, Alb and Ca. In the 200ppm group, males showed a significant decrease in AST, while females showed a significant increase in TC. In the 50 ppm group, there were no significant changes in any parameters in either sex.

Table B.6.8.1-7: Blood chemistry changes following treatment with 2-(trifluoromethyl)-benzamide, expressed as % of control

Item	Sex and Dose level (ppm)					
	Males			Females		
	50	200	800	50	200	800
AST	107	↓**82**	↓**80**	86	86	↓↓**68**
ALP	96	85	↓↓**74**	98	102	↓↓**64**
TG	82	82	↓↓**58**	111	106	↑**141**
TC	110	122	↑↑**187**	113	↑↑**127**	↑↑**199**

UN	110	106	↑↑**113**	100	91	107
CRE	103	102	109	97	93	↓↓**82**
TP	99	98	↑↑**110**	99	101	↑↑**112**
Alb	101	99	↑↑**108**	100	100	↑↑**109**
A/G	106	103	96	102	98	↓**93**
Glu	106	96	↓↓**77**	95	99	97
Cl	100	99	↓↓**98**	99	99	↓**98**
Ca	102	104	↑↑**109**	102	102	↑**107**

↓↑: $p < 0.05$, ↑↑ ↓↓: $p < 0.01$ (Dunnett's test or Steel's test)

Terminal findings: In males and females of the 800 ppm group, enlargement, accentuated lobular pattern and dark colouration of the liver were observed. The incidence of discolouration in the kidneys was increased significantly in males, and 2 males showed enlargement of the kidneys, while 1 male showed enlargement of the thyroid glands, but there were no similar changes in females. In the 200 ppm group, there were no statistically significant changes, but some changes were thought to be related to administration of the test item: accentuated lobular pattern in 1 male and 1 female, and discolouration of the kidneys in 2 males. In the 50 ppm group, there were no abnormalities in males or females. Other than the above, one male in the 200 ppm group showed hepatodiaphragmatic nodule in the liver and cyst in the kidneys, while one female in the 800 ppm group showed cystic ovarian bursa in the ovary, but these were considered unrelated to treatment, as they were each observed only in 1 animal and they are changes that are observed sporadically as spontaneous changes in normal rats.

Table B.6.8.1-7: Necropsy findings in male and female rats following treatment with 2-(trifluoromethyl)-benzamide

Organ / Findings	Sex and Dose level (ppm)							
	Males				Females			
	0	**50**	**200**	**800**	**0**	**50**	**200**	**800**
Liver: [N=]	[10]	[10]	[10]	[10]	[10]	[10]	[10]	[10]
Accentuated lobular pattern	0	0	1	↑**5**	0	0	1	↑**5**
Enlargement	0	0	0	↑↑**6**	0	0	0	1
Dark coloration	0	0	0	3	0	0	0	↑**5**
Kidneys: [N=]	[10]	[10]	[10]	[10]	[10]	[10]	[10]	[10]
Discoloration	0	0	2	↑↑**8**	0	0	0	0
Enlargement	0	0	0	2	0	0	0	0
Thyroid glands: [N=]	[10]	[10]	[10]	[10]	[10]	[10]	[10]	[10]
Enlargement	0	0	0	1	0	0	0	0

[N=]: The number of animals examined.

The values in the table indicate the number of animals that showed the change.

↑: $p < 0.05$, ↑↑: $p < 0.01$ (Fisher's exact probability method)

Organ weights: In the 800 ppm group, males and females showed significant increases in the absolute and relative weights of the liver. The significant decrease in the absolute weight of the brain reflected the reduced body weight. Males showed significant increases in the absolute and relative weights of kidneys, while the mildly increased relative kidney weight in females reflected the reduced terminal body weight. Male thyroid glands and testis and absolute weight of the epididymis and females showed significant increases in the relative weight of the kidneys and heart. Males and females in 200

ppm showed a significant increase in the relative weight of the liver, and males showed significant increases in the absolute and relative weights of the kidneys and in the relative weight of the thyroid glands. Males in 50 ppm showed a significant increase in the relative weight of the kidneys and significant decreases in the absolute and relative weights of the spleen. Females in the 50 ppm group showed no significant increases or decreases in any organ. The significant decreases in the absolute and relative weights of the spleen that were observed in males in the 50 ppm group were judged to be sporadic and un-related to treatment, as they were not dose-related. (See following table).

Table B.6.8.1-8: Organ weight changes in male and female rats treated with 2-(trifluoromethyl)-benzamide, expressed as % of control

		Sex and Dose level (ppm)					
		Males			Females		
		50	200	800	50	200	800
Final body weight		100	97	↓**92**	101	100	92
Liver	(absolute weight)	101	107	↑↑**146**	105	110	↑↑**143**
	(relative weight)	101	↑↑**111**	↑↑**159**	104	↑↑**110**	↑↑**155**
Kidneys	(absolute weight)	108	↑**114**	↑↑**117**	105	104	102
	(relative weight)	↑**108**	↑↑**117**	↑↑**127**	103	104	↑**110**
Thyroid glands	(absolute weight)	100	117	↑↑**125**	102	113	101
	(relative weight)	101	↑**120**	↑↑**136**	101	113	110
Brain	(absolute weight)	100	96	↓**94**	101	99	↓**95**
Heart	(absolute weight)	97	97	99	103	101	↑**114**
Spleen	(absolute weight)	↓**84**	87	92	99	103	88
	(relative weight)	↓**84**	90	101	98	103	96
Testis	(absolute weight)	101	107	↑**109**			
	(relative weight)	100	109	↑↑**117**			

For testis/epididymis, organ weight was calculated excluding 1 animal in the control group that showed histopathological abnormalities.

↓↑: $p < 0.05$,↑↑: $p < 0.01$ (Dunnett's test or Steel's test)

Histopathology and immunohistochemistry: In males and females of the 800ppm group, the incidence of centrilobular hepatocellular hypertrophy in the liver was increased significantly. In males at the same group, the incidence of excessive hyaline droplet accumulation in tubular epithelial cells, renal tubular degeneration/necrosis and basophilic change of renal tubules in the kidney were significantly increased. One male showed cellular infiltration in the kidney and 3 males showed follicular cell hypertrophy in the thyroid gland. In males and females of the 200 ppm group, the incidence of centrilobular hepatocellular hypertrophy in the liver was significantly increased. Males in the same group showed significant increases in the incidence of excessive hyaline droplet accumulation in tubular epithelial cells, renal tubular degeneration/necrosis and basophilic change of renal tubules. Two males showed cellular infiltration in the kidney and 1 male showed follicular cell hypertrophy in the thyroid gland. In males of the 50 ppm group, the incidence of excessive hyaline droplet accumulation in tubular epithelial cells in the kidney was significantly increased and 3 males showed basophilic change of renal tubules and 1 male showed cellular infiltration in the kidney. Females in this group showed no changes in any organ.

Table B.6.8.1-9: Histopathological changes in male and female rats treated with 2-(trifluoromethyl)-benzamide

Organ / Findings	Sex and Dose level (ppm)							
	Males				Females			
	0	**50**	**200**	**800**	**0**	**50**	**200**	**800**
Liver: [N=]	[10]	[10]	[10]	[10]	[10]	[10]	[10]	[10]
Hypertrophy, hepatocyte, centrilobular	0	0	↑↑**10**	↑↑**10**	0	0	↑↑**10**	↑↑**10**
Kidney: [N=]	[10]	[10]	[10]	[10]	[10]	[10]	[10]	[10]
Excessive accumulation of hyaline droplets, tubular epithelial cell	0	↑↑**10**	↑↑**10**	↑↑**10**	0	0	0	0
Necrosis / degeneration, renal tubules	0	1	↑**5**	↑↑**10**	0	0	0	0
Basophilic, renal tubules	0	3	↑↑**7**	↑↑**7**	0	0	0	0
Cell infiltration	0	1	2	1	0	0	0	0
Thyroid gland: [N=]	[10]	[10]	[10]	[10]	[10]	[10]	[10]	[10]
Hypertrophy, follicular cell	0	0	1	3	0	0	0	0

[N=]: The number of animals examined. Figures show the number of animals with each lesion.

↑: $p < 0.05$, ↑↑: $p < 0.01$ (Fisher's exact probability method)

Immunostaining using anti-α2u-globulin antibody confirmed that the hyaline droplets in tubular epithelial cells in the male rat kidney contained α2u-globulin.

Discussion

The minor haematological changes in females at 800 ppm (6-8% reduction from control in some red cell parameters) were indicative of a mild normocytic, normochromic anaemia. Increases in reticulocyte count indicated compensatory haematopoiesis.

Increased liver weight with centrilobular hepatocyte hypertrophy was likely a reflection of increased metabolic activity as an adaptive response. The effect however was considered to be treatment related and adverse. Some effect on liver function was suggested by changes in parameters associated with lipid metabolism and by decreases in AST and ALP at ≥200 ppm, but there was no effect on the related and more liver-specific enzyme ALT, while AST and ALP are distributed widely in other tissues; and therefore overall these changes might be secondary to a nutritional imbalance.

At ≥200 ppm the males showed increased thyroid weight, also with discernible follicular cell hypertrophy in a minority of those animals, but there were no clear effects in females. This was likely also to be associated with an adaptive metabolic response.

Changes in the kidneys of males were ascribed to the α_{2u}-globulin nephropathy that is specific to male rats and not relevant for human risk, as confirmed by the immunohistochemical investigation of the hyaline droplets. Blood chemistry showed increased urea nitrogen at 800 ppm in males, but as there was no change in creatinine or glomerular lesion incidence this was considered not to indicate any general lowering of filtering function. Increased relative kidney weight in females at 800 ppm was considered to be associated with the lower final body weight, there being no histological change, nor any changes in electrolytes.

Conclusions

The NOAEL for M-101 in the diet of rats was 50 ppm, equivalent to an average of 4.2 mg/kg/day for males, 4.4 mg/kg/day for females. This excludes the α_{2u}-globulin deposition in male kidneys, which is not relevant for human risk assessment.

In vitro genotoxicity studies – Ames test, 2-(trifluoromethyl)-benzamide

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study. The study is considered acceptable.

reference	:	Inagaki, K (2011)	vehicle	:	DMSO
Report number	:	LSRC-T11-079A (T-3200)	doses	:	61.7, 185, 556, 1667 and 5000 µg/ plate
test substance	:	2-(trifluoromethyl)-benzamide	GLP	:	yes
Test system	:	*Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2 uvrA	guideline	:	In accordance with OECD 471 (1997)
			acceptability	:	Yes

Executive summary

The metabolite 2-(trifluoromethyl)-benzamide (M-101, 100% purity) was tested in a reverse mutation test using the pre-incubation procedure, with and without metabolic activation, in *Salmonella typhimurium* strains TA 98, TA 100, TA 1535, TA 1537, plus *Escherichia coli* WP2 uvrA. S9 fraction was used as the metabolic activation system (rat liver, induced with phenobarbital and 5,6-benzoflavone). Dose levels in the main experiment were 61.7, 185, 556, 1667 and 5000 µg/plate, the vehicle being DMSO. Appropriate vehicle controls and positive controls were included.

There was no significant increase in revertants in any of the bacterial strains, either with or without the S9 metabolic activation system, at any dosage of the test item, while the positive controls each showed the expected mutagenic activity.

It was concluded that 2-(trifluoromethyl)-benzamide (M-101) was non-mutagenic in the bacterial test systems at up to 5000 µg/plate.

Materials and methods

Test item:	2-(trifluoromethyl)-benzamide (M-101)
Batch No.:	336876
Purity:	100.0%
Expiry:	11 July 2011
Bacteria:	*Salmonella typhimurium* strains TA100, TA1535, TA98, TA1537 (histidine auxotrophs); *Escherichia coli* strain WP2 *uvrA* (tryptophan dependent)
Metabolic activation:	Sprague-Dawley rat liver S-9 fraction, induced with phenobarbital and 5,6-benzoflavone (Oriental Yeast Co., Ltd)
Vehicle:	Dimethylsulphoxide (DMSO, ≥99% purity)

Positive controls:	AF-2: 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (100.2% purity)
	9-AA: 9-aminoacridine (>97% purity)
	Sodium azide (99.7% purity)
	2-AA: 2-aminoanthracene (97.5% purity)
Study start and completion:	31 May 2011 to 02 August 2012

The bacterial strains were derived from frozen stocks at the laboratory, screened periodically for essential characteristics: Histidine auxotrophy for *S. typhimurium* strains and tryptophan auxotrophy for the *E. coli* strain; sensitivity to UV light (uvrA, uvrB); susceptibility to crystal violet for *S. typhimurium* strains (*rfa*); presence/absence of ampicillin-resistant plasmid pKM101 for TA100 and TA98; reactivity to positive controls; number of spontaneous revertants.

The nutrient broth medium was sterilized 2.5% Nutrient Broth No. 2 (Oxoid Ltd, UK).

Minimum glucose agar plate medium consisted of Vogel-Bonner minimum medium E (0.02% magnesium sulphate-7hydrate, 0.2% citric acid monohydrate, 1% potassium phosphate, 0.35% hydrogen ammonium phosphate-4hydrate), 2% glucose and 1.5% agar.

Top agar for the *S. typhimurium* strains was a mixture of sterilized 0.5 mM L-histidine with 0.5 mM D-biotin, soft agar (0.6% agar and 0.5% sodium chloride), in a ratio of 1:10 (v/v). For the *E. coli* it was a mixture of the sterilized 0.5 mM L-tryptophan with the soft agar.

The S9 mix was prepared immediately before use by mixing with cofactors in distilled water and was stored at -80ºC and used within 6 months of preparation. The composition was as follows:

S9	0.1 mL
$MgCl_2$	8 µmol
Glucose-6-phosphate (G-6-P)	5 µmol
NADH	4 µmol
NADPH	4 µmol
KCl	33 µmol
Sodium phosphate buffer	100 mM

The test item was dissolved in DMSO (≥99% purity) at the maximum concentration of 50 mg/mL, which was then diluted as necessary to prepare each required concentration. The solutions were prepared just before usage.

The gene mutation test was performed as two independent tests: a dose finding experiment and the main experiment, using the pre-incubation method. There were 3 plates for each dose level and for the negative (vehicle) control. Positive control plates were also prepared for each test.

Dose levels for the dose-finding experiment were 19.5, 78.1, 313, 1250 and 5000 µg/plate. For the main experiment they were 61.7, 185, 556, 1667 and 5000 µg/plate. DMSO was the negative (vehicle) control, and AF-2, 9-AA, 2-AA and NaN_3 were used as positive controls. AF-2, 9-AA and 2-AA were dissolved in DMSO, NaN_3 in sterilized distilled water. The solutions were frozen at -20ºC and used within 18 months of preparation.

Bacterial culture was inoculated into nutrient broth medium and cultured for 6 or 8 h at 37ºC with shaking, until the stationary phase. The viable cell number was calculated from turbidity. The test item (0.1 mL) was mixed with 0.1 mL of the bacterial culture and 0.5 mL of 0.1 M sodium phosphate buffer (pH 7.4) for the test without metabolic activation. For the test with metabolic activation, 0.5 mL of S9 mix was added to the fresh bacterial culture. After incubation for 20 min at 37ºC with shaking, 2 mL of top agar at 48-50ºC was added. The homogeneous mixture was laid on the minimum glucose agar plate medium (30 mL/plate). After confirmation of presence/absence of precipitate, the plates were inverted and cultured for 48 h at 37ºC. Negative and positive controls were treated in the same manner. After incubation the revertant colonies were counted using a colony counter. Cytotoxicity was observed with a stereomicroscope and graded from slight diminution to complete clearing of the background lawn. The culture was also observed macroscopically for precipitate. A sterility test was also set up for each experiment.

Acceptance criteria:

Mean revertant colonies in the negative control group within the range of historical control data;

Mean revertant colonies in the positive control group within the range of historical control data;

No contamination with saprophytic bacteria in the sterility test.

Evaluation criteria:

The test was judged positive if:

The mean number of revertant colonies was clearly higher than in the negative control group;

The increase in revertant colonies was dose-dependent;

The increase in revertants was reproducible.

Results

Dose finding experiment:

2-(trifluoromethyl)-benzamide clearly did not increase the number of revertant colonies in any bacterial strain in the presence or absence of metabolic activation system, compared to that of the negative control group. There was neither cytotoxicity nor precipitation in any bacterial strain in the presence or absence of metabolic activation system. Based on these results, the maximum dose level in the main experiment was set at 5000 µg/plate for all bacterial strains with and without metabolic activation.

Table B.6.8.1-10: Revertant colonies following exposure to 2-(trifluoromethyl)-benzamide in the dose finding experiment, without S9

Test or control substance	Dose (µg/plate)	Number of revertant colonies/plate (Average ± Standard Deviation, n=3)				
		Base-pair substitution type			Frameshift type	
		TA100	TA1535	WP2 *uvrA*	TA98	TA1537
DMSO	-	97 ± 2	10 ± 3	19 ± 4	16 ± 2	5 ± 3
M-101	19.5	103 ± 15	9 ± 2	21 ± 5	12 ± 4	4 ± 0

	78.1	99 ± 5	12 ± 3	19 ± 2	16 ± 4	4 ± 1
	313	101 ± 17	6 ± 2	24 ± 2	13 ± 5	3 ± 2
	1,250	108 ± 6	8 ± 1	20 ± 3	15 ± 5	5 ± 1
	5,000	111 ± 16	8 ± 2	21 ± 8	16 ± 4	5 ± 1
AF-2	0.01	437 ± 36	NT	NT	NT	NT
	0.02	NT	NT	256 ± 12	NT	NT
	0.1	NT	NT	NT	294 ± 27	NT
NaN_3	0.5	NT	288 ± 34	NT	NT	NT
9-AA	80	NT	NT	NT	NT	267 ± 20

DMSO: dimethylsulfoxide, M-101: 2-(Trifluoromethyl)benzamide, AF-2: 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide,

NaN_3: sodium azide, 9-AA: 9-aminoacridine

NT: Not tested

Table B.6.8.1-11: Revertant colonies following exposure to 2-(trifluoromethyl)-benzamide in the dose finding experiment, with S9

Test or control substance	Dose (µg/plate)	Number of revertant colonies/plate (Average ± Standard Deviation, n=3)				
		Base-pair substitution type			Frameshift type	
		TA100	TA1535	WP2 *uvrA*	TA98	TA1537
DMSO	-	91 ± 20	6 ± 2	24 ± 3	23 ± 3	5 ± 3
M-101	19.5	81 ± 7	10 ± 2	20 ± 6	21 ± 2	5 ± 2
	78.1	88 ± 1	12 ± 1	19 ± 3	17 ± 6	5 ± 3
	313	93 ± 20	9 ± 2	19 ± 3	25 ± 12	4 ± 2
	1,250	88 ± 3	8 ± 2	22 ± 1	27 ± 1	4 ± 2
	5,000	77 ± 10	9 ± 3	20 ± 2	23 ± 4	3 ± 4
2-AA	0.5	NT	NT	NT	222 ± 25	NT
	1	628 ± 35	NT	NT	NT	NT
	2	NT	194 ± 8	NT	NT	108 ± 19
	10	NT	NT	364 ± 27	NT	NT

DMSO: dimethylsulfoxide, M-101: 2-(Trifluoromethyl)benzamide, 2-AA: 2-aminoanthracene

NT: Not tested

Main experiment:

2-(Trifluoromethyl)-benzamide clearly did not increase the number of revertant colonies in any bacterial strain in the presence or absence of metabolic activation system, compared to that of the negative control group. There was neither cytotoxicity nor precipitation in any bacterial strain in the presence or absence of metabolic activation system. These results of the main experiment clearly reproduced those of the dose-finding experiment.

Table B.6.8.1-12: Revertant colonies following exposure to 2-(trifluoromethyl)-benzamide in the main finding experiment, without S9

Test or control substance	Dose (µg/plate)	Number of revertant colonies/plate (Average ± Standard Deviation, n=3)				
		Base-pair substitution type			Frameshift type	
		TA100	TA1535	WP2 *uvrA*	TA98	TA1537
DMSO	-	103 ± 2	10 ± 1	22 ± 5	10 ± 5	5 ± 2
M-101	61.7	98 ± 9	7 ± 1	23 ± 3	12 ± 1	6 ± 1
	185	111 ± 8	6 ± 1	23 ± 5	14 ± 7	7 ± 1
	556	112 ± 18	11 ± 3	28 ± 5	14 ± 2	5 ± 2
	1,667	103 ± 9	7 ± 4	18 ± 3	16 ± 4	5 ± 1
	5,000	106 ± 7	10 ± 2	20 ± 3	11 ± 3	5 ± 1
AF-2	0.01	382 ± 13	NT	NT	NT	NT
	0.02	NT	NT	264 ± 3	NT	NT
	0.1	NT	NT	NT	295 ± 17	NT
NaN_3	0.5	NT	197 ± 9	NT	NT	NT
9-AA	80	NT	NT	NT	NT	436 ± 243

DMSO: dimethylsulfoxide, M-101: 2-(Trifluoromethyl)benzamide, AF-2: 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide,

NaN3: sodium azide, 9-AA: 9-aminoacridine

NT: Not tested

Table B.6.8.1-13: Revertant colonies following exposure to 2-(trifluoromethyl)-benzamide in the main finding experiment, with S9

Test or control substance	Dose (µg/plate)	Number of revertant colonies/plate (Average ± Standard Deviation, n=3)				
		Base-pair substitution type			Frameshift type	
		TA100	TA1535	WP2 *uvrA*	TA98	TA1537
DMSO	-	86 ± 13	9 ± 3	19 ± 4	20 ± 3	7 ± 1
M-101	61.7	97 ± 11	9 ± 2	24 ± 7	22 ± 5	4 ± 2
	185	86 ± 6	6 ± 1	20 ± 2	18 ± 3	5 ± 2
	556	86 ± 6	8 ± 4	26 ± 1	18 ± 6	6 ± 2
	1,667	97 ± 6	11 ± 3	17 ± 2	20 ± 5	7 ± 5
	5,000	88 ± 5	7 ± 2	20 ± 5	20 ± 6	7 ± 5
2-AA	0.5	NT	NT	NT	218 ± 9	NT
	1	629 ± 27	NT	NT	NT	NT
	2	NT	166 ± 29	NT	NT	134 ± 15
	10	NT	NT	202 ± 9	NT	NT

DMSO: dimethylsulfoxide, M-101: 2-(Trifluoromethyl)benzamide, 2-AA: 2-aminoanthracene

NT: Not tested

In both the dose-finding experiment and the main experiment, the data of the negative control group and the positive control groups were within the range of the historical values, and the positive control values were clearly higher than the negative control values. There was no contamination of

saprophytic bacteria. Based on these results, it was judged that all the tests were conducted appropriately and were sufficient for evaluation.

Conclusion:

It was concluded that 2-(trifluoromethyl)-benzamide (M-101) was non-mutagenic in the bacterial test systems at up to 5000 µg/plate.

In vitro genotoxicity studies - mammalian cell gene mutation test, 2-(trifluoromethyl)-benzamide

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study. The study was performed just before the current OECD guideline (2016) was adopted. However, no deviations from the current guideline were noted. The study is considered acceptable.

reference	:	Lloyd, M (2016a)	vehicle	:	DMSO
Report number	:	8337791 (T-3250)	doses	:	150 to 1892 µg/mL
test substance	:	2-(Trifluoromethyl)benzamide	GLP	:	yes
Test system	:	mouse lymphoma L5178Y cells	guideline	:	In accordance with OECD 490 (2015)
			acceptability	:	acceptable

Executive summary

M-101 (2-(trifluoromethyl)-benzamide), 99.3% purity, was assayed for the ability to induce mutation at the *tk* locus (5-trifluorothymidine [TFT] resistance) in mouse lymphoma cells using a fluctuation protocol. The study consisted of a cytotoxicity experiment followed by one mutation experiment, each conducted in the absence and presence of metabolic activation by an Aroclor 1254-induced rat liver post-mitochondrial fraction (S-9). The test item was formulated in dimethyl sulphoxide (DMSO). A 3 h treatment incubation period was used in the presence of S-9, while 3 and 24 h periods were applied in the absence of S-9.

In the cytotoxicity experiment with 3 h treatment, 6 concentrations from 59.13 to 1892 µg/mL (up to 10 mM equivalent) were tested. The highest concentration gave 66% and 56% relative total growth (RTG) in the absence and presence of S-9 respectively. With 24 h treatment, 9 concentrations were tested in the absence of S-9 only, from 7.391 to 1892 µg/mL, where the highest concentration to provide >10% RTG was 946 µg/mL, giving 40% RTG.

In the mutation experiment with 3 h treatment, 7 concentrations from 150 to 1892 µg/mL were tested. Two days after treatment, the highest concentration analyzed for viability and TFT resistance was 1892 µg/mL, which gave 105% and 95% RTG in the absence and presence of S-9 respectively. With 24 h treatment, 10 concentrations from 150 to 1902 µg/mL were tested in the absence of S-9 only, and two days after treatment the highest concentration analyzed for viability and TFT resistance was 1500 µg/mL, which gave 22% RTG (although 19% RTG was observed at 1350 µg/mL). Vehicle and positive controls were included, and mutant frequencies (MF) fell within acceptable ranges: clear increases occurred with methyl methane sulphonate (MMS, without S-9) and benzo[a]pyrene (Bp, with S-9), demonstrating validity of the study.

When tested at up to 10 mM for 3 h in the absence and presence of S-9, no increases in MF exceeding the Global Evaluation Factor (GEF) of 126 mutants per 10^6 viable cells (compared to concurrent vehicle controls) were observed in any treated cultures under either treatment condition. A weakly significant linear trend ($p \leq 0.05$) was observed for the 3 h treatment in the absence of S-9, but the maximum increase in MF observed in any treated culture was approximately 30 (at 1200 µg/mL), which was well below the GEF of 126 and therefore the trend was considered not biologically relevant.
It was concluded that M-101 (2-(trifluoromethyl)-benzamide) did not induce mutation at the *tk* locus of L5178Y mouse lymphoma cells when tested at up to 10 mM for 3 h in the absence and presence of a rat liver metabolite activation system (S-9), and at up to toxic concentrations for 24 h in the absence of S-9.

Materials and Methods

Test item:	M-101 (2-(trifluoromethyl)-benzamide)
Batch No.:	336876
Purity:	100%
Description:	White crystalline powder
Expiry:	25 March 2017
Storage:	Refrigerated, in the dark
Vehicle:	Dimethyl sulphoxide (DMSO), anhydrous, analytical grade
Positive controls:	Methyl methane sulphonate (MMS), Benzo[a]pyrene (BP), from Sigma-Aldrich
Cells:	L5178Y *tk+/-* (3.7.2C) mouse lymphoma cells, from Burroughs Wellcome Co.
Metabolic activation:	MolTox S-9 from Molecular Toxicology Inc., USA
Culture medium:	RPMI 1640 containing L-glutamine and HEPES
Study start and completion:	17 February 2016 to 21 July 2016

M-101 was soluble in DMSO at up to (at least) 190.2 mg/mL. The solubility limit in culture medium was in excess of 1902 µg/mL, as indicated by precipitation that persisted for 22 h at 37ºC. A maximum concentration of 1902 µg/mL was selected for the study, equivalent to 10 mM. Test item solutions were prepared with the aid of vortex mixing under subdued light, with serial dilution from the maximum required concentration. These solutions were used within 3.5 h of preparation, and no analyses for stability, homogeneity or achieved concentrations were undertaken. Negative (vehicle) controls comprised treatments with DMSO diluted 100-fold in the medium. The positive controls were used at 5 to 20 µg/mL (MMS) and 2 to 3 µg/mL (BP).
The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254. The batches of MolTox™ S-9 were stored frozen in aliquots at <-50°C prior to use. Each batch was checked by the manufacturer for sterility, protein content, ability to convert known promutagens to bacterial mutagens and cytochrome P-450-catalyzed enzyme activities (alkoxyresorufin-O-dealkylase activities). The final concentration of liver homogenate in the test system was 2%.

Cytotoxicity experiment:

Treatment of cell cultures for the cytotoxicity Range-Finder Experiment was as described below for the Mutation Experiment. The final treatment culture volume was 20 mL. In the absence of S-9, 3 and 24 h treatment incubation periods were used, while in the presence of S-9 a 3 h treatment incubation was used.

Changes in osmolality of more than 50 mOsm/kg and fluctuations in pH of more than one unit may be responsible for an increase in mutant frequencies (Scott *et al*., 1991; Brusick, 1986). Osmolality and pH measurements were taken on post-treatment incubation medium during the cytotoxicity experiment.

Mutation experiment:

For 3 h treatments in the absence and presence of S-9, at least 10^7 cells in a volume of 19.8 mL tissue culture medium (cells in RPMI 10 diluted with RPMI A [no serum] to give a final concentration of 5% serum) were used. For 24 hour treatment in the absence of S-9 at least 4 x 10^6 cells in a volume of 19.8 mL RPMI 10 were used. The 24 hour treatment tissue culture flasks were gassed with 5±1% v/v CO_2 in air. For all treatments 0.2 mL vehicle, test article or positive control solution was added. For 3 hour treatments, S-9 mix or 150 mM KCl was added, as described. Each treatment, in the absence or presence of S-9, was in duplicate (single cultures only used for positive control treatments) and the final treatment culture volume was 20 mL.

After 3 h incubation at 37±1°C with gentle agitation, cultures were centrifuged, washed with the appropriate tissue culture medium, centrifuged again and resuspended in 50 mL RPMI 10 medium. After static incubation at 37±1°C for 24 h, cultures were centrifuged, washed with the appropriate tissue culture medium, centrifuged again and resuspended in fresh RPMI 10 medium (20 mL). Cell densities were determined using a Coulter counter and adjusted to 2 x 10^5 cells/mL. The solubility of the test article in culture was assessed by eye at the beginning and end of treatment.

Cultures were maintained in flasks for a period of 2 days during which the *tk*$^{-/-}$ mutation would be expressed. During the expression period, subculturing was performed as required to retain an appropriate number of cells/flask. From observations on recovery and growth of the cultures during the expression period, the following cultures were selected to be plated for viability and TFT resistance as shown in the following table.

Table B.6.8.1-14: Concentrations of 2-(trifluoromethyl)-benzamide used during the mutation experiment

Mutation Experiment		
3 hour (µg/mL)		**24 hour (µg/mL)**
- S-9	+ S-9	- S-9
0	0	0
150	150	150
300	300	300
600	600	600
900	900	900
1200	1200	1050
1500	1500	1200
1892	1892	1350

		1500 1700 1892
MMS 15 MMS 20	B[a]P 2 B[a]P 3	MMS 5 MMS 7.5

Plating for viability: At the end of the expression period the cell densities in the selected cultures were determined using a Cell counter or haemocytometer and adjusted to approximately 8 cells/mL. For each concentration, 0.2 mL were plated into each well of a 96 well microtitre plate for determination of viability (cloning efficiency). All 96 well plates were incubated at 37±1°C in a humidified incubator gassed with 5±1% (v/v) CO_2 in air until scoreable (6 to 7 days). Wells containing viable clones were identified by eye using background illumination and counted.

Plating for mutation: At the end of the expression period, cell concentrations in the selected cultures were determined using a Coulter counter and adjusted to give 1 x 10^4 cells/mL in readiness for plating for TFT resistance. Samples from these were diluted to 8 cells/mL, then 0.2 mL of the final concentration of each culture was placed into each well of 2 x 96-well microtitre plates (192 wells averaging 1.6 cells/well). The plates were incubated at 37±1ºC in a humidified incubator gassed with 5±1% v/v CO_2 in air until scoreable (8 days). Wells containing viable clones were identified by eye using background illumination and counted.

Plating for TFT resistance: At the end of the expression period, the cell densities in the selected cultures were adjusted to 1 x 10^4 cells/mL. TFT (300 µg/mL) was diluted 100-fold into these suspensions to give a final concentration of 3 µg/mL. Using an eight-channel pipette, 0.2 mL of each suspension was placed into each well of four 96-well microtitre plates (384 wells at 2 x 10^3 cells/well). Plates were incubated at 37±1°C in a humidified incubator gassed with 5±1% v/v CO_2 in air until scoreable (13 days) and wells containing clones were identified as above and counted. In addition, the number of wells containing large colonies and the number containing small colonies were scored for the negative and positive controls and for concentrations of test article where a marked increase in mutant frequency (exceeding the GEF) was observed.

Data analysis:

Suspension Growth (SG), a measure of the growth in suspension during treatment and the expression period:

$$SG = a \times b \times c$$

$$\text{where } a = \left(\frac{D_0 \text{ post-treatment cell count}}{\text{Pre-treatment cell density}} \right)$$

$$\text{where } b = \left(\frac{D_1 \text{ cell count}}{\text{Cell count set up on } D_0 \text{ post-treatment}} \right)$$

$$\text{where } c = \left(\frac{D_2 \text{ cell count}}{\text{Cell count set up on } D_1} \right)$$

For 3 h treatments, ‘a’ is taken as 1; D_0, D_1, D_2 are Days 0, 1 and 2

Relative Suspension Growth (RSG), a measure of the growth in suspension during treatment and the expression period relative to the mean control:

$$\text{RSG (\%)} = \left(\frac{\text{Individual SG value}}{\text{Mean control SG value}} \right) \text{x } 100$$

Cloning Efficiency (CE), is viability as a measure of the cells' ability to clone:
For microtitre plate tests, calculations are based on P(0), the proportion of wells in which a colony has not grown:

$$\text{P(0)} = \left(\frac{\text{Number of wells with no colony}}{\text{Total number of wells}} \right)$$

The CE for each culture was calculated according to the following calculation:

$$\text{CE} = \left(\frac{\text{- ln P(0)}}{\text{Number of cells per well*}} \right) \text{x } 100$$

* Number of cells per well is 1.6 cells/well on average on all viability plates.

Relative Total Growth (RTG) is the measure of cytotoxicity relative to the control, taking into account all cell growth, and cell loss during the treatment period and the 2 day expression period (RSG), and the cells' ability to clone 2 days after treatment (viability):

$$\text{RTG} = \text{RSG x } \left(\frac{\text{Individual Viability Value}}{\text{Mean Control Viability Value}} \right)$$

Mutant Frequency (MF) was calculated from the number of TFT resistant mutants expressed per 10^6 viable cells as follows:

$$\frac{\text{- ln P(0) for mutant plates}}{\text{Number of cells per well* x (viability/100)}}$$

* Number of cells per well is 2000 cells/well on average on all mutant plates.

Small and large colony mutant frequencies were calculated in an identical manner, using the relevant number of empty wells for small and large colonies, as appropriate. Heterogeneity between replicate cultures was assessed and where considered to be excessive may have resulted in exclusion of a concentration.
The relevance of increases in mutant frequencies (total wells with clones), by comparison with concurrent controls and the global evaluation factor (GEF), was assessed according to the recommendations of the Mouse Lymphoma Workgroup, Aberdeen, 2003. Linear regression was performed on ranked mutant frequency against ranked concentration to test for a linear trend. The linear trend was performed on the overall MF per concentration level, not individual replicates (i.e. one

MF value per concentration). The test for linear trend is one-tailed, therefore negative trend was not considered relevant. The positive control was excluded from the test for linear trend.
For microwell assays, the GEF is defined as 126 mutants per 10^6 viable cells.

Acceptance criteria:
The assay was considered valid if all the following criteria were met:

1. The mean mutant frequencies in the vehicle control cultures fell within the normal range (50 to 170 mutants per 10^6 viable cells)
2. At least one positive control showed either an absolute increase in mean total MF of at least 300×10^{-6} (at least 40% of this should be in the small colony MF), or an increase in small colony mutant frequency of at least 150×10^{-6} above the concurrent vehicle control
3. The RTG for the positive controls was greater than 10%
4. The mean CE of the vehicle controls from the Mutation Experiment was between the range 65% to 120% on Day 2
5. The mean SG of the vehicle controls from the Mutation Experiment was between the range 8 to 32 following 3 hour treatments or between 32 and 180 following 24 hour treatments.

Evaluation criteria:
For valid data, the test item was considered to be mutagenic in the assay if all of these criteria were met:

1. The MF of any test concentration exceeded the sum of the vehicle control mutant frequency plus GEF
2. The linear trend test was statistically significant
3. Any observed response was reproducible under the same treatment conditions.

The test item was considered as negative in this assay if none of the above criteria were met.

Results

Cytotoxicity:
In the 3 h treatment, 6 concentrations were tested, in the absence and presence of S-9, ranging from 59.13 to 1892 µg/mL (equivalent to 10 mM at the highest concentration tested). No precipitate was observed at the time of treatment or following the treatment incubation period. The highest concentration tested (1892 µg/mL) gave 66% and 56% RTG in the absence and presence of S-9, respectively.
In the 24 h treatment, 9 concentrations were tested in the absence of S-9, ranging from 7.391 to 1892 µg/mL. No precipitate was observed at the time of treatment or following the treatment incubation period. The highest concentration to provide >10% RTG was 946 µg/mL, which gave 40% RTG.

No marked changes in osmolality or pH were observed in the 3 or 24 h experiments at the highest concentration tested (1892 µg/mL), compared to the concurrent vehicle controls.

Mutation experiment:
In the 3 h treatments, 7 concentrations, ranging from 150 to 1892 µg/mL, were tested in the absence and presence of S-9. Two days after treatment all concentrations in the absence and presence of S-9 were selected to determine viability and TFT resistance. The highest concentration analyzed was 1892 µg/mL, which gave 101% and 95% RTG in the absence and presence of S-9, respectively.

Table B.6.8.1-15: Mutation Experiment - 3 Hour Treatment in the Absence and Presence of S-9

3 hour treatment -S-9			**3 hour treatment +S-9**		
Concentration µg/mL	**%RTG**	**MF §**	**Concentration µg/mL**	**%RTG**	**MF §**
0	100	78.35	0	100	113.53
150	102	70.54	150	121	73.55
300	103	56.03	300	123	89.26
600	116	102.10	600	139	60.86
900	113	96.21	900	107	78.18
1200	94	108.63	1200	108	110.99
1500	76	97.03	1500	102	89.64
1892	101	101.45	1892	95	85.27
MMS 15	75	566.89	B[a]P 2	78	356.59
MMS 20	52	623.50	B[a]P 3	74	536.17

3 hour in the absence of S-9. Linear trend test on mutant frequency: p-value = 0.0428 (* P < 0.05)
3 hour in the presence of S-9. Linear trend test on mutant frequency: p-value = 0.5
MF § Mutant Frequency
%RTGPercent Relative Total Growth

In the Mutation Experiment, 24 hour treatment ten concentrations, ranging from 150 to 1892 µg/mL were tested in the absence of S-9. No precipitate was observed at the time of treatment or following the treatment incubation period. Two days after treatment all concentrations were selected to determine viability and TFT resistance. However, the highest two concentrations selected (1700 and 1892 µg/mL) were later rejected from analysis owing to extreme toxicity. The highest concentration analyzed was 1500 µg/mL, which gave 22% RTG, although 19% RTG was observed in cultures treated at 1350 µg/mL (see following table).

Table B.6.8.1-16: Mutation Experiment - 24 Hour Treatment in the Absence of S-9

Concentration µg/mL	%RTG	MF §
0	100	81.72
150	79	76.79
300	84	81.17
600	76	67.94
900	56	92.29
1050	44	62.55
1200	30	73.37
1350	19	108.46
1500	22	83.73
MMS 5	40	1160.03
MMS 7.5	42	1343.28

24 hour in the absence of S-9. Linear trend test on mutant frequency: p-value = 0.2728

MF § Mutant Frequency

%RTG Percent Relative Total Growth

The acceptance criteria were met and the study was accepted as valid.

When tested up to 10 mM for 3 hours in the absence and presence of S-9 and up to toxic concentrations for 24 hours in the absence of S-9, no increases in MF that exceeded the GEF of 126 mutants per 10^6 viable cells (compared to concurrent vehicle controls) were observed in any treated cultures under any treatment condition. A weakly significant linear trend ($p \leq 0.05$) was observed for the 3 hour treatment in the absence of S-9, but as the maximum increase in MF observed in any treated culture was approximately 30 (at 1200 µg/mL), which was well below the GEF of 126, the observation was considered not biologically relevant.

In addition, for the negative and positive controls, the number of wells containing small colonies and the number containing large colonies were scored. Thus the small and large colony MF could be estimated and the proportion of small mutant colonies could be calculated. For the vehicle controls, the proportion of small colony mutants in the absence and presence of S-9 ranged from 34% to 61%. Marked increases in the number of both small and large colony mutants were observed following treatment with the positive control chemicals MMS and B[a]P.

Conclusion

M-101 (2-(trifluoromethyl)-benzamide) did not induce mutation at the *tk* locus of L5178Y mouse lymphoma cells when tested at up to 10 mM for 3 h in the absence and presence of a rat liver metabolite activation system (S-9), and at up to toxic concentrations for 24 h in the absence of S-9.

In vitro genotoxicity studies – chromosome aberration assay, 2-(trifluoromethyl)-benzamide

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study. The study was performed just before the current OECD guideline (2016) was adopted. However, no deviations from the current guideline were noted. The study is considered acceptable.

reference	:	Lloyd, M (2016b)	vehicle	:	DMSO
Report number	:	8337788 (T-3246)	doses	:	750 to 1892 µg/mL
test substance	:	2-(trifluoromethyl)-benzamide	GLP	:	yes
Test system	:	Human lymphocytes	guideline	:	In accordance with OECD 473 (2014)
			acceptability	:	acceptabel

Executive summary

M-101 (2-(trifluoromethyl)-benzamide), 100% purity, was tested in an in vitro chromosome aberration assay using duplicate human lymphocyte cultures prepared from the pooled blood of three male donors in a single experiment. Treatments were performed both in the absence and presence of metabolic activation (S-9) from Aroclor 1254-induced rats. The test item was formulated in anhydrous dimethyl sulphoxide (DMSO). The concentrations tested ranged in small steps from 150 to 1892 µg/mL (equivalent to 10 mM), as determined by a preliminary cytotoxicity experiment.

Treatments were conducted 48 hours following mitogen stimulation by phytohaemagglutinin (PHA). The test item concentrations for chromosome analysis were selected by evaluating the effect of M-101 on mitotic index. Chromosome aberrations were analysed at three or four concentrations from 750 to 1892 µg/mL. Appropriate negative (vehicle) control cultures were included in the test system in the under each treatment condition. The proportion of cells with structural aberrations in these cultures fell within the 95th percentile of the current historical vehicle control (normal) ranges. Mitomycin C (MMC) and cyclophosphamide (CPA) were employed as positive control chemicals in the absence and presence of rat liver S-9 respectively. Cells receiving these were sampled 20 hours after the start of treatment, and both compounds induced statistically significant increases in the proportion of cells with structural aberrations. All acceptance criteria were considered met and the study was accepted as valid.

Treatment of cells with M-101 for 3+17 hours in the absence and presence of S-9, or for 20+0 hours in the absence of S-9, resulted in frequencies of aberrant cells that were similar to the concurrent vehicle controls at all concentrations analysed. The aberration frequencies (excluding gaps) were within the normal ranges in all treated cultures analyzed under the three treatment conditions. Small, sporadic increases in the frequency of cells with endoreduplication or polyploidy, marginally exceeding the concurrent controls and the normal ranges, were observed in a total of four individual cultures (including one vehicle control culture) across the three treatment conditions. However, these increases were small in magnitude and were not reproducible between replicate cultures, and therefore these isolated observations were considered not biologically relevant.

It was concluded that M-101 (2-(trifluoromethyl)-benzamide) did not induce structural chromosome aberrations in human peripheral blood lymphocytes when tested up to a concentration equivalent to 10 mM (limit dose) in the absence or presence of a metabolic activation system (S-9).

Materials and Methods

Test item:	2-(trifluoromethyl)-benzamide (M-101)
Batch No.:	336876
Purity:	100.0%
Expiry:	25 March 2017
Appearance:	Off-white crystalline powder
Storage:	Refrigerated, in the dark
Vehicle:	Anhydrous dimethyl sulphoxide (DMSO), analytical grade
Positive controls:	Mitomycin C (MMC), cyclophosphamide (CPA), from Sigma-Aldrich
Cells:	From blood from 3 healthy non-smoking males
Metabolic activation:	MolTox S-9 from Molecular Toxicology Inc., USA
Culture medium:	RPMI containing HEPES
Study start and completion:	17 February 2016 to 21 June 2016

M-101 was soluble in DMSO at up to (at least) 190.2 mg/mL. It was soluble in culture medium at in excess of 1902 µg/mL, as indicated by precipitation that persisted for 22 h at 37ºC. A maximum concentration of 1892 µg/mL was selected for the study, equivalent to 10 mM. Test item solutions were prepared with the aid of vortex mixing under subdued light, with serial dilution from the maximum required concentration. These solutions were used within 4 h of preparation, and no analyses for stability, homogeneity or achieved concentrations were undertaken. The positive controls were used at 0.05 to 0.4 µg/mL (MMC) and 1 to 2 µg/mL (CPA).

The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254. The batches of MolTox^{TM} S-9 were stored frozen in aliquots at <-50°C prior to use. Each batch was checked by the manufacturer for sterility, protein content, ability to convert known promutagens to bacterial mutagens and cytochrome P-450-catalyzed enzyme activities (alkoxyresorufin-O-dealkylase activities). The S-9 mix was prepared with G6P (180 mg/mL), NADP (25 mg/mL), KCl (150 mM) and rat liver S-9 mixed in the ratio 1:1:1:2. For all cultures treated in the presence of S-9, an aliquot of the mix was added to each cell culture to achieve the required final concentration of test article in a total of 10 mL. The final concentration of the liver homogenate in the test system was 2%. Cultures treated in the absence of S-9 received an equivalent volume of 150 mM KCl.

Blood from three healthy, non-smoking male volunteers from a panel of donors was used for each experiment. No donor was suspected of any virus infection or exposed to high levels of radiation or hazardous chemicals. All donors were non-smokers and not heavy consumers of alcohol, and none were taking any form of medication. The measured cell cycle time of the donors used fell within the range 13±2 hours. For each experiment, an appropriate volume of whole blood was drawn from the peripheral circulation into heparinized tubes on the day of culture initiation. Blood was stored refrigerated and was pooled using equal volumes from each donor prior to use. Whole blood cultures were established in sterile disposable centrifuge tubes by placing 0.4 mL of pooled heparinised blood into 9.0 mL pre-warmed (37±1°C) HEPES-buffered RPMI medium containing 10% (v/v) heat inactivated fetal calf serum and 0.52% penicillin / streptomycin, so that the final volume following

addition of S-9 mix or KCl and the test item in its chosen vehicle was 10mL. The mitogen phytohaemagglutinin (PHA, reagent grade) was included in the culture medium at a concentration of approximately 2% of culture to stimulate the lymphocytes to divide. Blood cultures were incubated at 37±1°C for approximately 48 hours and rocked continuously.

S-9 mix or KCl (0.5 mL per culture) was added as appropriate. Cultures were treated with the test item or vehicle control or positive control (0.1 mL per culture) and final culture volume was 10 mL. (Positive control treatments were not included for cytotoxicity range-finding). Cultures were incubated at 37±1°C for the designated exposure time. The scheme is shown in the table below.

Table B.6.8.1-17: Treatment scheme

		Number of Cultures			
Treatment	**S-9**	**Cytotoxicity Range-Finder**		**Chromosome Aberration Experiment**	
		3+17*	**20+0***	**3+17***	**20+0***
Vehicle control	- +	2 2	2	4 4	4
Test article	- +	1 1	1	2 2	2
Positive controls	- +			2 2	2
* Hours treatment + hours recovery					

For removal of the test article, cells were pelleted by centrifugation, washed twice with sterile saline (pre-warmed in an incubator set to 37±1°C), and resuspended in fresh pre-warmed medium containing fetal calf serum and penicillin/streptomycin. Osmolality and pH measurements on post-treatment incubation medium were taken in the cytotoxicity range-finder experiment.

Approximately 2 hours prior to harvest, colchicine was added to give a final concentration of approximately 1 µg/mL to arrest dividing cells in metaphase. At the defined sampling time cultures were centrifuged, the supernatant was carefully removed and cells were resuspended in 4 mL pre-warmed hypotonic (0.075 M) KCl and incubated at 37±1ºC to allow cell swelling to occur. Cells were fixed by dropping the KCl suspension into fresh, cold methanol/glacial acetic acid (3:1, v/v). The fixative was changed by centrifugation and resuspension, a procedure that was repeated as necessary until the cell pellets were clean.

Lymphocytes were kept in fixative for a minimum of 3 hours at 2-8ºC prior to slide preparation to ensure that cells were adequately fixed. Cells were centrifuged and resuspended in a minimal amount of fresh fixative (if required) to give a milky suspension. Several drops of 45% (v/v) aqueous acetic acid were added to each suspension to enhance chromosome spreading and several drops of suspension were transferred onto clean, labelled microscope slides and dried on a hot plate, then stained in filtered 4% (v/v) Giemsa at pH 6.8, then rinsed, dried and mounted.

Slides from the cytotoxicity experiment were examined and the Mitotic Index (MI) determined i.e. the percentage of cells in mitosis. Mitotic inhibition (MIH) was calculated as:

MIH (%) = [1 - (mean MIT/mean MIC)] x 100%

(where T = treatment and C = vehicle control)

Slides from sufficient concentrations from each treatment group were scored to determine whether chemically induced MIH had occurred. This was defined as a clear decrease in MI compared with

vehicle controls (based on at least 1000 cells counted, where possible), preferably concentration-related. A suitable range of concentrations was selected for the chromosome aberration experiment based on these toxicity data.

The highest concentration selected for chromosome aberration analysis following all treatment conditions was the highest concentration tested (1892 µg/mL) equivalent to 10 mM. Slides from this highest concentration and two or three lower concentrations were taken for microscopic analysis, such that a range of cytotoxicity was covered. For each treatment regime, two vehicle control cultures were analysed for chromosome aberrations. Slides from positive control treatments were checked to ensure that the system was operating satisfactorily. One concentration from each positive control, giving satisfactory responses in terms of quality and quantity of mitoses and extent of chromosomal damage, was analyzed.

A minimum of 300 metaphases per concentration were analyzed for chromosome aberrations. Only cells with 44 to 48 chromosomes were accepted for analysis. Any cell with more than 48 chromosomes (*ie* polyploid or endoreduplicated) observed during this evaluation was noted and recorded separately. Structural aberrations were classified according to the ISCN scheme (1995). Under this scheme, a gap is defined as a discontinuity less than the width of the chromatid with no evidence of displacement of the fragment, and a deletion is defined as a discontinuity greater than the width of the chromatid and/or evidence of displacement of the fragment.

After completion of scoring and decoding of slides the numbers of aberrant cells in each culture were categorised as follows:

1. Cells with structural aberrations including gaps
2. Cells with structural aberrations excluding gaps
3. Polyploid or endoreduplicated cells

The totals for category 2 in vehicle control cultures were compared with the 95th percentile of the current historical vehicle control (normal) ranges to determine whether the assay was acceptable or not. The proportions of cells with structural chromosome aberrations excluding gaps were compared with the proportion in vehicle controls by using Fisher's exact test. In addition, a Cochran-Armitage Trend Test was performed to aid determination of concentration-response relationships. Probability values of $p \leq 0.05$ were accepted as significant. The proportions of cells in categories 1 and 3 were examined in relation to the historical vehicle control range. The proportions of aberrant cells in each replicate were used to establish acceptable heterogeneity between replicates by means of a binomial dispersion test, probability values of $p \leq 0.05$ being accepted as significant.

Acceptance criteria:

The assay was considered valid if all the following criteria were met:

1. The binomial dispersion test demonstrated acceptable heterogeneity between replicate cultures;
2. The proportion of cells with structural aberrations (excluding gaps) in vehicle control cultures fell within the normal ranges;
3. At least 300cells were analysed at each concentration, unless 15 or more cells showing structural aberrations (per slide) other than gaps only were observed during analysis;

4. The positive control chemicals induced statistically significant increases in the proportion of cells with structural chromosome aberrations (excluding gaps). Both replicate cultures at the positive control concentration analysed under each treatment condition demonstrated structural aberration cell frequencies (excluding gaps) that clearly exceeded the normal vehicle control ranges;
5. The maximum concentration analysed under each treatment condition was the maximum concentration tested, a concentration inducing approximately 50% cytotoxicity or the lowest concentration at which precipitate was observed at the end of the treatment period.

Evaluation criteria:

The test item was considered to induce clastogenic events if:

1. A statistically significant increase in the proportion of cells with structural aberrations (excluding gaps) was observed (p≤0.05);
2. The incidence of cells with structural aberrations (excluding gaps) at such a concentration that exceeded the normal range in both replicate cultures;
3. A concentration-related increase in the proportion of cells with structural aberrations (excluding gaps) was observed (positive trend test).

The test item was considered positive in this assay if all of the above criteria were met. It was considered negative if none of the above criteria were met.

Results

Table B.6.8.1-18: Chromosome aberrations in human lymphocytes following 2-(trifluoromethyl)-benzamide treatment

Treatment	Concentration (µg/mL)	Cytotoxicity (%)[$]	% Cells with Aberrations (Excluding Gaps)	Historical control range (%)[#]	Statistical significance
3+17 hour - S9	Vehicle[a]	-	0.33	0.00 – 3.00	-
	1200	3	0.33		NS
	1500	4	0.00		NS
	1892	19	0.00		NS
	*MMC, 0.40	52	24.09		p≤0.001
3+17 hour +S9	Vehicle[a]	-	0.00	0.00 – 2.00	-
	1200	9	0.00		NS
	1500	0	0.33		NS
	1892	15	0.0		NS
	*CPA, 1.00	7	6.33		p≤0.001
20+0 hour - S9	Vehicle[a]	-	0.00	0.00 – 2.90	-
	750	9	0.67		NS
	1350	34	0.67		NS
	1700	47	0.00		NS
	1892	34	1.00		NS
	*MMC, 0.10	50	15.69		p≤0.001

a Vehicle control was DMSO

* Positive control

95th percentile of the observed range

$ Based on mitotic index

NS Not Significant

Treatment of cells with M-101 for 3+17 hours in the absence and presence of S-9 or for 20+0 hours in the absence of S-9 resulted in frequencies of aberrant cells that were similar to the concurrent vehicle controls at all concentrations analyzed. The aberration frequencies (excluding gaps) fell within the normal ranges in all treated cultures analyzed under the three treatment conditions.

Small, sporadic increases in the frequency of cells with endoreduplication or polyploidy, marginally exceeding the concurrent controls and the normal ranges, were observed in a total of four individual cultures (including one vehicle control culture) across the three treatment conditions. However, these increases were small in magnitude and were not reproducible between replicate cultures and therefore these isolated observations were considered not biologically relevant.

Conclusion

M-101 (2-(trifluoromethyl)-benzamide) did not induce structural chromosome aberrations in human peripheral blood lymphocytes when tested up to a concentration equivalent to 10 mM (limit dose) in the absence or presence of a metabolic activation system (S-9).

B.6.8.1.2 Studies with 2-(trifluoromethyl)-benzoic acid

Acute oral toxicity, 2-(trifluoromethyl)-benzoic acid

Previous evaluation	Newly submitted for the purpose of renewal
Evaluation of the RMS	The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study. The study is considered acceptable. Based on the study no classification is required for acute oral toxicity.

reference	:	[redacted] (2016)	exposure	:	via gavage
Report number	:	B-7984 (T-3244)	doses	:	2000 mg/kg bw
test substance	:	2-(trifluoromethyl)-benzoic acid	GLP	:	yes
species	:	Sprague Dawley rats [Crl:CD(SD)]	guideline	:	In accordance with OECD 423
group size	:	3 females/ dose	acceptability	:	Acceptable

Executive summary

The acute oral toxicity of M-102 (2-(trifluoromethyl)-benzoic acid) was investigated in groups of 3 female Sprague-Dawley rats, using a dosage of 2000 mg/kg body weight (limit dose) in two successive dosing steps, based on the acute toxic class method.

No mortality occurred and there were no abnormal clinical signs in any animal. Regular body weight gain was observed in all but one animal, which showed depressed gain on day 1 after dosing, but then gained weight thereafter. There were no gross pathological findings at necropsy after the 14-day post-dosing observation period.

It was concluded that the acute oral LD_{50} for M-102 (2-(trifluoromethyl)-benzoic acid) in rats was >2000 mg/kg body weight, and that the test item was therefore classified as 'Category 5, Unclassified' under the GHS CLP criteria.

Materials and Methods

Test item:	2-(trifluoromethyl)-benzoic acid (M-102)
Batch No.:	UQTVJ (Tokyo Chemical Industry Co., Ltd.)
Purity:	99.8%
Expiry:	08 July 2023
Appearance:	Not stated
Storage:	Refrigerated, in the dark
Vehicle:	1% w/v methylcellulose (MC) aqueous solution
Animals:	Sprague-Dawley female rats, Crl:CD strain [redacted]), 8 weeks of age, 202-207 g at dose administration
Study start and completion:	25 February 2016 to 26 May 2016

Just before use, a requisite amount of the test item was weighed and suspended in 1% MC solution at the specified concentration. As the expectation of toxicity for this test item was low, the initial dosage was set at 2000 mg/kg, with a dose volume of 10 mL/kg body weight. Each group of animals was constituted with 3 females, which were fasted overnight prior to dosing. The dose was administered once to each animal by gavage on day 0, then the animals were permitted feed again after the 4 h post-dosing clinical observation. The animals were observed frequently for the first 6 h after dosing (5, 15 and 30 min, then 1, 2, 4 and 6 h), then once daily for 14 days, for any clinical signs of reaction to treatment. Body weights were recorded on the day of administration (immediately before dosing) and on days 1, 3, 7 and 14 thereafter. After the final observation on day 14 the animals were euthanized and subjected to gross necropsy with macroscopic examination of the organs and tissues of the cranial, thoracic and abdominal cavities. No tissues were preserved.
Following administration of 2000 mg/kg to the first group of 3 females and subsequent observation of no mortality, a second group of 3 females received the same dosage.

Results

No mortality occurred after either the first or second dosing occasions, and no abnormal clinical signs were observed in any animal during the post-dosing observation period.
Animals in the first group showed regular body weight gain throughout. Among the 3 females in the second group, one showed depression of weight gain on day 1 after dosing, but showed regular weight gain thereafter.

Table B.6.8.1-19: Body weight of female rats treated with 2-(trifluoromethyl)-benzoic acid, first group at 2000 mg/kg

Animal	Day after administration	Gain

No.	0		1		3		7		14		0-14
1101	203		216		232		242		255		52
1102	202		221		231		237		253		51
1103	207		217		232		241		254		47
Mean	204		218		232		240		254		50
S.D.	3		3		1		3		1		3

Table B.6.8.1-20: Body weight of female rats treated with 2-(trifluoromethyl)-benzoic acid, second group at 2000 mg/kg

Animal No.	Day after administration										Gain 0-14
	0		1		3		7		14		
2101	203		214		230		235		249		46
2102	202		215		227		232		248		46
2103	202		205		227		231		241		39
Mean	202		211		228		233		246		44
S.D.	1		6		2		2		4		4

There were no abnormal macroscopic findings in any animal at necropsy.

Conclusion

The acute oral LD_{50} for M-102 (2-(trifluoromethyl)-benzoic acid) in rats was >2000 mg/kg body weight, and that the test item was therefore classified as 'Category 5, Unclassified' under the GHS CLP criteria.

Oral 28-day study, 2-(trifluoromethyl)-benzoic acid

Previous evaluation	Newly submitted for the purpose of renewal
Evaluation of the RMS	The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study. The study deviates slightly from OECD 407: - The weight of the prostate + seminal vesicles with coagulating glands was not recorded during gross necropsy. However, histopathological analysis was performed. - In addition to the requirements laid down in OECD 407 immunohistochemical examinations were also performed. The study is considered acceptable.

reference	:	[redacted] (2010)	exposure	:	via the diet
Report number	:	LSRC-T10-039A (T-3184)	doses	:	600, 3,000 and 15,000 ppm
test substance	:	2-(trifluoromethyl)-benzoic acid	GLP	:	Yes
species	:	Sprague Dawley (Crl:CD(SD))	guideline	:	In accordance with OECD 407
group size	:	10/ sex/ group	acceptability	:	acceptable

Executive summary

M-102 (2-(trifluoromethyl)-benzoic acid, 100% purity) was mixed into powdered basal diet at concentrations of 0 (control), 600, 3000 or 15000 ppm and administered to test groups consisting of 10 male and 10 female Sprague Dawley rats, for 28 or 29 days. Achieved mean test item intake was 51.6-53.7, 252-269 and 1316-1359 mg/kg/day for males and females at the respective diet

concentrations. During the treatment period, mortality and clinical signs were observed daily, and detailed clinical observations, body weights and food consumption were recorded weekly. Functional observations were conducted before treatment and in the 4th week of treatment for all animals. Ophthalmological examination was conducted before treatment for all animals and in the 4th week of treatment for all animals in the control and 15000 ppm dose groups. Urinalysis, haematology, blood chemistry, necropsy and organ weight measurements were conducted at the 4th week of treatment for all animals. Histopathological examination was performed for all animals in the control and 15000 ppm dose groups, while only organs with gross pathological findings were examined for the 600 and 3000 ppm groups.

Males receiving 15000 ppm showed increased liver weight. These males also showed an apparent increase of motor activity, but this was considered in context unlikely to be an effect of treatment. In blood chemistry, changes consistent with effects on the liver were noted in these animals, such as increase of ALT, Alb and A/G ratio and decrease of TG. Meanwhile, females at this dose level showed decreases of RBC, Hb and Ht in haematology, suggestive of mild anaemia. There was no evidence of treatment effect in either sex at the 600 or 3000 ppm dosages.

A NOAEL for 2-(trifluoromethyl)-benzoic acid (M-102) administered in the diet to rats for 28-29 days was 3000 ppm, equivalent to 252 or 269 mg/kg/day for males and females respectively.

Materials and Methods

Test item:	2-(trifluoromethyl)-benzoic acid (M-102)
Batch No.:	20090816
Purity:	100.0%
Expiry:	10 June 2013
Appearance:	Off-white crystals
Storage:	Refrigerated, in the dark
Diet:	MF powdered diet (Oriental Yeast Co., Ltd, Japan)
Animals:	Sprague-Dawley rats, Crl:CD strain ([redacted], 5 weeks of age, 148-164 g (males) and 126-146 g (females) at the start of administration
Study start and completion:	01 January 2010 to 07 July 2010

The rats were distributed into four groups, each of 10 males and 10 females, to receive diets containing test item at concentrations of 0 (control), 600, 3000 or 15000 ppm for 28 or 29 consecutive days (first day of administration = day 0). The dosages were selected on the basis of earlier studies, wherein after 7 days of administration to rats of the same strain at 15000 ppm there was no effect on general condition, body weight gain or food consumption in either sex, while the LD_{50} was ≥2000 mg/kg. Based on this, 15000 ppm was selected as the highest dose for the present study, equivalent to the limit dose of 1000 mg/kg/day.

Test diets were prepared by first grinding the test item in a mortar, adding some diet and grinding the mixture further, then adding this mixture to further diet in a mixing machine to produce the highest required concentration. Aliquots of this were diluted with further diet in the mixer to produce the lower

concentrations. The formulated diets were stored at room temperature in closed containers and used within 2 weeks of preparation. Samples were taken from each preparation, and those from the first preparation and representative samples from later in the study were analyzed for achieved concentration, homogeneity and stability.

The animals were observed daily for mortality and overt clinical signs. Detailed clinical observations were conducted once weekly:

Cage-side: Body position/posture, abnormal vocalization, tremor, convulsion, abnormal behavior, abnormal gait.

Hand-held: Ease of removal from cage, ease of handling, muscle tone, palpebral closure, lacrimation, exophthalmos, pupil diameter, salivation, secreta, piloerection, condition of fur, body temperature, abnormal respiratory sound, changes in the skin, and changes in the visible mucosa.

Open field: Rearing, abnormal vocalization, tremor, convulsion, spontaneous movement, abnormal behaviour, abnormal gait, grooming, breathing, defecation, urination.

Functional observations were conducted before the start of treatment and on day 27 or 28 of administration:

Sensory reactions: Visual response, touch response, auditory response, pain response, air-righting reflex, landing foot splay of hindlimb, grip strength (forelimb and hindlimb), and spontaneous locomotor activity (1 h in an automated system).

Body weights were recorded on days 0, 7, 14, 21 and 28 of administration, then terminally after fasting on day 29 or 30. Food consumption was determined for each week of administration, as g/rat/day.

Ophthalmology was conducted, including use of a slit lamp, for all animals at the start of administration and for the control and high dosage groups on day 28. As there were no treatment related effects, the lower dosage groups were not examined on day 28.

Urinalysis was conducted for all animals on day 28 or 29, examining pH, protein, glucose, ketones, bilirubin, urobilinogen, occult blood, specific gravity, leucocytes and sediment.

Just prior to necropsy the animals were anaesthetized and blood was collected from the posterior aorta for haematology and blood chemistry:

Haematology: White cell count, red cell count, haemoglobin, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count, differential white cell count, prothrombin time (PT), activated partial thromboplastin time (APTT) and reticulocyte count.

Blood chemistry: Total protein (TP), albumin (Alb), A/G ratio, glucose (Glu), total cholesterol (TC), triglyceride (TG), total bilirubin (T-Bil), urea nitrogen (UN), creatinine (CRE), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltranspeptidase (γ-GTP), alkaline phosphatase (ALP), sodium (Na), potassium (K), calcium (Ca), chlorides (Cl) and inorganic phosphorus (P).

Organ weights were recorded at necropsy for all animals:

Liver, kidneys, adrenal glands, thymus, spleen, heart, brain, thyroid including parathyroids (weighted post-fixation), testes, ovaries.

The following organs were preserved:

Skin, lymph nodes (cervical lymph node and mesenteric lymph node), aorta, salivary glands, bone and bone marrow (sternum and femur), thymus, trachea, lungs and bronchi, heart, thyroid glands (including parathyroid glands), oesophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, liver, pancreas, spleen, kidneys, adrenal glands, urinary bladder, brain (including medulla oblongata, cortex of cerebellum and cortex of cerebrum, except rhinencephalon), pituitary, sciatic nerves, skeletal muscles (femoral), spinal cord (cervical, thoracic and lumbar), eyeballs, harderian gland, testes, epididymides, seminal vesicle /coagulation gland, prostate, ovaries, uterus, vagina, mammary gland, and any macroscopic lesions at necropsy.

Fixation was in 10% neutral buffered formalin. Testes were first fixed in buffered formalin and acetic acid, and the eyeballs in Davidson's fluid, overnight before transfer to 10% neutral buffered formalin (NBF).

H&E stained specimens were prepared after paraffin embedding and sectioning. For histopathological examination, all organs were examined for the control group and the high dose group, while only the organs that showed histopathological abnormalities in the high dose group were examined for the middle and low dose groups. Pathological findings were classified by severity (–: no effects, ±: minimal, +: slight, 2+: moderate, and 3+: severe).

Statistical analyses: For the body weight, food consumption, organ weight (including relative weight), results of haematological and blood biochemistry examinations, grip strength, spontaneous movement and landing foot splay of hindlimbs, group mean with standard deviations were calculated. They were firstly evaluated by Bartlett's test for homogeneity of variance. When group variances were homogeneous ($p \leq 0.05$), Dunnett's multiple comparison test was conducted to determine which group was significantly different from the control. When group variances were heterogeneous ($p \leq 0.05$), Steel's multiple comparison test was conducted between each test item group and the control group. Data from the detailed clinical observations, the results of visual response, touch response, auditory response, pain response and air-righting reflex, and the urinalysis data that were obtained from survivors at the end of the administration period were evaluated by Steel's multiple comparison test between the control group and each test item group. However, the dichotomous response data in detailed clinical observations, clinical signs, the results of ophthalmological examination, gross pathological findings and histopathological findings were evaluated by Fisher's Exact Probability test. Significance of differences from control were recognized at $p \leq 0.05$.

Results

Test diets were formulated to within ±6.3% of nominal concentrations, with coefficients of variation (CV) for the means of 7 to 9.7%. Storage stability was also satisfactory, with residual concentrations of 107 and 109% at room temperature.

Overall mean achieved dosages of test item were: 51.6 mg/kg/day (male) and 53.7 mg/kg/day (females) at 600 ppm, 252 and 269 mg/kg/day at 3000 ppm, 1316 and 1359 mg/kg/day at 15000 ppm.

There was no mortality and no treatment related overt clinical signs. Among the more detailed clinical observations, males at 15000 ppm showed a significant decrease in defaecation in week 4 of dosing,

while females at 3000 ppm showed significantly increased grooming in week 1 of dosing. There were no significant changes at 600 ppm. See tables below.

In week 4 of administration, males in the 3000 and 15000 ppm groups and females in all treated groups showed significant increases in spontaneous locomotor activity in the first 10 minutes of measurement. In addition, males in the 15000 ppm group showed significant increases in the 10 minutes of the 5th period (40 to 50 minutes after the start of measurement) and in the total for 1 hour of measurement. Males in the 600 ppm group showed no significant increases or decreases in spontaneous locomotor activity.

Table B.6.8.1-21: Significant changes of spontaneous locomotor activity in male and female rats treated with 2-(trifluoromethyl)-benzoic acid, expressed as % of the control

Sex		**Males**			**Females**		
Dose level (ppm)		**600**	**3,000**	**15,000**	**600**	**3,000**	**15,000**
Spontaneous locomotor activity	0-10 min	114	↑**119**	↑↑**125**	↑**116**	↑↑**123**	↑↑**120**
	40-50 min	86	101	↑**348**	123	237	219
	Total	104	118	↑**142**	111	127	130

Results obtained in week 4 after the start of administration

↑: $p<0.05$, ↑↑: $p<0.01$ (Dunnett's test)

Analysis and interpretation of these locomotor activity data is complicated by the wide variation in individual animal activity within groups, resulting in high measures of central tendency. However, if total activity scores over the 1-hour test period are considered in terms of percentage change between pre-treatment and post-treatment for each animal, partitioning the substantial (perhaps more biologically meaningful) changes from the lesser changes (a threshold of approximately +100% appears appropriate for this set of results), it is then apparent that only 3 or 4 animals out of the 10 achieved or exceeded this threshold in any one treated group, as against 2 animals out of 10 in the control group. There were no associated meaningful changes in related open-field parameters (spontaneous movement, exploratory behaviour, rearing, grooming), and the apparent finding that males at 15000 ppm produced fewer faecal boluses than in the other groups is contrary to any possible interpretation that the increased motor activity might reflect increased 'anxiety', because anxiety typically increases faecal output in response to novel surroundings; and indeed the faecal boli did not correlate with activity levels. Overall, it was considered that the apparent changes in motor activity levels were unlikely to be associated with the test item.

For the sensory reactions (visual response, touch response, auditory response, pain response, air-righting reflex), landing foot spray of hindlimbs and grip strength (forelimbs and hindlimbs), there were no significant changes in males or females in any dose group.

There were no meaningful, dose related intergroup differences in body weight and food consumption during the study.

Table B.6.8.1-22: Body weight gain in male rats treated with 2-(trifluoromethyl)-bezoic acid

Test substance	**Dose (ppm)**		**Body weight gain (g/week)**
			Week

			1		2		3		4	
2-Trifluoromethylbenzoic acid	0	Mean	58.17		62.13		53.42		35.85	
		S.D.	7.37		12.30		7.85		4.47	
		N	10		10		10		10	
	600	Mean	57.16		60.43		49.94		33.63	
		S.D.	6.09		8.79		11.60		11.10	
		N	10		10		10		10	
	3,000	Mean	58.63		59.98		48.30		36.57	
		S.D.	4.96		6.72		11.09		8.51	
		N	10		10		10		10	
	15,000	Mean	57.27		63.51		43.53		27.17	
		S.D.	5.62		9.61		8.51		8.29	
		N	10		10		10		10	

Table B.6.8.1-23: body weight gain in female rats treated with 2-(trifluoromethyl)-benzoic acid

Test substance	Dose (ppm)		Body weight gain (g/week)							
			Week							
			1		2		3		4	
2-Trifluoromethylbenzoic acid	0	Mean	37.26		22.85		25.45		13.28	
		S.D.	2.93		3.96		4.50		4.00	
		N	10		10		10		10	
	600	Mean	34.04		19.05		21.42		14.98	
		S.D.	3.86		3.93		5.70		4.35	
		N	10		10		10		10	
	3000	Mean	35.62		24.11		26.84		12.79	
		S.D.	4.65		6.41		6.31		4.75	
		N	10		10		10		10	
	15000	Mean	36.74		21.69		25.90		14.50	
		S.D.	7.85		5.08		7.37		5.46	
		N	10		10		10		10	

There were no ophthalmological findings in the study.

There were no significant changes among the urinalysis parameters.

Among the haematology parameters, females at 15000 ppm showed significant decreases in red blood cell count, haemoglobin concentration and haematocrit, possibly indicating very mild normocytic normochromic anaemia, although there was no accompanying change in reticulocyte numbers. There were no meaningful changes at 600 or 3000 ppm.

Table B.6.8.1-24: Significant changes in haematology in male and female rats treated with 2-(trifluoromethyl)-benzoic acid, expressed as % of control

Sex	Males			Females		
Dose level (ppm)	**600**	**3,000**	**15,000**	**600**	**3,000**	**15,000**
Red blood cell count	101	99	101	98	97	↓ **94**
Hemoglobin concentration	101	100	101	99	97	↓ **95**
Hematocrit	101	100	101	97	96	↓ **94**

↓: p<0.01 (Dunnett's test)

In blood chemistry, significant increases in alanine aminotransferase (ALT), albumin (Alb) and albumin/globulin ratio (A/G) and a significant decrease in triglyceride (TG) were noted for 15000 ppm males. These changes were consistent with effects on the liver, although there were no histopathological correlates (see below). There were no significant changes at 600 or 3000 ppm. In females, significant decreases in urea nitrogen (UN) and total bilirubin (T.Bil) were noted at 15000 ppm, and a significant decrease in T.Bil also at 3000 ppm. These changes were not associated with evidence of severe liver effect, and in view also of their small magnitude they were considered of no toxicological significance. In 600 ppm females only a significant increase in chloride (Cl) was recorded. The increase of chloride observed in females treated 600 ppm 2-(trifluoromethyl)-benzoic acid is not considered to be treatment related since a dose-response effect is lacking.

Table B.6.8.1-25: Significant changes in blood chemistry in male and female rats treated with 2-(trifluoromethyl)-benzoic acid, expressed as % of control

Sex	Males			Females		
Dose level (ppm)	**600**	**3,000**	**15,000**	**600**	**3,000**	**15,000**
ALT	97	97	**135*** **	104	100	96
TG	95	81	**65** **	95	73	98
Alb	100	103	**106***	97	100	100
A/G	100	100	**117*** **	100	92	100
UN	105	106	103	93	95	**86** **
T. Bil	83	83	83	86	**71***	**71***
Cl	100	100	101	**101***	101	100

*: p<0.05, **: p<0.01, ***: p<0.001 (Dunnett's test)

At necropsy there were no treatment related macroscopic findings. However, organ weight analysis confirmed significant increase in liver weight at 15000 and 3000 ppm. Considering that in animals treated with 3000 ppm flutolanil the relative liver weight was marginal (circa 10%) in the abence of any histopathological or clinical effects and considering that a increased liver weight was only observed inmales, this effect was not considered adverse. A very small increase in kidney weight was also recorded in males at 15000 ppm, with no histopathological correlate. There were no other meaningful intergroup differences among organ weights. A significant increase of the relative weight of the spleen was observed in males. However, a significant difference in the absolute spleen weight was not observed and the effect observed in relative spleen weight was not found to be dose related. Therefore this effect is not considered to be treatment related.

Table B.6.8.1-26: Significant organ weight changes in male and female rats treated with 2-(trifluoromethyl) benzoic acid, expressed as % control

Sex		Males			Females		
Dose level (ppm)		**600**	**3,000**	**15,000**	**600**	**3,000**	**15,000**
Liver	(absolute weight)	104	110	**134*** **	96	104	**122** **
	(relative weight)	106	**112** **	**141*** **	100	103	**122*** **
Spleen	(relative weight)	**113***	**117** **	109	98	104	102
Kidney	(relative weight)	103	108	**113*** **	101	95	105

↑: *: p < 0.05 (Dunnett's test), ↑: **: p < 0.01, ***: p<0.001 (Dennett's Dunnett's or Steel's test)

There were no meaningful changes in the incidence of findings in the histopathological examinations. There were only isolated incidental findings, with no dosage relationship.

Conclusions

Administration of 15000 ppm M-102 to rats in the diet for at least 28 days produced signs of effects on the liver, and also of a mild anaemia in females. There were no significant/meaningful effects at ≥3000 ppm.

A NOAEL for 2-(trifluoromethyl)-benzoic acid (M-102) administered in the diet to rats for 28-29 days was 3000 ppm, equivalent to 252 or 269 mg/kg/day for males and females respectively.

In vitro genotoxicity studies - Ames test, 2-(trifluoromethyl)-benzoic acid

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study. The study is considered acceptable.

reference	:	Lloyd, M (2016c)	vehicle	:	DMSO
Report number	:	8337790 (T-3245)	doses	:	up to 5000 µg/plate
test substance	:	2-(trifluoromethyl)-benzoic acid	GLP	:	yes
Test system	:	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA102	guideline	:	In accordance with OECD 471 (1997)
			acceptability	:	acceptable

Executive summary

The metabolite M-102 (2-(trifluoromethyl)-benzoic acid), 99.3% purity, was tested in a reverse mutation test, with and without metabolic activation, in *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, TA1537 and TA1538. S9 fraction was used as the metabolic activation system (rat liver, induced with Aroclor 1254). Dose levels were 80, 160, 300, 625, 1250, 2500 and 5000 µg/plate, the vehicle being DMSO. Appropriate vehicle controls and positive controls were included.

There was no significant increase in revertants in any of the bacterial strains, either with or without the S9 metabolic activation system, at any dosage of the test item, while the positive controls each showed the expected mutagenic activity.

It was concluded that M-102 (2-(trifluoromethyl)-benzoic acid) was non-mutagenic in the bacterial test systems at up to 5000 µg/plate, under the conditions of the study.

Materials and methods

Test item:	2-(trifluoromethyl)-benzoic acid (M-102)
Batch No.:	2AE4201P
Purity:	99.3%
Appearance:	Off-white powder
Expiry:	19 April 2020
Bacteria:	*Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, TA1537, TA1538

Metabolic activation:	Sprague-Dawley rat liver S-9 fraction, induced with Aroclor 1254 (MolTox™, from Molecular Toxicology Inc., USA)
Vehicle:	Dimethylsulphoxide (DMSO)
Positive controls:	AAN: 2-aminoanthracene (5 µg/plate for TA100, TA1535, TA1537, with S9; 20 µg/plate for TA102 with S9) 2NF: 2-nitrofluorene (5 µg/plate for TA98, minus S9) NaN_3: Sodium azide (2 µg/plate for TA100, TA1535, minus S9) AAC: 9-aminoacridine (50 µg/plate for TA1537, minus S9) MMC: Mitomycin C (0.2 µg/plate for TA102, minus S9) B[a]P: Benzo[a]pyrene (10 µg/plate for TA98, with S9)
Study start and completion:	17 February 2016 to 14 June 2016

Preliminary solubility data indicated that M-102 was soluble in dimethyl sulphoxide (DMSO) at a concentration in excess of 100 mg/mL. Test item stock solutions were prepared by formulating M-102 under subdued lighting in DMSO with the aid of vortex mixing, to give the maximum required concentration. Subsequent dilutions were made using DMSO. The solutions were protected from light and used within approximately 4.5 hours of initial formulation. Experiment 1 used a dosage range of 5 to 5000 µg/plate, with 0.1 mL additions of test item solution. Experiment 2 used 80, 160, 300, 625, 1250, 2500 and 5000 µg/plate, with 0.1 mL of test item solution for plate-incorporation (without S9), or 0.05 mL for pre-incubation treatments (with S9). No analyses for achieved concentration of test item were conducted. Control treatments used the same addition volumes per plate, vehicle controls comprising DMSO.

The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254. The batches of MolTox™ S-9 were stored frozen at <-50°C, and thawed just prior to use. Each batch was checked by the manufacturer for sterility, protein content, ability to convert ethidium bromide and cyclophosphamide to bacterial mutagens and cytochrome P-450-catalysed enzyme activities (alkoxyresorufin-O-dealkylase activities). Inocula were taken from frozen cultures that had been checked for strain characteristics (histidine dependence, *rfa* character, *uvrB* character and resistance to ampicillin or ampicillin plus tetracycline).

Treatments were carried out both in the absence and presence of S-9 by addition of either buffer solution or 10% S-9 mix respectively.

L-histidine HCl (20 µg) in 250 mM $MgCl_2$ and D-biotin (24.4 µg) were added at the time of plating, by supplementing the S-9 mix or buffer solution for plate incorporation treatments or the molten agar for pre-incubation treatments.

M-102 was tested for mutation (and toxicity) in five strains of *S. typhimurium* (TA98, TA100, TA1535, TA1537 and TA102) in two separate experiments, at the concentrations detailed previously, using triplicate plates without and with S-9. Vehicle controls were included in quintuplicate and positive controls were included in triplicate in both assays without and with S-9. These platings were achieved by the following sequence of additions to molten agar at 46±1°C:

0.1 mL bacterial culture
0.1 mL test item solution or control
0.5 mL 10% S-9 mix or buffer solution

This was followed by pouring onto Vogel-Bonner E agar plates. When set, these were inverted and incubated at 37±1ºC, protected from light, for 2 or 3 days. The plates were then examined for evidence of toxicity to the background lawn, and where possible revertant colonies were counted (either automatically with a colony counter, or manually if necessary).
As the results of Experiment 1 were negative, treatments in the presence of S-9 in Experiment 2 included a pre-incubation step. Quantities of test item or control solution, bacteria and S-9 mix detailed above, were mixed together and incubated for 20 min at 37±1°C, with shaking, before the addition of 2.5 mL molten agar at 46±1°C. Plating of these treatments then proceeded as for the normal plate-incorporation procedure. Volume additions for the Experiment 2 pre-incubation treatments were reduced to 0.05 mL, considering that the vehicle (DMSO) is known to be close to toxic levels at 0.1 mL in this system.
Assay validity acceptance criteria:

The vehicle control counts fell within the laboratory's historical control ranges;

The positive control chemicals induced increases in revertant numbers of ≥1.5-fold (in strain TA102), ≥2-fold (in strains TA98 and TA100) or ≥3-fold (in strains TA1535 and TA1537) the concurrent vehicle control, confirming discrimination between different strains and an active S-9 preparation.

Evaluation criteria for a positive result:

A concentration related increase in revertant numbers was ≥1.5-fold (in strain TA102), ≥2-fold (in strains TA98 or TA100) or ≥3-fold (in strains TA1535 or TA1537) the concurrent vehicle control values;
And the positive trend/effects were reproducible.

Results

In Experiment 1, evidence of toxicity in the form of slight thinning of the background bacterial lawn was observed at 5000 µg/plate for strains TA98, TA100, TA1535, TA1537 in the absence and presence of S-9, and at 1600 µg/plate and above for strain TA102 in the absence and presence of S-9.
In Experiment 2, evidence of toxicity in the form of slight thinning of the background bacterial lawn and/or a reduction in revertant numbers to ≤0.5-fold the concurrent vehicle control value, was observed at 2500 µg/plate and above for strains TA98, TA100 and TA1537 in the absence of S-9 and strain TA102 in the absence and presence of S-9, and at 5000 µg/plate for strain TA1535 in the absence of S-9 and for strain TA98 in the presence of S-9.
The test item was completely soluble in the aqueous assay system at all concentrations treated, in each of the experiments.

Mean vehicle control counts fell within the laboratory's historical ranges, while the positive control chemicals all induced increases in revertant numbers of≥1.5-fold (in strain TA102), ≥2-fold (in strains TA98 and TA100) or ≥3-fold (in strains TA1535 and TA1537) the concurrent vehicle controls, confirming discrimination between different strains, and an active S-9 preparation. The study therefore demonstrated correct strain and assay functioning and was accepted as valid.

Following M-102 treatments of all the test strains in the absence and presence of S-9, no increases in revertant numbers were observed that were ≥1.5-fold (in strain TA102), ≥2-fold (in strains TA98 and TA100) or ≥3-fold (in strains TA1535 and TA1537) the concurrent vehicle control. This study was considered therefore to have provided no evidence of any M-102 mutagenic activity in the assay system.

Table B.6.8.1-27: Mutagenicity data obtained in experiment 1, in the absence of S9

Strain	Compound	Conc. Level (µg/plate)	Mean	SD	Fold Increase
TA98	DMSO	-	23.8	3.9	-
	M-102	5	28.0	2.6	1.2
		16	30.0	7.8	1.3
		50	27.0	5.0	1.1
		160	25.0	6.0	1.1
		500	27.7	4.0	1.2
		1600	29.0	3.0	1.2
		5000	23.7	10.6	1.0
	2NF	5	1020.7	64.0	42.9
TA100	DMSO	-	136.0	10.2	-
	M-102	5	128.3	7.6	0.9
		16	124.7	7.5	0.9
		50	126.0	0.0	0.9
		160	131.0	8.5	1.0
		500	127.3	7.5	0.9
		1600	111.7	1.5	0.8
		5000	100.0	10.4	0.7
	NaN_3	2	1610.0	76.5	11.8
TA1535	DMSO	-	26.0	6.6	-
	M-102	5	30.0	0.0	1.2
		16	31.3	10.8	1.2
		50	21.7	6.8	0.8
		160	27.7	7.5	1.1
		500	24.3	9.7	0.9
		1600	17.7	8.1	0.7
		5000	17.3	6.1	0.7
	NaN_3	2	1551.7	103.0	59.7
TA1537	DMSO	-	15.4	5.3	-
	M-102	5	19.0	6.6	1.2
		16	11.7	3.1	0.8
		50	10.0	5.6	0.6
		160	16.7	2.1	1.1
		500	14.0	2.0	0.9
		1600	11.3	1.2	0.7
		5000	8.7	4.7	0.6
	AAC	50	711.7	100.7	46.2
TA102	DMSO	-	282.8	24.0	-
	M-102	5	282.0	11.5	1.0
		16	262.0	15.9	0.9
		50	283.3	9.1	1.0

		160	268.3	6.5	0.9
		500	254.0	12.1	0.9
		1600	238.3	6.8	0.8
		5000	201.7	17.6	0.7
	MMC	0.2	697.7	12.1	2.5

Table B.6.8.1-28: Mutagenicity data obtained in experiment 1, in the presence of S9

Strain	Compound	Conc. Level (µg/plate)	Mean	SD	Fold Increase
TA98	DMSO	-	35.8	3.3	-
	M-102	5	44.0	7.0	1.2
		16	45.7	8.1	1.3
		50	48.0	7.5	1.3
		160	33.7	2.5	0.9
		500	34.3	6.8	1.0
		1600	40.3	4.7	1.1
		5000	31.7	4.6	0.9
	B[a]P	10	648.3	240.6	18.1
TA100	DMSO	-	141.2	19.0	-
	M-102	5	156.3	7.6	1.1
		16	163.0	10.4	1.2
		50	160.7	5.1	1.1
		160	139.0	12.1	1.0
		500	142.7	3.1	1.0
		1600	144.7	11.9	1.0
		5000	141.3	12.6	1.0
	AAN	5	2336.3	39.5	16.5
TA1535	DMSO	-	19.8	5.8	-
	M-102	5	11.7	6.8	0.6
		16	20.3	4.5	1.0
		50	19.7	5.5	1.0
		160	18.0	3.6	0.9
		500	16.3	5.5	0.8
		1600	18.3	8.1	0.9
		5000	15.7	8.1	0.8
	AAN	5	307.3	13.6	15.5
TA1537	DMSO	-	24.4	2.9	-
	M-102	5	21.0	4.6	0.9
		16	27.7	9.5	1.1
		50	23.7	2.5	1.0
		160	26.0	1.0	1.1
		500	26.3	4.5	1.1
		1600	26.0	1.0	1.1
		5000	20.7	5.0	0.8
	AAN	5	277.3	64.0	11.4
TA102	DMSO	-	263.4	25.1	-
	M-102	5	290.0	16.7	1.1
		16	284.7	14.5	1.1
		50	279.0	13.2	1.1
		160	291.0	9.6	1.1
		500	267.7	17.9	1.0
		1600	220.7	26.1	0.8
		5000	160.0	5.3	0.6
	AAN	20	1586.7	67.2	6.0

Table B.6.8.1-29: Mutagenicity data obtained in experiment 2, in the absence of S9

Strain	Compound	Conc. Level	Mean	SD	Fold Increase

		(µg/plate)			
TA98	DMSO	-	19.2	8.3	-
	M-102	80	16.7	4.9	0.9
		160	13.0	2.6	0.7
		300	21.7	3.2	1.1
		625	16.3	3.5	0.9
		1250	12.7	3.2	0.7
		2500	16.3	3.5	0.9
		5000	7.7	2.5	0.4
	2NF	5	1199.7	82.1	62.5
TA100	DMSO	-	96.6	18.2	-
	M-102	80	107.0	7.0	1.1
		160	109.7	13.2	1.1
		300	92.3	12.6	1.0
		625	99.3	17.9	1.0
		1250	102.3	8.7	1.1
		2500	60.0	36.4	0.6
		5000	79.3	15.0	0.8
	NaN_3	2	668.0	24.2	6.9
TA1535	DMSO	-	14.0	5.2	-
	M-102	80	10.0	1.0	0.7
		160	8.7	4.0	0.6
		300	17.7	2.3	1.3
		625	11.7	2.1	0.8
		1250	13.3	2.1	1.0
		2500	11.0	4.4	0.8
		5000	4.3	3.5	0.3
	NaN_3	2	529.7	13.5	37.8
TA1537	DMSO	-	10.2	3.6	-
	M-102	80	7.3	3.1	0.7
		160	10.0	1.0	1.0
		300	9.0	2.6	0.9
		625	12.0	3.6	1.2
		1250	7.0	2.6	0.7
		2500	4.7	1.5	0.5
		5000	1.7	1.2	0.2
	AAC	50	200.7	32.5	19.7
TA102	DMSO	-	255.2	12.3	-
	M-102	80	229.3	25.3	0.9
		160	257.0	21.5	1.0
		300	241.3	17.5	0.9
		625	226.3	12.9	0.9
		1250	232.3	19.3	0.9
		2500	133.0	17.7	0.5
		5000	106.3	24.5	0.4
	MMC	0.2	873.3	23.1	3.4

Table B.6.8.1-30: Mutagenicity data obtained in experiment 2, in the presence of S9

Strain	Compound	Conc. Level (µg/plate)	Mean	SD	Fold Increase
TA98	DMSO	-	26.2	4.4	-
	M-102	80	23.3	5.5	0.9
		160	29.7	8.1	1.1
		300	32.3	2.3	1.2
		625	22.7	9.9	0.9
		1250	28.0	5.2	1.1
		2500	22.3	3.1	0.9
		5000	13.3	2.5	0.5

	B[a]P	10	469.3	34.1	17.9
TA100	DMSO	-	138.6	12.1	-
	M-102	80	130.7	15.3	0.9
		160	140.7	8.1	1.0
		300	118.0	6.1	0.9
		625	138.0	15.0	1.0
		1250	127.7	12.7	0.9
		2500	121.7	11.5	0.9
		5000	115.0	14.5	0.8
	AAN	5	1037.7	142.0	7.5
TA1535	DMSO	-	16.4	5.3	-
	M-102	80	17.3	1.2	1.1
		160	19.3	4.0	1.2
		300	15.7	2.1	1.0
		625	20.3	6.4	1.2
		1250	18.3	4.9	1.1
		2500	18.7	2.1	1.1
		5000	14.0	5.6	0.9
	AAN	5	228.0	91.9	13.9
TA1537	DMSO	-	14.8	2.9	-
	M-102	80	20.3	8.1	1.4
		160	16.0	3.6	1.1
		300	16.0	4.4	1.1
		625	11.0	4.4	0.7
		1250	12.3	5.9	0.8
		2500	15.3	6.4	1.0
		5000	10.3	2.5	0.7
	AAN	5	233.3	45.6	15.8
TA102	DMSO	-	313.0	22.3	-
	M-102	80	291.0	31.4	0.9
		160	314.7	28.0	1.0
		300	316.0	46.5	1.0
		625	297.7	5.1	1.0
		1250	291.0	31.2	0.9
		2500	218.3	42.8	0.7
		5000	143.7	31.2	0.5
	AAN	20	1694.0	217.0	5.4

Conclusion

M-102 did not induce mutation in five histidine-requiring strains (TA98, TA100, TA1535, TA1537 and TA102) of *Salmonella typhimurium* in the absence or presence of a rat liver metabolic activation system (S-9) at concentrations up to 5000 µg/plate. It was therefore non-mutagenic under the conditions of the study.

In vitro genotoxicity studies – mammalian cell gene mutation test, 2-(trifluoromethyl)-benzoic acid, study 1

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study. The study was performed just before the current OECD guideline (2016) was adopted. However, no deviations from the current guideline were noted. The study is considered acceptable.

reference	:	Lloyd, M (2016d)	vehicle	:	DMSO
Report number	:	8337792 (T-3248)	doses	:	150 to 1902 µg/mL
test substance	:	2-(trifluoromethyl)-benzoic acid	GLP	:	yes
Test system	:	Mouse lymphoma L5178Y cells	guideline	:	In accordance with OECD 490 (2015)
			acceptability	:	acceptable

Executive summary

M-102 (2-(trifluoromethyl)-benzoic acid), 99.3% purity, was assayed for the ability to induce mutation at the *tk* locus (5-trifluorothymidine [TFT] resistance) in mouse lymphoma cells using a fluctuation protocol. The study consisted of a cytotoxicity experiment followed by one mutation experiment, each conducted in the absence and presence of metabolic activation by an Aroclor 1254-induced rat liver post-mitochondrial fraction (S-9). The test item was formulated in dimethyl sulphoxide (DMSO). A 3 h treatment incubation period was used in the presence of S-9, while 3 and 24 h periods were applied in the absence of S-9.

In the cytotoxicity experiment with 3 h treatment, 6 concentrations from 59.44 to 1902 µg/mL (up to 10 mM equivalent) were tested. The highest concentration gave 87% and 92% relative total growth (RTG) in the absence and presence of S-9 respectively. With 24 h treatment, 9 concentrations were tested in the absence of S-9 only, from 7.43 to 1902 µg/mL, where the highest concentration to provide >10% RTG was 951 µg/mL, giving 37% RTG.

In the mutation experiment with 3 h treatment, 7 concentrations from 150 to 1902 µg/mL were tested. Two days after treatment, the highest concentration analyzed for viability and TFT resistance was 1902 µg/mL, which gave 65% and 47% RTG in the absence and presence of S-9 respectively. With 24 h treatment, 10 concentrations from 150 to 1902 µg/mL were tested in the absence of S-9 only, and two days after treatment the highest concentration analyzed for viability and TFT resistance was 1200 µg/mL, which gave 13% RTG. Vehicle and positive controls were included, and mutant frequencies (MF) fell within acceptable ranges: clear increases occurred with methyl methane sulphonate (MMS, without S-9) and benzo[a]pyrene (Bp, with S-9), demonstrating validity of the study.

When tested at up to 10 mM for 3 h in the absence and presence of S-9, no increases in MF exceeding the Global Evaluation Factor (GEF) of 126 mutants per 10^6 viable cells (compared to concurrent vehicle controls) were observed in any treated cultures under either treatment condition. A weakly significant linear trend ($p \leq 0.05$) was observed for the 3 h treatment in the absence of S-9, but the maximum increase in MF observed in any treated culture was 34, well below the GEF of 126 and observed at only one concentration (900 µg/mL), and therefore the linear trend was considered not biologically relevant.

When tested up to toxic concentrations for 24 h in the absence of S-9, increases in MF of approximately 175 and 246 mutants per 10^6 viable cells (compared to concurrent controls), exceeding the GEF of 126, were observed at the highest two concentrations analyzed (1050 and 1200 µg/mL, respectively) and there was a highly significant linear trend ($p \leq 0.001$). These increases were observed at concentrations giving 18% and 13% RTG at 1050 and 1200 µg/mL, respectively, and were therefore close to the upper limit of cytotoxicity.

It was concluded that M-102 induced mutation at the *tk* locus of L5178Y mouse lymphoma cells when tested up to toxic concentrations for 24 h in the absence of a rat liver metabolic activation system (S-9). Further, a preponderance of small colonies in the increases suggested a clastogenic effect,

rather than gene mutation. In the same test system, M-102 did not induce mutation when tested up to a maximum concentration equivalent to 10 mM for 3 h in the absence or presence of S-9.

Materials and Methods

Test item:	M-102 (2-(trifluoromethyl)-benzoic acid, or α,α,α-trifluoro-*o*-toluic acid)
Batch No.:	2AE4201P
Purity:	99.3%
Description:	Off-white powder
Expiry:	19 April 2020
Storage:	Refrigerated, in the dark
Vehicle:	Dimethyl sulphoxide (DMSO), anhydrous, analytical grade
Positive controls:	Methyl methane sulphonate (MMS), Benzo[a]pyrene (BP), from Sigma-Aldrich
Cells:	L5178Y *tk*+/- (3.7.2C) mouse lymphoma cells, from Burroughs Wellcome Co.
Metabolic activation:	MolTox S-9 from Molecular Toxicology Inc., USA
Culture medium:	RPMI 1640 containing L-glutamine and HEPES
Study start and completion:	17 February 2016 to 12 August 2016

M-102 was soluble in DMSO at up to (at least) 191.31 mg/mL. It was soluble in culture medium at approximately 956.6 to 1913 µg/mL, as indicated by precipitation that persisted for 22 h at 37ºC. A maximum concentration of 1902 µg/mL was selected for the study, equivalent to 10 mM. Test item solutions were prepared with the aid of vortex mixing under subdued light, with serial dilution from the maximum required concentration. These solutions were used within 4 h of preparation, and no analyses for stability, homogeneity or achieved concentrations were undertaken. Negative (vehicle) controls comprised treatments with DMSO diluted 100-fold in the medium. The positive controls were used at 5 to 20 µg/mL (MMS) and 2 to 3 µg/mL (BP).

The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254. The batches of MolTox^{TM} S-9 were stored frozen in aliquots at <-50°C prior to use. Each batch was checked by the manufacturer for sterility, protein content, ability to convert known promutagens to bacterial mutagens and cytochrome P-450-catalyzed enzyme activities (alkoxyresorufin-O-dealkylase activities). The final concentration of the liver homogenate in the test system was 2%.

Cytotoxicity experiment:

Treatment of cell cultures for the cytotoxicity Range-Finder Experiment was as described below for the Mutation Experiment. The final treatment culture volume was 20 mL. In the absence of S-9, 3 and 24 h treatment incubation periods were used, while in the presence of S-9 a 3 h treatment incubation was used.

Changes in osmolality of more than 50 mOsm/kg and fluctuations in pH of more than one unit may be responsible for an increase in mutant frequencies and therefore, osmolality and pH measurements on post-treatment incubation medium were taken in the cytotoxicity experiment.

Mutation experiment:

For 3 hour treatments in the absence and presence of S-9, at least 10^7 cells in a volume of 18.8 mL tissue culture medium (cells in RPMI 10 diluted with RPMI A [no serum] to give a final concentration of 5% serum) were used. For 24 hour treatment in the absence of S-9 at least 4 x 10^6 cells in a volume of 19.8 mL RPMI 10 were used. The 24 hour treatment tissue culture flasks were gassed with 5±1% v/v CO_2 in air. For all treatments 0.2 mL vehicle, test article or positive control solution was added. For 3 hour treatments, S-9 mix or 150 mM KCl was added, as described. Each treatment, in the absence or presence of S-9, was in duplicate (single cultures only used for positive control treatments) and the final treatment culture volume was 20 mL.

After 3 h incubation at 37±1°C with gentle agitation, cultures were centrifuged, washed with the appropriate tissue culture medium, centrifuged again and resuspended in 50 mL RPMI 10 medium. After static incubation at 37±1°C for 24 h, cultures were centrifuged, washed with the appropriate tissue culture medium, centrifuged again and resuspended in fresh RPMI 10 medium (20 mL). Cell densities were determined using a Coulter counter and adjusted to 2 x 10^5 cells/mL. The solubility of the test article in culture was assessed by eye at the beginning and end of treatment.

Cultures were maintained in flasks for a period of 2 days during which the *tk*$^{-/-}$ mutation would be expressed. During the expression period, subculturing was performed as required to retain an appropriate number of cells/flask. From observations on recovery and growth of the cultures during the expression period, the following cultures were selected to be plated for viability and TFT resistance as shown in the following table.

Table B.6.8.1-31: Concentrations of 2-(trifluoromethyl)-benzoic acid used during the mutation experiment

Mutation Experiment		
3 hour (µg/mL)		**24 hour (µg/mL)**
- S-9	+ S-9	- S-9
0 150 300 600 900 1200 1500 1902	0 150 300 600 900 1200 1500 1902	0 150 300 600 900 1050 1200 1350 1500 1700 1902
MMS 15 MMS 20	B[a]P 2 B[a]P 3	MMS 5 MMS 7.5

Plating for viability: At the end of the expression period the cell densities in the selected cultures were determined using a Cell counter or haemocytometer and adjusted to approximately 8 cells/mL. For each concentration, 0.2 mL were plated into each well of a 96 well microtitre plate for determination of

viability (cloning efficiency). All 96 well plates were incubated at 37±1°C in a humidified incubator gassed with 5±1% (v/v) CO_2 in air until scoreable (6 to 7 days). Wells containing viable clones were identified by eye using background illumination and counted.

Plating for mutation: At the end of the expression period, cell concentrations in the selected cultures were determined using a Coulter counter and adjusted to give 1 x 10^4 cells/mL in readiness for plating for TFT resistance. Samples from these were diluted to 8 cells/mL, then 0.2 mL of the final concentration of each culture was placed into each well of 2 x 96-well microtitre plates (192 wells averaging 1.6 cells/well). The plates were incubated at 37±1ºC in a humidified incubator gassed with 5±1% v/v CO_2 in air until scoreable (8 days). Wells containing viable clones were identified by eye using background illumination and counted.

Plating for TFT resistance: At the end of the expression period, the cell densities in the selected cultures were adjusted to 1 x 10^4 cells/mL. TFT (300 µg/mL) was diluted 100-fold into these suspensions to give a final concentration of 3 µg/mL. Using an eight-channel pipette, 0.2 mL of each suspension was placed into each well of four 96-well microtitre plates (384 wells at 2 x 10^3 cells/well). Plates were incubated at 37±1°C in a humidified incubator gassed with 5±1% v/v CO_2 in air until scoreable (13 days) and wells containing clones were identified as above and counted. In addition, the number of wells containing large colonies and the number containing small colonies were scored for the negative and positive controls and for concentrations of test article where a marked increase in mutant frequency (exceeding the GEF) was observed.

Data analysis:

Suspension Growth (SG), a measure of the growth in suspension during treatment and the expression period:

$$SG = a \times b \times c$$

$$\text{where } a = \left(\frac{D_0 \text{ post-treatment cell count}}{\text{Pre-treatment cell density}} \right)$$

$$\text{where } b = \left(\frac{D_1 \text{ cell count}}{\text{Cell count set up on } D_0 \text{ post-treatment}} \right)$$

$$\text{where } c = \left(\frac{D_2 \text{ cell count}}{\text{Cell count set up on } D_1} \right)$$

For 3 h treatments, ‘a’ is taken as 1; D_0, D_1, D_2 are Days 0, 1 and 2

Relative Suspension Growth (RSG), a measure of the growth in suspension during treatment and the expression period relative to the mean control:

$$RSG\ (\%) = \left(\frac{\text{Individual SG value}}{\text{Mean control SG value}} \right) \times 100$$

Cloning Efficiency (CE), is viability as a measure of the cells’ ability to clone:

For microtitre plate tests, calculations are based on P(0), the proportion of wells in which a colony has not grown:

$$P(0) = \left(\frac{\text{Number of wells with no colony}}{\text{Total number of wells}} \right)$$

The CE for each culture was calculated according to the following calculation:

$$CE = \left(\frac{-\ln\ P(0)}{\text{Number of cells per well*}} \right) x\,100$$

* Number of cells per well is 1.6 cells/well on average on all viability plates.

Relative Total Growth (RTG) is the measure of cytotoxicity relative to the control, taking into account all cell growth, and cell loss during the treatment period and the 2 day expression period (RSG), and the cells' ability to clone 2 days after treatment (viability):

$$RTG = RSG\ x\ \left(\frac{\text{Individual Viability Value}}{\text{Mean Control Viability Value}} \right)$$

Mutant Frequency (MF) was calculated from the number of TFT resistant mutants expressed per 10^6 viable cells as follows:

$$\frac{-\ln P(0) \text{ for mutant plates}}{\text{Number of cells per well*}\ x\ (\text{viability}/100)}$$

* Number of cells per well is 2000 cells/well on average on all mutant plates.

Small and large colony mutant frequencies were calculated in an identical manner, using the relevant number of empty wells for small and large colonies, as appropriate. Heterogeneity between replicate cultures was assessed and where considered to be excessive may have resulted in exclusion of a concentration.

The relevance of increases in mutant frequencies (total wells with clones), by comparison with concurrent controls and the global evaluation factor (GEF), was assessed according to the recommendations of the Mouse Lymphoma Workgroup, Aberdeen, 2003. Linear regression was performed on ranked mutant frequency against ranked concentration to test for a linear trend. The linear trend was performed on the overall MF per concentration level, not individual replicates (i.e. one MF value per concentration). The test for linear trend is one-tailed, therefore negative trend was not considered relevant. The positive control was excluded from the test for linear trend.

For microwell assays, the GEF is defined as 126 mutants per 10^6 viable cells.

Acceptance criteria:

The assay was considered valid if all the following criteria were met:

6. The mean mutant frequencies in the vehicle control cultures fell within the normal range (50 to 170 mutants per 10^6 viable cells)
7. At least one positive control showed either an absolute increase in mean total MF of at least 300 x 10^{-6} (at least 40% of this should be in the small colony MF), or an increase in small colony mutant frequency of at least 150 x 10^{-6} above the concurrent vehicle control

8. The RTG for the positive controls was greater than 10%
9. The mean CE of the vehicle controls from the Mutation Experiment was between the range 65% to 120% on Day 2
10. The mean SG of the vehicle controls from the Mutation Experiment was between the range 8 to 32 following 3 hour treatments or between 32 and 180 following 24 hour treatments.

Evaluation criteria:

For valid data, the test item was considered to be mutagenic in the assay if all of these criteria were met:

4. The MF of any test concentration exceeded the sum of the vehicle control mutant frequency plus GEF
5. The linear trend test was statistically significant
6. Any observed response was reproducible under the same treatment conditions.

The test item was considered as negative in this assay if none of the above criteria were met.

Results

Osmolality and pH

No marked or meaningful changes in osmolality or pH were observed in the 3 and 24 hour experiment at the highest three concentrations tested (475.5, 951 and 1902 µg/mL), compared to the concurrent vehicle controls.

Cytotoxicity

In the cytotoxicity experiment, 3 h treatment, 6 concentrations were tested in the absence and presence of S-9 ranging from 59.44 to 1902 µg/mL (equivalent to 10 mM at the highest concentration tested). Upon addition of the test item to the cultures, precipitate was observed at the highest concentration tested (1902 µg/mL), but none was observed following the 3 hour treatment incubation period. The highest concentration tested (1902 µg/mL) gave 87% and 92% RTG in the absence and presence of S-9, respectively.

In the cytotoxicity experiment, 24 h treatment, 9 concentrations were tested in the absence of S-9, ranging from 7.430 to 1902 µg/mL. Upon addition of the test item to the cultures, precipitate was observed at the highest concentration tested (1902 µg/mL), but none was observed following the 24 hour treatment incubation period. The highest concentration to provide >10% RTG was 951 µg/mL, which gave 37% RTG.

In the mutation experiment (3 h treatments) seven concentrations, ranging from 150 to 1902 µg/mL, were tested in the absence and presence of S-9. Upon addition of the test item to the cultures, precipitate was observed at the highest concentration tested (1902 µg/mL), but none was observed following the 3 h treatment incubation period. Two days after treatment all concentrations in the absence and presence of S-9 were selected to determine viability and TFT resistance. The highest concentration analyzed was 1902 µg/mL, which gave 65% and 47% RTG in the absence and presence of S-9, respectively.

Table B.6.8.1-32: Mutation Experiment - 3 Hour Treatment in the Absence and Presence of S-9

3 hour treatment -S-9			3 hour treatment +S-9		
Concentration µg/mL	**%RTG**	**MF §**	**Concentration µg/mL**	**%RTG**	**MF §**
0	100	128.62	0	100	103.94
150	97	101.96	150	95	115.41
300	100	107.31	300	104	106.05
600	93	126.01	600	101	102.30
900	79	162.21	900	109	101.97
1200	81	135.75	1200	104	122.82
1500	75	136.12	1500	94	110.44
1902 P	65	130.82	1902 P	47	127.59
MMS 15	52	1034.72	B[a]P 2	61	510.42
MMS 20	39	1067.57	B[a]P 3	55	620.83

3 hour in the absence of S-9. Linear trend test on mutant frequency: p-value = 0.0428 (* P < 0.05)

3 hour in the presence of S-9. Linear trend test on mutant frequency: p-value = 0.1302

P Precipitation observed at the time of treatment

MF § Mutant Frequency

%RTG Percent Relative Total Growth

In the mutation experiment (24 h treatment) 10 concentrations, ranging from 150 to 1902 µg/mL were tested in the absence of S-9. Upon addition of the test item to the cultures, precipitate was observed at the highest two concentrations tested (1700 and 1902 µg/mL), but none was observed following the 24 h treatment incubation period. Two days after treatment all concentrations were selected to determine viability and TFT resistance. However, the highest four concentrations selected (1350 to 1902 µg/mL) were later rejected from analysis due to extreme toxicity. The highest concentration analyzed was 1200 µg/mL, which gave 13% RTG.

Table B.6.8.1-33: Mutation Experiment - 24 Hour Treatment in the Absence of S-9

Concentration µg/mL	**%RTG**	**MF §**
0	100	67.95
150	97	55.92
300	82	81.40
600	84	82.32
900	45	174.37
1050	18	242.60 #
1200	13	313.58 #
MMS 5	61	957.31
MMS 7.5	48	1408.33

Linear trend test on mutant frequency: p-value = 0.0002 (* P < 0.001)

MF § Mutant Frequency

%RTG Percent Relative Total Growth

The MF of the test concentration exceeded the sum of the mean control MF plus GEF

The acceptance criteria were met and the study was therefore accepted as valid.

When tested up to 10 mM for 3 h in the absence and presence of S-9, no increases in MF that exceeded the Global Evaluation Factor (GEF) of 126 mutants per 10^6 viable cells (compared to concurrent vehicle controls) were observed in any treated cultures under either treatment condition. A

weakly significant linear trend (p≤0.05) was observed for the 3 h treatment in the absence of S-9, but the maximum increase in MF observed in any treated culture (approximately 34, at 900 µg/mL) was well below the GEF of 126 and observed at only one concentration, therefore the linear trend was considered not biologically relevant.

When tested up to toxic concentrations for 24 h in the absence of S-9, increases in MF of approximately 175 and 246 mutants per 10^6 viable cells (compared to concurrent controls), which exceeded the GEF of 126, were observed at the highest two concentrations analyzed (1050 and 1200 µg/mL, respectively) and there was a highly significant linear trend (p≤0.001). These increases were observed at concentrations giving 18% and 13% RTG at 1050 and 1200 µg/mL, respectively, and therefore both were close to the upper limit of cytotoxicity.

In addition, for the negative and positive controls and concentrations of test item that exceeded the GEF, the number of wells containing small colonies and the number containing large colonies were scored. For the vehicle controls, the proportion of small colony mutants in the absence and presence of S-9 ranged from 48% to 58%. Marked increases in the number of both small and large colony mutants were observed following treatment with the positive control chemicals MMS and BP. At the concentrations of M-102 that exceeded the GEF following the 24 h treatment in the absence of S-9, increases in both small and large colony MF were observed and there was an increase in the proportion of small colonies.

Table B.6.8.1-34: Mutation experiment, without and with S-9, 3 h treatment: Small and large colony mutant frequencies

Concentration µg/mL	Small Colonies Mutants		MF	Large Colonies Mutants		MF	Proportion of Small Colony Mutants
	Ym	Nm		Ym	Nm		
0	669	768	70.95	696	768	50.61	0.58
MMS 15	159	384	624.04	314	384	142.43	0.81
MMS 20	171	384	632.39	311	384	164.82	0.79
With S-9:							
0	691	768	46.35	684	768	50.82	0.48
BP 2	239	384	257.48	295	384	143.17	0.64
BP 3	216	384	279.54	276	384	160.45	0.64

Table B.6.8.1-35: Mutation experiment, without S-9, 24 h treatment: Small and large colony mutant frequencies

Concentration µg/mL	Small Colonies Mutants		MF	Large Colonies Mutants		MF	Proportion of Small Colony Mutants
	Ym	Nm		Ym	Nm		
0	718	768	36.27	727	768	29.56	0.55
1050	589	768	154.29	685	768	66.50	0.70
1200	550	768	199.99	673	768	79.10	0.72
MMS 5	188	384	535.06	303	384	177.49	0.75
MMS 7.5	170	384	646.06	279	384	253.27	0.72

Ym = Wells without colonies (mutant); Nm = Total wells (mutant); MF = Mutant frequency

Conclusions

It was concluded that M-102 induced mutation at the *tk* locus of L5178Y mouse lymphoma cells when tested up to toxic concentrations for 24 h in the absence of a rat liver metabolic activation system (S-9). Further, a preponderance of small colonies in the increases suggested a clastogenic effect, rather than gene mutation. In the same test system, M-102 did not induce mutation when tested up to a maximum concentration equivalent to 10 mM for 3 h in the absence or presence of S-9.

In vitro genotoxicity studies – mammalian cell gene mutation test, 2-(trifluoromethyl)-benzoic acid, study 2

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study. The study was performed just before the current OECD guideline (2016) was adopted. However, no deviations from the current guideline were noted. The study is considered acceptable.

reference	:	Lloyd, M (2016e)	vehicle	:	DMSO
Report number	:	8347523 (T-3249)	doses	:	150 to 1902 µg/mL
test substance	:	2-(trifluoromethyl)-benzoic acid	GLP	:	yes
Test system	:	Mouse lymphoma L5178Y cells	guideline	:	In accordance with OECD 490 (2015)
			acceptability	:	acceptable

Executive summary

This complementary study was conducted in response to the result of the previous study (above), where an apparently positive mutagenic/clastogenic response occurred after 24 h exposure in the absence of a metabolic activation system. The present study used modified experimental conditions, to examine the possibility that an excessive change of pH on addition of the test item might have affected the mutation frequency.

M-102 (2-(trifluoromethyl)-benzoic acid), 99.3% purity, was assayed for the ability to induce mutation at the *tk* locus (5-trifluorothymidine [TFT] resistance) in mouse lymphoma cells using a fluctuation protocol. The study consisted of one Mutation Experiment, conducted in the absence and presence of metabolic activation by an Aroclor 1254-induced rat liver post-mitochondrial fraction (S-9). The test item was formulated in anhydrous analytical grade dimethyl sulphoxide (DMSO). In the present study the culture medium was without HEPES buffer, but procedures were otherwise the same as in the previous study. The pH in cultures was measured immediately after addition of the test item, for the three highest concentrations tested under each experimental condition, using additional cultures especially set up for the purpose. Measurement of osmolality and pH were also made for post-treatment cultures at the same three concentrations under each treatment condition.

A 3 hour treatment incubation period was used for the treatment performed in the presence of S-9. In the absence of S-9, 3 hour and 24 hour treatment incubation periods were used. In the 3 hour treatments, seven concentrations from 150 to 1902 µg/mL were tested in the absence and presence of S-9. Two days after treatment the highest concentration analyzed to determine viability and TFT resistance was 1902 µg/mL, which gave 67% and 72% RTG in the absence and presence of S-9, respectively. In the 24 h treatment, 10 concentrations from 150 to 1902 µg/mL were tested in the

absence of S-9. Two days after treatment the highest concentration analyzed to determine viability and TFT resistance was 1500 µg/mL, which gave 10% RTG.

Vehicle and positive control treatments were included in the for each treatment condition. Mutant frequencies (MF) in vehicle control cultures fell within acceptable ranges and clear increases in mutation were induced by the positive control chemicals Methyl methane sulphonate (without S-9) and Benzo[a]pyrene (with S-9), and therefore the study was accepted as valid.

M-102 tested at up to 10 mM for 3 h in the absence and presence of S-9 produced no increases in MF exceeding the Global Evaluation Factor (GEF) of 126 mutants per 10^6 viable cells (compared to concurrent controls) in any treated cultures under either treatment condition. A statistically significant linear trend ($p \leq 0.01$) was observed in the absence of S-9, but the maximum increase in MF observed in any treated culture was approximately 26 (at 1902 µg/mL, the highest concentration tested), which was well below the GEF of 126, and therefore this observation was considered not biologically relevant.

When tested at up to toxic concentrations for 24 h in the absence of S-9, there were also no increases in MF exceeding the GEF of 126 (compared to concurrent controls) in any treated cultures. There was a statistically significant linear trend ($p \leq 0.01$) with a maximum increase in MF observed in any treated culture of approximately 95 (at 1500 µg/mL, the highest concentration analyzed, giving 10% RTG), but as this fell below the GEF of 126 the trend was considered not biologically relevant.

Materials and methods

Refer to Lloyd (2016d) above for these details, but note that HEPES buffer was excluded from the culture medium, buffering capacity being maintained by gassing with 5% CO_2 in air. The pH was measured immediately after addition of the test item, in cultures especially set up in parallel for this purpose.

The same concentration range was used in the present study for the 3h treatments (150 to 1902 µg/mL), but the range after 24 h treatment selected for viability/TFT resistance plating was limited at the upper end (150 to 1700 µg/mL), excluding 1902 µg/mL on this occasion. Additional cultures were prepared in parallel with the three highest concentrations for each experimental condition (1200, 1500 and 1902 µg/mL for 3 h treatments, and 1500, 1700 and 1902 µg/mL for the 24 h treatment); these were used solely for pH measurement on addition of the test item and were not incubated for the respective periods.

Study start and completion: 05 July 2016 to 16 August 2016

Results

Osmolality and pH

No marked changes in osmolality were observed in post-treatment media at the highest three concentrations analyzed under each treatment condition, compared to the concurrent vehicle controls. Immediately following addition of the test article, there was a decrease in pH of approximately 1 unit at the highest 1 to 2 concentrations tested, compared to the pH prior to test article addition. In the post-treatment media samples, increases in pH of approximately 0.3-0.4 unit were seen at all concentrations tested following the 3 hour treatments in the absence and presence of S-9, but the

increases were very small in magnitude (<0.2 unit) following the 24 hour treatment in the absence of S-9.

In the 3 h treatments, 7 concentrations from 150 to 1902 µg/mL were tested in the absence and presence of S-9. Upon addition of the test item to the cultures, precipitate was observed at 1902 µg/mL, but following the 3 h treatment incubation period, no precipitate was observed. Two days after treatment all concentrations in the absence and presence of S-9 were selected to determine viability and TFT resistance. The highest concentration analyzed was 1902 µg/mL, which gave 67% and 72% RTG in the absence and presence of S-9, respectively.

Table B.6.8.1-36: Mutation Experiment - 3 Hour Treatment in the Absence and Presence of S-9

3 hour treatment -S-9			3 hour treatment +S-9		
Concentration µg/mL	**%RTG**	**MF**	**Concentration µg/mL**	**%RTG**	**MF**
0	100	75.33	0	100	91.87
150	87	81.17	150	85	89.22
300	102	83.26	300	87	79.69
600	80	76.35	600	94	58.97
900	86	84.05	900	87	71.15
1200	124	97.61	1200	59	80.76
1500	63	93.52	1500	75	89.60
1902 P	67	101.71	1902 P	72	99.45
MMS 15	28	593.59	B[a]P 2	62	573.56
MMS 20	38	761.32	B[a]P 3	37	811.53

3 hour in the absence of S-9. Linear trend test on mutant frequency: p-value = 0.001 (** P < 0.01)

3 hour in the presence of S-9. Linear trend test on mutant frequency: p-value = 0.3257

P Precipitation observed at the time of treatment

MF Mutant Frequency

%RTG Percent Relative Total Growth

*, **, *** Test for linear trend (one-sided), significant at 5%, 1% and 0.1% level respectively

In the 24 h treatment, 10 concentrations from 150 to 1902 µg/mL were tested in the absence of S-9. Upon addition of the test item to the cultures, precipitate was observed at 1902 µg/mL, but following the 24 h treatment incubation period, no precipitate was observed. Two days after treatment the highest concentration tested (1902 µg/mL) was considered too toxic for selection to determine viability and TFT resistance, but all other concentrations were selected. However, the highest concentration remaining (1700 µg/mL) was later rejected from analysis owing to extreme toxicity (<10% RTG). The highest concentration analyzed was 1500 µg/mL, which gave 10% RTG.

Table B.6.8.1-37: Mutation Experiment - 24 Hour Treatment in the Absence of S-9

Concentration µg/mL	**%RTG**	**MF**
0	100	71.94
150	96	53.44
300	96	59.31
600	57	89.81
900	46	68.39
1050	29	109.17

1200	19	156.74
1350	16	123.29
1500	10	167.35
MMS 5	45	834.75
MMS 7.5	34	1178.88

Linear trend test on mutant frequency: p-value = 0.0019 (** P < 0.01)

MF Mutant Frequency

%RTG Percent Relative Total Growth

*, **, *** Test for linear trend (one-sided), significant at 5%, 1% and 0.1% level respectively

The acceptance criteria were met and the study was accepted as valid.

M-102 tested at up to 10 mM for 3 h in the absence and presence of S-9 produced no increases in MF exceeding the GEF of 126 mutants per 10^6 viable cells (compared to concurrent controls) in any treated cultures under either treatment condition. A statistically significant linear trend (p≤0.01) was observed in the absence of S-9, but the maximum increase in MF observed in any treated culture was approximately 26 (at 1902 µg/mL, the highest concentration tested), which was well below the GEF of 126, and therefore this observation was considered not biologically relevant.

When tested up to toxic concentrations for 24 h in the absence of S-9, there were also no increases in MF exceeding the GEF of 126 (compared to concurrent controls) in any treated cultures. There was a statistically significant linear trend (p≤0.01) and the maximum increase in MF observed in any treated culture was approximately 95 (at 1500 µg/mL, the highest concentration analyzed, giving 10% RTG), but as this fell below the GEF of 126, the trend was considered not biologically relevant.

In addition, for the negative and positive controls, the number of wells containing small colonies and the number containing large colonies were scored, so that the small and large colony MF could be estimated and the proportion of small mutant colonies calculated. For the vehicle controls, the proportion of small colony mutants in the absence and presence of S-9 ranged from 40% to 49%. Marked increases in the number of both small and large colony mutants were observed following treatment with the positive control chemicals MMS and B[a]P.

Table B.6.8.1-38: Mutation experiment, without and with S-9, 3 h treatment: Small and large colony mutant frequencies

Concentration	**Small Colonies**			**Large Colonies**			**Proportion**
µg/mL	**Mutants**		**MF**	**Mutants**		**MF**	**of Small**
	Ym	Nm		Ym	Nm		**Colony Mutants**
0	713	768	35.51	711	768	36.85	0.49
MMS 15	241	384	325.02	304	384	162.99	0.67
MMS 20	215	384	447.02	311	384	162.51	0.73
With S-9:							
0	718	768	35.44	694	768	53.33	0.40
B[a]P 2	246	384	253.13	277	384	185.67	0.58
B[a]P 3	234	384	345.59	269	384	248.33	0.58

Table B6.8.1-39: Mutation experiment, without S-9, 24 h treatment: Small and large colony mutant frequencies

Concentration	Small Colonies			Large Colonies			Proportion
µg/mL	Mutants		MF	Mutants		MF	of Small
	Ym	Nm		Ym	Nm		Colony Mutants
0	719	768	30.99	706	768	39.56	0.44
MMS 5	222	384	428.35	294	384	208.77	0.67
MMS 7.5	192	384	598.34	290	384	242.36	0.71

Ym = Wells without colonies (mutant); Nm = Total wells (mutant); MF = Mutant frequency

Discussion

It was evident that, using HEPES-free medium, increases in MF were seen following the 24 h treatment in the absence of S-9 and there was a statistically significant linear trend ($p \leq 0.01$), as was the case in the previous study. However, the increases were smaller in magnitude than in the previous study, they did not reach the GEF of 126, and therefore they did not constitute a positive result according to the evaluation criteria described in the OECD guideline 490 (2015).

Conclusion

M-102 (2-(trifluoromethyl)-benzoic acid) did not induce mutation at the *tk* locus of L5178Y mouse lymphoma cells when tested up to a maximum concentration equivalent to 10 mM for 3 h in the absence and presence of a rat liver metabolic activation system (S-9), or when tested up to toxic concentrations for 24 h in the absence of S-9, under the conditions of the study.

In vitro genotoxicity studies – mammalian chromosomal aberration test, 2-(trifluoromethyl)-benzoic acid

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study. The study was performed just before the current OECD guideline (2016) was adopted. However, no deviations from the current guideline were noted. The study is considered acceptable.

reference	:	Lloyd, M (2016f)	vehicle	:	DMSO
Report number	:	8337789 (T-3247)	doses	:	600 to 1902 µg/mL
test substance	:	2-(trifluoromethyl)-benzoic acid	GLP	:	yes
Test system	:	human lymphocytes	guideline	:	In accordance with OECD 473 (2014)
			acceptability	:	acceptable

Executive summary

M-102 (2-(trifluoromethyl)-benzoic acid), 100% purity, was tested in an in vitro chromosome aberration assay using duplicate human lymphocyte cultures prepared from the pooled blood of three male donors in a single experiment. Treatments were performed both in the absence and presence of metabolic activation (S-9) from Aroclor 1254-induced rats. The test item was formulated in anhydrous dimethyl sulphoxide (DMSO). The concentrations tested ranged up to 1902 µg/mL (equivalent to 10 mM), as determined by a preliminary cytotoxicity experiment.

Treatments were conducted 48 hours following mitogen stimulation by phytohaemagglutinin (PHA). The test item concentrations for chromosome analysis were selected by evaluating the effect of M-101 on mitotic index. Chromosome aberrations were analysed at three or four concentrations up to 1902 µg/mL. Appropriate negative (vehicle) control cultures were included in the test system in the under each treatment condition. The proportion of cells with structural aberrations in these cultures fell within the 95th percentile of the current historical vehicle control (normal) ranges. Mitomycin C (MMC) and cyclophosphamide (CPA) were employed as positive control chemicals in the absence and presence of rat liver S-9 respectively. Cells receiving these were sampled 20 hours after the start of treatment, and both compounds induced statistically significant increases in the proportion of cells with structural aberrations. All acceptance criteria were considered met and the study was accepted as valid.

Treatment of cells with M-102 for 3+17 hours in the absence and presence of S-9, or for 20+0 hours in the absence of S-9, resulted in frequencies of aberrant cells that were similar to the concurrent vehicle controls at all concentrations analysed. The aberration frequencies (excluding gaps) were within the normal ranges in all treated cultures analyzed under the three treatment conditions. A weak statistically significant linear trend ($p \leq 0.05$) was observed for the 20+0 hour treatment in the absence of S-9, but as the aberration frequencies (excluding gaps) were within the normal ranges in all treated cultures analyzed and no statistically significant frequency in aberration frequencies (excluding gaps) were observed, this observation was considered not biologically relevant. No notable increases in the frequency of cells with numerical aberrations, exceeding the concurrent controls and the normal ranges, were observed in any cultures treated with M-102 under any of the three treatment conditions.

It was concluded that M-102 (2-(trifluoromethyl)-benzoic acid) did not induce structural chromosome aberrations in human peripheral blood lymphocytes when tested up to a concentration equivalent to 10 mM (limit dose) in the absence or presence of a metabolic activation system (S-9).

Materials and Methods

Test item:	M-102 (2-(trifluoromethyl)-benzoic acid, or α,α,α-trifluoro-*o*-toluic acid)
Batch No.:	2AE4201P
Purity:	99.3%
Expiry:	19 April 2020
Appearance:	Off-white powder
Storage:	Refrigerated, in the dark
Vehicle:	Anhydrous dimethyl sulphoxide (DMSO), analytical grade
Positive controls:	Mitomycin C (MMC), cyclophosphamide (CPA), from Sigma-Aldrich

Cells:	From blood from 3 healthy non-smoking males
Metabolic activation:	MolTox S-9 from Molecular Toxicology Inc., USA
Culture medium:	RPMI containing HEPES
Study start and completion:	17 February 2016 to 21 June 2016

M-102 was soluble in DMSO at up to (at least) 191.3 mg/mL. It was soluble in culture medium at up to approximately 956.6 to 1913 µg/mL, as indicated by precipitation that persisted for 22 h at 37ºC. A maximum concentration of 1902 µg/mL was selected for the study, equivalent to 10 mM. Test item solutions were prepared with the aid of vortex mixing under subdued light, with serial dilution from the maximum required concentration. These solutions were used within 4 h of preparation, and no analyses for stability, homogeneity or achieved concentrations were undertaken. The positive controls were used at 0.05 to 0.4 µg/mL (MMC) and 1 to 2 µg/mL (CPA).

The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254. The batches of MolTox^{TM} S-9 were stored frozen in aliquots at <-50°C prior to use. Each batch was checked by the manufacturer for sterility, protein content, ability to convert known promutagens to bacterial mutagens and cytochrome P-450-catalyzed enzyme activities (alkoxyresorufin-O-dealkylase activities). The S-9 mix was prepared with G6P (180 mg/mL), NADP (25 mg/mL), KCl (150 mM) and rat liver S-9 mixed in the ratio 1:1:1:2. For all cultures treated in the presence of S-9, an aliquot of the mix was added to each cell culture to achieve the required final concentration of test article in a total of 10 mL. The final concentration of the liver homogenate in the test system was 2%. Cultures treated in the absence of S-9 received an equivalent volume of 150 mM KCl.

Blood from three healthy, non-smoking male volunteers from a panel of donors was used for each experiment. No donor was suspected of any virus infection or exposed to high levels of radiation or hazardous chemicals. All donors were non-smokers and not heavy consumers of alcohol, and none were taking any form of medication. The measured cell cycle time of the donors used fell within the range 13±2 hours. For each experiment, an appropriate volume of whole blood was drawn from the peripheral circulation into heparinized tubes on the day of culture initiation. Blood was stored refrigerated and was pooled using equal volumes from each donor prior to use. Whole blood cultures were established in sterile disposable centrifuge tubes by placing 0.4 mL of pooled heparinised blood into 9.0 mL pre-warmed (37±1°C) HEPES-buffered RPMI medium containing 10% (v/v) heat inactivated fetal calf serum and 0.52% penicillin / streptomycin, so that the final volume following addition of S-9 mix or KCl and the test item in its chosen vehicle was 10mL. The mitogen phytohaemagglutinin (PHA, reagent grade) was included in the culture medium at a concentration of approximately 2% of culture to stimulate the lymphocytes to divide. Blood cultures were incubated at 37±1°C for approximately 48 hours and rocked continuously.

S-9 mix or KCl (0.5 mL per culture) was added as appropriate. Cultures were treated with the test item or vehicle control or positive control (0.1 mL per culture) and final culture volume was 10 mL. (Positive control treatments were not included for cytotoxicity range-finding). Cultures were incubated at 37±1°C for the designated exposure time. The scheme is shown in the table below.

Table B.6.8.1-40: Treatment scheme

		Number of Cultures			
Treatment	**S-9**	**Cytotoxicity Range-Finder**		**Chromosome Aberration Experiment**	
		3+17*	**20+0***	**3+17***	**20+0***
Vehicle control	- +	2 2	2	4 4	4
Test article	- +	1 1	1	2 2	2
Positive controls	- +			2 2	2
* Hours treatment + hours recovery					

For removal of the test article, cells were pelleted by centrifugation, washed twice with sterile saline (pre-warmed in an incubator set to 37±1°C), and resuspended in fresh pre-warmed medium containing fetal calf serum and penicillin/streptomycin. Osmolality and pH measurements on post-treatment incubation medium were taken in the cytotoxicity range-finder experiment.

Approximately 2 hours prior to harvest, colchicine was added to give a final concentration of approximately 1 µg/mL to arrest dividing cells in metaphase. At the defined sampling time cultures were centrifuged, the supernatant was carefully removed and cells were resuspended in 4 mL pre-warmed hypotonic (0.075 M) KCl and incubated at 37±1ºC to allow cell swelling to occur. Cells were fixed by dropping the KCl suspension into fresh, cold methanol/glacial acetic acid (3:1, v/v). The fixative was changed by centrifugation and resuspension, a procedure that was repeated as necessary until the cell pellets were clean.

Lymphocytes were kept in fixative for a minimum of 3 hours at 2-8ºC prior to slide preparation to ensure that cells were adequately fixed. Cells were centrifuged and resuspended in a minimal amount of fresh fixative (if required) to give a milky suspension. Several drops of 45% (v/v) aqueous acetic acid were added to each suspension to enhance chromosome spreading and several drops of suspension were transferred onto clean, labelled microscope slides and dried on a hot plate, then stained in filtered 4% (v/v) Giemsa at pH 6.8, then rinsed, dried and mounted.

Slides from the cytotoxicity experiment were examined and the Mitotic Index (MI) determined i.e. the percentage of cells in mitosis. Mitotic inhibition (MIH) was calculated as:

MIH (%) = [1 - (mean MIT/mean MIC)] x 100%

(where T = treatment and C = vehicle control)

Slides from sufficient concentrations from each treatment group were scored to determine whether chemically induced MIH had occurred. This was defined as a clear decrease in MI compared with vehicle controls (based on at least 1000 cells counted, where possible), preferably concentration-related. A suitable range of concentrations was selected for the chromosome aberration experiment based on these toxicity data.

The highest concentration selected for chromosome aberration analysis following 3+17 h treatment was the highest concentration tested (1902 µg/mL) equivalent to 10 mM, while the highest concentration tested following 20+0 h treatment was that achieving 50-60% mitotic inhibition. Analysis of slides from highly toxic concentrations was avoided. Slides from the highest selected concentration and two or three lower concentrations were taken for microscopic analysis, such that a range of

cytotoxicity was covered. Where possible, the positive control concentrations analyzed did not exceed the cytotoxicity limits for the test article concentration selection, but the CPA (2 µg/mL) positive control analyzed following the 3+17 h treatment in the presence of S-9 gave 62% MIH, which marginally exceeded 60%. This did not affect the interpretation of the data. For each treatment regime, two vehicle control cultures were analysed for chromosome aberrations. Slides from positive control treatments were checked to ensure that the system was operating satisfactorily. One concentration from each positive control, giving satisfactory responses in terms of quality and quantity of mitoses and extent of chromosomal damage, was analyzed.

A minimum of 300 metaphases per concentration were analyzed for chromosome aberrations. Only cells with 44 to 48 chromosomes were accepted for analysis. Any cell with more than 48 chromosomes (*ie* polyploid or endoreduplicated) observed during this evaluation was noted and recorded separately. Structural aberrations were classified according to the ISCN scheme (1995). Under this scheme, a gap is defined as a discontinuity less than the width of the chromatid with no evidence of displacement of the fragment, and a deletion is defined as a discontinuity greater than the width of the chromatid and/or evidence of displacement of the fragment.

After completion of scoring and decoding of slides the numbers of aberrant cells in each culture were categorised as follows:

1. Cells with structural aberrations including gaps
2. Cells with structural aberrations excluding gaps
3. Polyploid or endoreduplicated cells

The totals for category 2 in vehicle control cultures were compared with the 95th percentile of the current historical vehicle control (normal) ranges to determine whether the assay was acceptable or not. The proportions of cells with structural chromosome aberrations excluding gaps were compared with the proportion in vehicle controls by using Fisher's exact test. In addition, a Cochran-Armitage Trend Test was performed to aid determination of concentration-response relationships. Probability values of $p \leq 0.05$ were accepted as significant. The proportions of cells in categories 1 and 3 were examined in relation to the historical vehicle control range. The proportions of aberrant cells in each replicate were used to establish acceptable heterogeneity between replicates by means of a binomial dispersion test, probability values of $p \leq 0.05$ being accepted as significant.

Acceptance criteria:

The assay was considered valid if all the following criteria were met:

1. The binomial dispersion test demonstrated acceptable heterogeneity between replicate cultures;
2. The proportion of cells with structural aberrations (excluding gaps) in vehicle control cultures fell within the normal ranges;
3. At least 300 cells were analysed at each concentration, unless 15 or more cells showing structural aberrations (per slide) other than gaps only were observed during analysis;
4. The positive control chemicals induced statistically significant increases in the proportion of cells with structural chromosome aberrations (excluding gaps). Both replicate cultures at the positive control concentration analysed under each treatment condition demonstrated structural aberration cell frequencies (excluding gaps) that clearly exceeded the normal vehicle control ranges;

5. The maximum concentration analysed under each treatment condition was the maximum concentration tested, a concentration inducing approximately 50% cytotoxicity, or the lowest concentration at which precipitate was observed at the end of the treatment period.

Evaluation criteria:

The test item was considered to induce clastogenic events if:

1. A statistically significant increase in the proportion of cells with structural aberrations (excluding gaps) was observed (p≤0.05);
2. The incidence of cells with structural aberrations (excluding gaps) at such a concentration that exceeded the normal range in both replicate cultures;
3. A concentration-related increase in the proportion of cells with structural aberrations (excluding gaps) was observed (positive trend test).

The test item was considered positive in this assay if all of the above criteria were met. It was considered negative if none of the above criteria were met.

Results

Table B.6.8.1-41: Chromosome aberrations in human lymphocytes following 2-(trifluoromethyl)-benzoic acid treatment

Treatment	Concentration (µg/mL)	Cytotoxicity (%)[$]	% Cells with Aberrations (Excluding Gaps)	Historical control range (%)[#]	Statistical significance
3+17 hour - S9	Vehicle[a]	0 (0.00%)	0 (0.00%)	0.00 – 3.00	-
	600	1 (0.33%)	1 (0.33%)		NS
	1200	3 (1.00%)	3 (1.005)		NS
	1902	2 (0.67%)	1 (0.33%)		NS
	*MMC, 0.40	56 (18.67%)	55 (18.33%)		p≤0.001
3+17 hour +S9	Vehicle[a]	0 (0.00%)	0 (0.00%)	0.00 – 2.00	-
	600	1 (0.33%)	1 (0.33%)		NS
	1200	1 (0.33%)	1 (0.33%)		NS
	1902	3 (1.00%)	2 (0.67%)		NS
	*CPA, 1.00	44 (15.88%)	43 (15.52%)		p≤0.001
20+0 hour - S9	Vehicle[a]	1 (0.33%)	1 (0.33%)	0.00 – 2.90	-
	200	0 (0.00%)	0 (0.00%)		NS
	400	2 (0.67%)	1 (0.33%)		NS
	600	4 (1.33%)	3 (1.00%)		NS
	650	8 (2.67%)	4 (1.33%)		NS
	*MMC, 0.10	29 (9.67%)	28 (9.33%)		p≤0.001

[a] vehicle control was DMSO

* positive control

[#] 95th percentile of the observed range

[$] based on the mitotic index

NS not significant

Treatment of cells with M-102 for 3+17 hours in the absence and presence of S-9 and for 20+0 hours in the absence of S-9 resulted in frequencies of aberrant cells that were similar to those observed in the concurrent vehicle controls at all concentrations analysed. The aberration frequencies (excluding gaps) were within the normal ranges in all treated cultures analysed under the three treatment conditions. A weak statistically significant linear trend ($p \leq 0.05$) was observed for the 20+0 hour treatment in the absence of S-9, but as the aberration frequencies (excluding gaps) were within the normal ranges in all treated cultures analyzed and no statistically significant frequency in aberration frequencies (excluding gaps) were observed, this observation was considered not biologically relevant.

No notable increases in the frequency of cells with numerical aberrations exceeding the concurrent controls and the normal ranges were observed in any cultures treated with M-102 under any of the three treatment conditions.

Conclusion

M-102 (2-(trifluoromethyl)-benzoic acid) did not induce structural chromosome aberrations in human peripheral blood lymphocytes when tested up to a concentration equivalent to 10 mM (limit dose) in the absence or presence of a metabolic activation system (S-9).

B.6.8.2 Supplementary studies on the active substance

Immunotoxicity study – T-cell dependent antibody assay

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	For this 4-week dietary T cell-dependent antibody assay with fllutolanil no OECD test guidance is available. However, the study is performed according to OPPTS, Series 870, Health Effects Testing Guideline, No. 870.7800 (August 1998) and the study is considered acceptable.

reference	:	[redacted] (2011)	exposure	:	Via the diet
Report number	:	8236937 (T-3203)	doses	:	0, 750, 3000 or 12000 ppm
test substance	:	flutolanil	GLP	:	Yes
species	:	Sprague-Dawley rats	guideline	:	No OECD guideline available
group size	:	10/ sex/ group	acceptability	:	acceptable

Executive summary

Flutolanil TGAI (technical grade active ingredient), 98.7% purity, was administered to groups of Sprague-Dawley rats (10/sex/group) by admixture with the diet at concentrations of 0 (negative control), 750, 3000 or 12000 ppm for 4 weeks. A further group of 5/sex received cyclophosphamide at 250 mg/kg by intraperitoneal injection for 4 days prior to sacrifice, and served as positive control.

Mortality, clinical observations, body weight and food consumption were monitored, then necropsy and organ weights. Immune function was assessed with an antibody-forming cell (AFC) assay using sheep red blood cells (SRBC).

There were no treatment related effects among any of the observations and parameters, while those animals receiving cyclophosphamide showed the expected decreases in spleen and thymus weights and had significantly ablated AFC responses, indicative of immunosuppression.
A NOAEL for rats receiving flutolanil TGAI in the diet for 4 weeks was concluded to be 12000 ppm (equivalent to a mean dosage of approximately 1000 mg/kg/day).

Materials and Methods

Test item:	Flutolanil TGAI
Batch No.:	9AE0011P
Purity:	98.7%
Expiry:	11 July 2013
Storage:	Room temperature, in the dark
Diet:	Certified Rodent Diet #8728CM (Harlan Laboratories, Inc.)
Sheep Red Blood Cells:	From Colorado Serum Company, diluted with sterile saline
Positive control:	Cyclophosphamide (Sigma) in sterile saline
Animals:	Sprague-Dawley rats, Crl:CD strain ([redacted], 48-54 days of age, 199-251 g (males) and 49-55 days of age, 160-209 g (females) at the start of administration
Study start and completion:	22 February 2011 to 29 September 2011

Test diet formulations were prepared once weekly and stored at room temperature. They were analyzed for achieved concentration, and homogeneity on Day 1 and Week 4, while stability was assessed from Day 1 preparations over a period of 10 days. Cyclophosphamide (CP) solution was prepared on the day before administration. The sheep red blood cells (SRBC) were prepared on the day before or on the day of administration, at a concentration in saline of approximately 4 x 10^8 cells/mL, stored refrigerated, then allowed to equilibrate to room temperature prior to injection.
Four groups of 10 male and 10 female Sprague-Dawley rats (Groups 1-4) were formed to receive flutolanil TGAI in the diet at concentrations of 0 (negative control), 750, 3000 or 12000 ppm for at least 4 weeks prior to sacrifice. A fifth group of 5 rats/sex (Group 5) was formed to receive cyclophosphamide (CP) at 2.5 mg/mL and 10 mL/kg by intraperitoneal injection for 4 consecutive days immediately prior to sacrifice. All animals were injected with SRBC preparation (0.5 mL/animal, approximately 2 x 10^8 cells) 4 days prior to sacrifice.
All animals were checked twice daily for mortality and examined at the cage side for clinical signs of toxicity, except on Days 1, 8, 15, 22 and 28, on which more detailed clinical evaluation was conducted. Body weight and food consumption were measured over these same weekly intervals. Terminal sacrifice was on Day 29, following overnight fasting. A detailed necropsy was performed and the weights of the brain, spleen and thymus were recorded. The spleen was collected into medium for use in the AFC assays.
An agarose plaque technique was used to assess the induction of splenic AFC specific for the T cell-dependent antigen SRBC, based on the method of Holsapple, 1995. Single-cell suspensions of each spleen were prepared, total cellularity and viability being determined. The splenocytes were mixed

with fresh SRBC and guinea-pig complement in a semisolid agarose matrix. Monolayers of this mixture were plated and the cultures incubated at approximately 37ºC for at least 3 h. AFC was quantified by visual inspection of the resulting haemolytic plaques.
Statistical analyses were conducted as necessary, using ANOVA, or ANCOVA (for organ weights). Levene's test was applied to test for homogeneity of variances, prior to comparison of treated groups with control using Dunnett's test.

Results

Dose analysis:
Achieved concentrations were within 6.4% of nominal, while relative standard deviations within 5% indicated good homogeneity. In terms of stability, the individual results (109.4 and 95.8%) of the 750 ppm concentration tested 10 days after storage varied by more than 5.0% from the mean of 102.4%; however, since these replicates were still similar (within 7.5% from the mean) and the overall mean value was greater than the original value, the results were considered valid and the preparations were considered stable for 10 days at room temperature, protected from light.
In-life observations:
There were no mortalities and no treatment related clinical signs of toxicity. There were no test item related changes in body weight, the small intergroup differences not correlating with the food consumption values, which also showed no meaningful changes.
Pathology:
There were no test item related macroscopic findings at necropsy, or in the weights of the brain, spleen or thymus.
Antibody-forming cell assay:
No test article-related effects on immune function were observed. All animals had AFC/million cells (AFCM) and AFC/spleen (AFCS) similar to or greater than controls. Although overall spleen cell count and viable cells/spleen for animals given 3000 and 12000 ppm were lower than controls and noted as statistically significant (males), these values were considered not toxicologically relevant by the study authors and were attributed to biological variation, as no differences in organ weights were apparent and these animals had mean AFCM values greater than controls with similar AFCS values, indicating the T-cell dependent antibody response was functionally similar to controls. However, the RMS concludes that an effect induced by flutolanil cannot be completely excluded since a clear dose-dependent response was observed in males that was statistically different from the control.

Table B.6.8.2-1: mean antibody-forming cell data

Group/ Sex		Cell Count (E3/uL)	PVSP Splenocyte Viability %	AVSP(E6) Viable Cells per Spleen	AFCM AFC/ 1e6 Cells	AFCS(E3) AFC/spleen
	Mean	41.27	100	411	1667	658
	SD	11.211	0.48	111.28	838.80	289.13
0 ppm/ M	N	10	10	10	10	10
	Mean	37.27	100	371	1869	641
	SD	10.778	0.69	107.68	788.92	191.73
750 ppm/ M	N	10	10	10	10	10
	Mean	27.91*	99	277*	2599	645
	SD	8.316	0.83	83.10	1385.03	198.32
3000 ppm/ M	N	10	10	10	10	10
	Mean	23.77*	100	237*	2436	566
	SD	6.306	0.51	63.02	767.48	203.44
12000 ppm/ M	N	10	10	10	10	10
	Mean	4.62*	99	46*	0*	0*
	SD	2.200	1.27	22.29	0.00	0.00
Cyclophosphamide/ M	N	4	5	4	4	5
	Mean	28.41	100	284	1695	481
	SD	6.240	0.3	62.3	1049.1	277.3
0 ppm/ F	N	10	10	10	10	10
	Mean	34.69	100	347	1550	530
	SD	7.506	0.3	75.2	699.0	264.1
750 ppm/ F	N	10	10	10	10	10
	Mean	27.28	100	272	2350	620
	SD	5.829	0.7	59.1	1572.1	362.2
3000 ppm/ F	N	10	10	10	10	10
	Mean	30.44	100	304	1886	528
	SD	5.519	0.4	54.9	1375.3	279.5
12000 ppm/ F	N	10	10	10	10	10
	Mean	4.11*	100	41*	102*	1*
	SD	3.161	0.0	31.6	228.2	2.2
Cyclophosphamide/ F	N	5	5	5	5	5
* = significant at $p \leq 0.05$ when compared to the control group.						

Conclusions

Although the study author indicated that administration of flutolanil TGAI in the diet to rats for 4 weeks at concentrations up to 12000 ppm had no effect on immune function as measured in the antibody-forming cell assay, the RMS concludes that an effect induced by flutolanil cannot be completely excluded. The dose-related reduction in overall spleen cell count and viable cells/ spleen might indicate a potential immune suppression. A NOAEL of 750 ppm was therefore concluded, equivalent to an overall mean dosage of 61.1 mg/kg/day for males, 74.6 mg/kg/day for females.

B.6.8.3 Studies on endocrine disruption

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The review submitted provides an assessment of flutolanil and its potential

	for endocrine disruption based on 1) studies reviewed in the DAR, 2) studies conducted for USEPA's endocrine disruptor screening program, 3) ToxCast in vitro studies and 4) a search of published literature. The review of Odum assessed the endocrine disrupting potential of flutolanil within the framework of European legislation. Summaries of the underlying studies included in the review of Odum (2016) which were not included in the DAR are presented below (B.6.8.3.1-B.6.8.3.10). Dose repeated toxicity studies with information relevant for endocrine systems that were evaluated in the DAR are also included in the current RAR. Also ToxCAst was evaluated and results were in line with that described in the review of Odum. Based on the review of Odum, it can be concluded that overall flutalonil does not show endocrine disrupting effects. This conclusion is in line with the weight of evidence assessment made by the USEPA (2015).

Reference	**CA 5.8.3/01: Odum, J *et al* (2016)**	**-**
Title	Assessment of flutolanil and its potential for endocrine disruption	
Report No.	NIC002-001	

Executive summary

The potential of flutolanil and any major metabolites to interact with endocrine systems in mammals and wildlife has been reviewed, to facilitate an assessment of whether flutolanil may be judged to be an endocrine disrupter (ED) within the framework of European legislation. The studies on which this assessment is based are those reviewed in the EC Draft Assessment Report on flutolanil (DAR, 2006), studies conducted for USEPA's Endocrine Disruptor Screening Program (USEPA, 2015), Toxcast *in vitro* studies (USEPA, 2016) and a search of published literature. The evidence shows that flutolanil does not interact with molecular endpoints known to cause endocrine activity. Flutolanil also had no endocrine activity in mammalian assays specific for endocrine disruption and in other regulatory studies with endpoints relevant for endocrine disruption. This weight of evidence indicates that it does not interact with mammalian endocrine systems in studies designed to detect effects relevant for human health. The major mammalian metabolites M-2 and M-4 have also been concluded to have no effect on endocrine systems. This conclusion was drawn by the study authors based on an EFSA conclusion (2008) stating the following: "It was concluded that the metabolites M-4 and M-2, being the main rat metabolites, had the same toxicological profile as flutolanil." Thus, when performing in vivo endocrine studies in rat also the endocrine effects caused by these metabolites will be determined. The RMS agrees with this conclusion since the metabolites M2 and M4 are considered as being the main rat metabolites. Formation of metabolite M2 and M4 in the metabolism of flutolanil was found to be >10% and therefore it can be concluded that the toxicological studies with flutolanil provide adequate information to cover for M2 and M4. In ecotoxicity assays, flutolanil gave equivocal results in the Fish Short-Term Reproduction Assay, most likely due to systemic toxicity. In acute and chronic regulatory ecotoxicity studies in fish there were no endocrine-related effects. There was no evidence of effects on amphibians. In reproduction studies in birds there was no evidence that flutolanil has endocrine activity. The human health studies demonstrate that flutolanil will not cause endocrine effects in wild mammals.

According to the criteria suggested by EFSA (2013), an ED is defined by the presence of i) an adverse effect in an intact organism, or a (sub)population; ii) an endocrine activity; iii) a plausible relationship between the two. Flutolanil does not produce an adverse effect relevant for human health and it does not cause adverse effects in wildlife species at concentrations below those where effects might be expected as a consequence of systemic toxicity. The weight of evidence indicates that it has no endocrine activity.
Overall, flutolanil does not fit either the interim EC criteria, or the recently published (but not yet ratified as final) 2016 EC criteria, with respect to human or environmental health and therefore is not an ED.

B.6.8.3.1 Studies on endocrine disruption, effects on estrogen pathway, study 1

Luciferase activity assay

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study is performed according to OECD guideline 455 and is considered acceptable.

reference	:	Akhurst (2011)	vehicle	:	DMSO
Report number	:	T-3202	doses	:	0 - 100 µM
test substance		Flutalonil	GLP	:	yes
test system	:	human cell line (HeLa-9903)	guideline	:	In accordance with OECD 455
			acceptability	:	Yes, supplemental study

Executive summary

The purpose of this study was to determine the potential for Flutolanil to function as an estrogen receptor α (ERα) ligand by activating an agonist response in a human cell line (HeLa-9903). ERα activation was determined using a luciferase activity assay system. The test was conducted on two independent occasions. The highest concentration of Flutolanil was 100 µM which was the highest soluble concentration. It was observed that Flutolanil was slightly cytotoxic at 100 µM, producing viability values of 77.36 and 79.55% of the solvent control value in the first and second tests, respectively. As these viability values were below 80%, the dose level of 100 µM was not used in the data analysis. The highest dose level used in the data analysis was 10 µM. In both tests, Flutolanil produced a negative response for ERα activation. The four reference chemicals produced the anticipated response for a strong estrogen (17β- estradiol), a weak estrogen (17α-estradiol), a weak agonist (17α-methyltestosterone) and a negative chemical (corticosterone). It was concluded that Flutolanil did not show evidence of ERα transcriptional activation in this test system.

Materials and methods

The test substance was dissolved in DMSO. In order to ensure that the solvent did not cause cytotoxicity, it was used at a final concentration of 0.1% v/v. The highest concentration of test substance in DMSO used was 100 µM, based on a test for solubility that showed the highest soluble concentration of Flutolanil in culture medium to be 100 µM. Six serial ten-fold dilutions in DMSO were then prepared from the 100 mM concentration, giving a dose range of seven concentrations. The responsiveness of the test system was assessed during each test using appropriate concentrations of a strong estrogen (E2, 17β-estradiol), a weak estrogen (17α-estradiol), a very weak agonist (17α-

methyltestosterone) and a negative chemical (corticosterone). Each reference chemical was dissolved in DMSO and then serial ten-fold dilutions prepared to give a test range of seven concentrations. The positive control was E2 at a final concentration of 1 nM. The vehicle control was DMSO, the solvent that was used to dissolve the test substance. It was used at a final concentration of 0.1% v/v.
The vehicle control was established by adding a 1.5 µL aliquot of DMSO to 500 µL of culture medium and then adding 50 µL aliquots of this dilution in medium to the assay plate. Six replicate wells were prepared. The positive control was established by adding a 1.5 µL aliquot of the E2 dilution at 1 µM to 500 µL of culture medium and then adding 50 µL aliquots of this dilution in medium to the assay plate to give 1 nM. Six replicate wells were prepared. Two test plates were established – one plate to be used for the luciferase assay and the second plate to investigate cytotoxicity using MTS. Each dilution of the test and reference chemicals and controls was added in 1.5 µL aliquots to 500 µL of culture medium. The dilution in culture medium was then added in 50 µL aliquots to the assay wells. This gave a total volume in each well of 150 µL. This was performed in triplicate for each dilution of the test substance and reference chemicals. The final concentrations of Flutolanil were 100 µM, 10 µM, 1 µM, 100 nM, 10 nM, 1 nM and 100 pM. Steady-Glo® Luciferase Assay System from Promega (E2520, lot number 309185) was used to perform the luciferase assay. The assay was conducted on two independent occasions using the same concentrations of Flutolanil in both tests.

Results

It was observed that Flutolanil was slightly cytotoxic at the highest test concentration used, 100 µM, which produced viability values of 77.36 and 79.55% of the solvent control value in the first and second tests, respectively. As these viability values were below 80%, the dose level of 100 µM was not used in the data analysis, including calculation of log PC10.
The luminescence values for log PC50, log PC10 and RPCMax for each test were calculated using the OECD 455 spreadsheet. Flutolanil produced a negative response with a RPCMax of <10%. The RPCMax was determined to be 6.1%. In this first test, all acceptance criteria for both the test and reference plates were met, except the following:

- The Hill slope for 17α-estradiol, 0.73, lay just outside the acceptance range of 0.9 – 2.0.
- The log PC10 for 17α-methyltestosterone, -8.10, lay just outside the acceptance range of -8.0 - -6.2.

In this second test, all acceptance criteria for both the test and reference plates were met except the following:

- The Hill slopes for 17α-estradiol, 0.87, lay just outside the acceptance range of 0.9 – 2.0.
- The log PC10 for 17α-methyltestosterone, -8.31, lay just outside the acceptance range of -8.0 – -6.2.
- Corticosterone produced a slight response with a log PC10 of -4.38 and RPCMax of 11.3%.

The coefficient of variation (CV) values of the luminescence reading triplicates were calculated for both tests. All CVs were <20%, with the exception of two values for corticosterone and one value for 17β-estradiol, for the reference chemicals of the first test, which gave CVs of 20%, 20% and 22%, respectively. The deviations from the acceptance criteria listed above were not considered to affect the

sensitivity of the assay. Overall, the performance of the reference chemicals was satisfactory and Flutolanil was observed to produce a reproducible negative response in both assays.

Conclusion

It was concluded that Flutolanil did not show evidence of ERα transcriptional activation in this test system.

B.6.8.3.2 Studies on endocrine disruption, effects on estrogen pathway, study 2

Uterotrophic assay for estrogenic activity

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study is performed according to OECD guideline 440 and is considered acceptable. The study was also evaluated as part of USEPA's endocrine disruptor screening program and was considered acceptable. The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study. Based on the uterotropic assay in rats it can be concluded that flutolanil is not estrogenic under the test conditions applied.

reference	:	[redacted] (2011)	exposure	:	Via oral gavage
Report number	:	LSRC-TII-0018A (T-3195)	doses	:	0, 500, or 1000 mg/kg bw/day
test substance	:	flutalonil	GLP	:	yes
species	:	Sprague Dawley rats	guideline	:	OECD 440
group size	:	6 females/ group	acceptability	:	yes

Executive summary

Flutolanil was tested in a Uterotrophic assay for estrogenic activity in 67-69-day old, ovariectomized female Sprague Dawley rats (n=6 per group). Flutolanil was administered daily via oral gavage at doses of 0 (vehicle; 0.5% methyl cellulose), 500, or 1000 mg/kg bw/day. The positive control group was dosed 17 α -ethynylestradiol (0.5 µg/kg bw/day) by s.c. injection. Dosing was for 3 days, after which wet and blotted uterine weights were determined. Livers were also removed and weighed. Body weights, body weight gains, and food consumption in the flutolanil treatment groups were comparable to the vehicle controls. There were no treatment-related effects on wet or blotted uterine weights, or liver weight, in the flutolanil treatment groups. The positive control group performed as expected. Flutolanil was negative for estrogenicity in the Uterotrophic assay.

Materials and methods

Female rats were subjected to ovariectomy at 6 weeks old, and then they were confirmed to be free from estrus cycling after 3-weeks of recovery period. They were assigned to following 4 experimental groups (6 animals each) on their postnatal day 67; vehicle control, positive control, and Flutolanil treated groups at high and low dose levels. From the day of assignment, animals in each group were given following oral gavage administration and subcutaneous injection for consecutive 3 days. Oral

gavage dosing of Flutolanil (98.7% purity) suspended in 0.5% methyl cellulose solution (MC) was given at 1000 mg/kg/day (a limit dose) and 500 mg/kg/day (its half dose) for high and low dose levels, respectively. For positive control group, 17α-ethynyl estradiol dissolved in corn oil was given by subcutaneous injection at a dose of 0.5 µg/kg/day, which was previously found to be approximately equivalent to ED70 to ED80 in this testing facility. Vehicle control group was given oral gavage dosing of MC and subcutaneous injection of corn oil. Other experimental groups were given oral gavage dosing and/or subcutaneous injection of comparative vehicle as necessary. Animals were observed daily for clinical sign, body weight and food consumption. Animals were euthanatized 24-hours after final treatment and their uterus was collected to weigh its wet weight and blotted weight. Liver weight was measured since it was target organ by Flutolanil. Also, necropsy findings were recorded.

Results

Group mean of relative wet uterine weight to terminal body weight in vehicle control group was 0.0363 %, therefore current study condition fulfills the prescribed performance criteria (< 0.04%) for variectomized model indicated in testing guidelines of the Uterotropic assay. In positive control group, their wet and blotted uterine weight showed expected increase with statistical significance. Therefore, the technical performance and sensitivity of this study was considered to be valid. Under such condition, in Flutolanil treated groups, no treatment-related change was observed in any of clinical sign, body weight, body weight gain, food consumption, necropsy findings. Both wet and blotted uterine weight was comparable with vehicle control group and no effect of treatment was identified at any dose levels of Flutolanil.

Conclusion

As a conclusion of this Uterotrophic assay in ovariectomized rats, Flutolanil is not estrogenic up to the limit dose at 1000 mg/kg/day.

B.6.8.3.3 Studies on endocrine disruption, effects on estrogen pathway, study 3

ER-binding assay

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study is not performed according to an OECD guideline or GLP and is published in publically available literature. Based on the study it can be concluded that flutolanil contains estrogenic activity. A drawback of this stud however is that the purity of flutalonil is unknown. Furthermore, the results might be considered equivocal since: - ERα protein levels were not quantified; based on western blot analysis Erα protein levels were found to be decreased when MCF-7 cells were exposed to flutolanil - A quantitative investigation of gene expression is lacking; PR and pS2 mRNA expression was determined but not quantified.

	- The number of replicates remains unclear. The study was evaluated using the ToxRTool to assess the realiability of the data and was assigned Klimisch score 3. The study shoud therefore not be used as a key study but based on the above, the study should be regarded as supplemental.

reference	:	Oh *et al.* (2007)	vehicle	:	
Report number	:	Published literature: *Sci Total Environ.* 388, 8-15	doses	:	
test substance		Flutalonil	GLP	:	no
test system	:	MCF-7 BUS estrogen receptor (ER)-positive human breast cancer cells and ER-negative MDA MB 231 cell lines	guideline	:	n.a.
			acceptability	:	Yes, supplemental study

Executive summary

Endocrine disruptors, when absorbed into the body, interfere with the normal function by mimicking or blocking the hormone system. To investigate compounds mimicking estrogen in the drinking water source of the residence of Seoul, the Pal-dang reservoir was monitored over a period of 5 years, between 2000 and 2004. Nine kinds of pesticide (carbaryl, DBCP, diazinon, fenitrothion, fenobucarb, flutolanil, iprobenphos, isoprothiolane and parathion) were found to exist in the river water sample. These compounds were detected at low concentrations in the water samples. The total concentration and those of each of these pesticides were below the permissible limits of the National Institute of Environmental Research (NIER), Korea. The estrogenic potencies of the nine pesticides were examined using an E-screen assay with MCF-7 BUS estrogen receptor (ER)-positive human breast cancer cells, with ER-negative MDA MB 231 cell lines also used to compare the results. From this, flutolanil and isoprothiolane were confirmed to have estrogenic activities as shown by the increasing MCF-7 BUS cell growth on their addition. In addition, the estrogen receptor alpha (ERα) protein, estrogen receptor-regulated progesterone receptor (PR) and pS2 mRNA levels on the addition of flutolanil and isoprothiolane were measured with MCF-7 BUS cells. It was observed that the levels of ERα protein decreased and those of the PR and pS2 genes increased on the addition of either flutolanil or isoprothiolane at concentrations of 10−4 M, in the same manner as with the addition of 17β-estradiol, which was used as the positive control. From these results, it was confirmed that flutolanil and isoprothiolane exhibit estrogenic activities, suggesting they might act through estrogen receptors.

B.6.8.3.4 Studies on endocrine disruption, effects on estrogen pathway, study 4

Aromatase assay

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study was evaluated as part of USEPA's endocrine disruptor screening program. The USEPA concluded that the study was performed according to test guideline OSCPP 890.1200. For this aromatase assay no OECD test guidance is available. Since the study was already evaluated

	by the USEPA and an OECD guideline is not available, the RMS did not re-evaluate the study. The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study. Based on the study outcome it can be concluded that flutalonil does not inhibit aromatase activity.

reference	:	Yasunaga (2011)	vehicle	:	
Report number	:	LSRC-M11-007A (T-3198)	doses	:	10^{-10} to 10^{-4} M
test substance		Flutalonil	GLP	:	yes
test system	:	human recombinant microsomal aromatase (CYP19)	guideline	:	OSCPP 890.1200
			acceptability	:	yes

Executive summary

Flutolanil was tested for aromatase (CYP19) inhibition in an assay using recombinant aromatase and tritiated androstenedione. For flutolanil, aromatase activity averaged 0.230 ± 0.017 nmol/mg/protein at the lowest tested concentration of 10^{-10} M and 0.242 ± 0.011 nmol/mg/protein at the highest successfully tested concentration of 10^{-4} M. The mean aromatase activity of 10–4 M flutolanil was 105.56% of control activity. The lowest portion of the response curve was greater than 75% activity at all concentrations of flutolanil i.e. no inhibition was observed. Based on the results from the average response curve, flutolanil was classified as a Non-inhibitor of aromatase activity according to the data interpretation procedure in the TG.

Materials and methods

Effect of positive control chemical (4-hydroxyandrostenedione, 4-OH ASDN) and flutolanil (purity 99.5%) on aromatase activity was examined using human recombinant microsomal aromatase (CYP19). Aromatase activity was determined by radiometry of 3H2O produced by aromatization of radiolabeled substrate, [1β-3H]-androstenedione ([3H]-ASDN).

At first, to confirm the basal aromatase activity of the microsome used, full and background activity a control study was done. Secondly, to confirm the validity of test system, a positive control study was done. Finally, the main study was done. At first, the main study was done with 10 concentrations of flutolanil (flutolanil conc.1~conc.10). However, because insolubility (cloudiness) was observed in the highest and second highest concentrations (flutolanil conc.1 and conc.2; final 10-3 and 10-3.5M), these two concentrations were excluded from the second and third runs.

In the aromatase assay the test components were added in 13 × 100 mm glass test tubes, and preincubated in water bath (37 ± 1°C) for 5 min before initiation of assay. Microsomal protein for the assay was prepared by diluting in assay buffer to 0.008 mg/mL, and preincubated in water bath (37 ± 1°C) for 5 min before initiation of assay. After preincubation, 0.008 mg of microsomal protein was added in each assay tube, vortexed gently, and initiated the aromatase reaction by incubating in the water bath (37 ± 1°C) for 15 min. The aromatase reaction was stopped by adding 2 mL of dichloromethane, and then each assay tube was vortexed for 5 sec and placed on ice for 5 min, and

vortexed for an additional 20-25 sec, followed by centrifuged at 200 × g, 4°C for 10 min. After centrifugation, the dichloromethane layers were removed, and then the aqueous layers were extracted again with 2 mL of dichloromethane. After centrifugation at 200 × g, 4°C for 10 min, the dichloromethane layers were removed. This extraction procedure was performed once again, and then the dichloromethane layers were removed. Finally, 0.5 mL of the final obtained aqueous layers were transferred into 20 mL liquid scintillation counting vials in duplicate, mixed with 10 mL of Ultima GoldTM XR, and measured 3H-counts by LSC. The radiochemical purity of [3H]-ASDN was determined using high performance liquid chromatography (HPLC) and microplate scintillation counter (MSC), Top Count NTX.

Results

The results in positive control study are shown in Table B.6.8.3.4-01. Aromatase activity was inhibited by 4-OH ASDN in dose-dependent manner. Average of calculated log (IC50) value, slope, top value (%) and bottom value (%) for 4-OH ASDN were -7.2, -1.0, 97 % and 1%, respectively. These results met the performance criteria (log (IC50) value; -7.3 ~ -7.0, slope; -1.2 ~ -0.8, top (%); 90 ~ 110% and bottom (%); -5 ~ 6%) as defined in the testing guideline. Therefore, the technical performance and sensitivity of this study were considered to be valid.

Table B.6.8.3.4-01: Parameters in positive control study

	Mean	SD	Criteria
Slope	-1.0	0.0	-1.2 ~ -0.8
Top (%)	97	2	90 ~ 110
Bottom (%)	1	0	-5 ~6
Log (IC50)	-7.2	0.1	-7.3 ~-7.0

Under the experimental conditions performed, flutolanil showed no effect on aromatase activity as shown in Summary Table B.6.8.3.4-02.

Table B.6.8.3.4-02: aromatase activity in the presence of flutolanil

Log conc. (M)	Number of runs	Percent of control activity (%)	
		Mean	SD
-4.0	3	105.56	5.33
-4.5	3	104.13	7.89
-5.0	3	104.9	3.99
-6.0	3	101.61	2.60
-7.0	3	101.77	5.22
-8.0	3	102.41	3.05
-9.0	3	98.26	1.64
-10.0	3	99.97	3.62

Conclusion

In conclusion, flutalonil was classified as a non-inhibitor of aromatase activity.

B.6.8.3.5 Studies on endocrine disruption, effects on estrogen pathway, study 5

ER-binding assay

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study is not performed according to an OECD guideline and is published in publically available literature. The study was evaluated using the ToxRTool to assess the realiability of the data and was assigned Klimisch score 1. The study is thus considered reliable. Flutalonil was found negative for ER and AR agonist and antagonist activity. Therefore, it can be concluded that no (anti-)estrogenic or (anti-)androgenic effect was observed for flutalonil under the experimental conditions applied.

reference	:	Kojima (2004)	vehicle	:	DMSO
Report number	:	Public literature: *Environ. Health Perspect.* 112(5), 524–531	doses	:	< 10^{-5} M
test substance		Flutalonil	GLP	:	no
test system	:	Chinese hamster ovary cells	guideline	:	n.a.
			acceptability	:	yes

Executive summary

200 pesticides were tested, including flutalonil, for agonism and antagonism to two human estrogen receptor (hER) subtypes, hERα and hERβ, and a human androgen receptor (hAR) by highly sensitive transactivation assays using Chinese hamster ovary cells. Flutolanil was tested at concentrations < 10–5 M. The results of the ER and AR assays show that flutalonil did not had estrogenic and antiandrogenic activities under the experimental conditions applied.

B.6.8.3.6 Studies on endocrine disruption, effects on estrogen pathway, study 6

Steroidogenesis assay

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study is performed according to OECD guidance 456 and is considered acceptable. The study was also evaluated as part of USEPA's endocrine disruptor screening program. The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study. Based on the study, it can be concluded that flutalonil did not affect testosterone and 17ß-estradiol production under the experimental conditions applied.

reference	:	Hamasake (2011)	vehicle	:	DMSO
Report number	:	LSRC-M11-100A (T-3204)	doses	:	0.0001 to 100 µM
test substance		Flutalonil	GLP	:	Yes
test system	:	human cell line-H295R	guideline	:	In accordance ith OECD 456

acceptability : yes

Executive summary

Flutolanil was tested in a steroidogenesis assay using the H295R cell line. Hormone concentrations in the assay were determined using an enzyme-linked immunosorbent assay (ELISA). Flutolanil was tested at concentrations from 0.0001 to 100 µM in 3 runs. Data at concentrations (≥ 3 µM) were discarded due to cytotoxicity. No effects on testosterone and estradiol concentrations were observed at flutolanil concentrations below cytotoxic concentrations. Flutolanil did not cause statistically significant and reproducible alterations in testosterone or estradiol production. It was therefore negative in the steroidogenesis assay.

Materials and methods

This study was performed to determine whether Flutolanil affects testosterone and 17ß-Estradiol production on a human adrenocotical carcinoma cell line, NCI-H295R. Flutolanil (purity 99.5%) was tested at concentrations from 0.0001 to 100 µM in 3 runs and was dissolved in DMSO (final concentration DMSO 0.1%). Testosterone and 17ß-estradiol were used to determine the quantity of hormones in samples. Reference substances (*i.e.* forskolin and prochloraz) were included to evaluate the sensitivity of test systems. ELISA kits were used for quantification of T and E2 in test medium.

The H295R cells were exposed to the test substances for 48 hours after which cell conditions were visually inspected. Then, medium were collected and stored in a freezer until needed. The cells in the plates were subjected to viability/cytotoxicity assay. Medium were subjected to hormone quantification with ELISA kits. Hormone measurement systems using the ELISA kits was validated by Spike recovery assay.

Results

Cytotoxicity was observed at cocnentrations ≥3 µM. Flutolanil did not significantly change testosterone and 17ß-estradiol production in the H295R cells at non-cytotoxic conditions (Table B.6.8.3.6-01).

Table B.6.8.3.6-01: Fold-changes of testosterone and 17ß-estradiol production in H295R cells

		Fold change relative to SC = 1				LIVE %	
		Testosterone		17ß-estradiol			
	Dose (µM)	Mean	SD	Mean	SD	mean	SD
Flutolanil (run 1)	SC	1.00	0.02	1.00	0.11	100	3
	0.0001	1.01	0.07	1.12	0.13	104	4
	0.001	1.02	0.03	1.24	0.28	101	8
	0.01	1.10	0.03	1.30	0.12	95	6
	0.1	1.10	0.12	1.12	0.26	90	4
	1	1.00	0.10	1.29	0.11	89	5
	10	-	-	-	-	75	5
	100	-	-	-	-	69	1
Flutolanil (run 2)	SC	1.00	0.16	1.00	0.02	100	1
	0.001	0.92	0.03	1.17	0.04	99	5
	0.01	1.07	0.09	1.21	0.17	94	3
	0.1	1.17	0.12	1.22	0.18	91	2
	0.3	1.13	0.20	1.09	0.23	95	4
	1	1.15	0.24	1.06	0.08	88	9

	3	-	-	-	-	80	9
	10	-	-	-	-	77	6
Flutolanil (run 3)	SC	1.00	0.02	1.00	0.12	100	6
	0.001	0.98	0.05	0.98	0.06	94	3
	0.01	1.02	0.03	0.97	0.02	90	2
	0.1	1.13	0.10	0.90	0.02	93	3
	0.3	1.15	0.04	0.95	0.09	100	2
	1	1.09	0.14	0.94	0.04	87	2
	3	1.14	0.11	1.06	0.02	82	2
	10	-	-	-	-	80	6

SC: solvent control, -: No data due to LIVE values 80% and less.

Conclusion

In this study, effects of Flutolanil on the productions of testosterone and 17ß-Estradiol in the H295R cells were evaluated. The test system was validated with regard to the recoveries of hormones, the hormone quantification methods and the test procedures. Flutolanil did not significantly affect the T and E2 production in the H295R cells at non-cytotoxic conditions.

B.6.8.3.7 Studies on endocrine disruption, effects on estrogen pathway, study 7

ER-binding assay

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study was evaluated as part of USEPA's endocrine disruptor screening program. The USEPA concluded that the study was performed according to test guideline OSCPP 890.1250. For this ER binding assay no OECD test guidance is available. Since the study was already evaluated by the USEPA and an OECD guideline is not available, the RMS did not re-evaluate the study. Based on the study outcome it can be concluded that flutalonil does not affect ER binding.

reference	:	[redacted] (2011)	vehicle	:	EtOH and DMSO
Report number	:	GA-27, 11-0063 (T-3205)	doses	:	10^{-10} to 10^{-4} M
test substance		Flutalonil	GLP	:	Yes
test system	:	uterine cytosol from Sprague Dawley rats	guideline	:	OSCPP 890.1250
			acceptability	:	yes

Executive summary

Flutolanil was tested in an Estrogen receptor (ER) binding assay using uterine cytosol from Sprague Dawley rats. In the saturation binding experiment, the dissociation constant (Kd) and maximum binding capacity (Bmax) for [3H]-17ß-estradiol demonstrated that the receptor preparation was adequate for use. In 3 runs of the competitive binding experiment, flutolanil displaced less than 25% of the radiolabeled estradiol from the ER over the range of soluble concentrations tested (10^{-10} to 10^{-4} M). Based on the results from the three assay runs, flutolanil was classified as "Not Interactive" in the ER Binding Assay.

Materials and methods

To examine the effect of Flutolanil on the ligand binding on the ER, rat uterus (obtained from Female SPF rats) was used for the ER and radiolabeled estrogen, [3H]-17beta-estradiol ([3H]-E2), was used for the specific ligand of ER.

At first, to confirm that the ER isolated from rat uterus function with appropriate affinity for the native ligand (E2), a saturation binding assay was done. Total and non-specific binding of increasing concentrations of [3H]-E2 were measured under conditions of equilibrium. From these values, specific bindings were calculated. Secondary, to determine whether Flutolanil has an effect on ligand binding on the ER, a competitive binding assay was done. Flutolanil (purity 99.5%) was tested at concentrations ranging between 10^{-10} and 10^{-4} M dissolved in ethanol. E2 was used as a positive control, 19-norethindrone as a weak positive control and octyltriethoxysilane was used as a negative control. Three replicates were done within one run and three non-concurrent runs were done.

Results

The test system was validated adequately and the classification of E2, 19-norethindrone and Octyltriethoxysilane were classified “Positive”, “Weak positive” and “Negative”, respectively. The results of reference chemicals are shown in Table B.6.8.3.7-01.

Table B.6.8.3.7-01: Results of competitive binding assay of reference chemicals

Chemical	Positive control (E2)	Weak positive control (19-norethindrone)	Negative control (octyltriethoxisilane)
Log IC50 (M)	-9.09 ~-9.04	-5.15 ~-5.01	N/A
IC50	8.10×10^{-10} ~ 9.14×10^{-10}	7.06×10^{-6} ~ 9.75×10^{-6}	N/A
RBA*	1	9.32×10^{-5} ~ 1.15×10^{-4}	N/A
Classification	Positive	Weak positive	negative

* RBA: relative binding affinity (IC50 of E2 = 1)

N/A: not applicable

Under the condition applied, Flutolanil showed no effect on the ligand binding on the ER over the range of concentration (10^{-4} ~ 10^{-10} M). The results are shown in Table B.6.8.3.7-02.

Table 6.8.3.7-02: results of competitive binding assay of flutolanil

Chemical	Test chemical (flutolanil)
Log IC50 (M)	N/A
IC50	N/A
RBA*	N/A
Classification	negative

* RBA: relative binding affinity (IC50 of E2 = 1)

N/A: not applicable

Conclusion

In conclusion, Flutolanil was classified as “Negative” for estrogen receptor binding.

B.6.8.3.8 Studies on endocrine disruption, effects on androgen/ anti-androgenic pathway, study 1

Hershberger assay

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study is performed according to OECD guidance 441 and is considered acceptable. The study was also evaluated as part of USEPA's endocrine disruptor screening program. The study report does not contain a GLP-certificate. However, the performing lab is known to be GLP certified also during the period of conducting the study. Based on the Hershberger assay it can be concluded that flutolanil is negative for androgenicity and anti-androgenicity.

reference	:	[redacted] (2011)	exposure	:	Via oral gavage
Report number	:	LSRC-T10-117A (T-3195)	doses	:	0, 500, or 1000 mg/kg bw/day
test substance	:	flutalonil	GLP	:	Yes
species	:	Sprague Dawley rats	guideline	:	OECD 440
group size	:	6 males/ group	acceptability	:	yes

Executive summary

Flutolanil was tested in a Hershberger assay in 57-day old, castrated male Sprague Dawley rats (n=6 per group). In the androgenic phase, flutolanil was administered daily via oral gavage at doses of 0 (vehicle; 0.5% methyl cellulose), 500, or 1000 mg/kg bw/day. The androgenic positive control group was dosed testosterone propionate (TP) (0.4 mg/kg bw/day) by subcutaneous (s.c.) injection. In the antiandrogenic phase, flutolanil was administered daily via oral gavage at doses of 0 (vehicle; 0.5% methyl cellulose), 500, or 1000 mg/kg bw/day in conjunction with a daily dose of reference androgen TP (0.4 mg/kg bw/day, s.c.). TP alone was used as the anti-androgenic negative control. The antiandrogenic positive control group was dosed with flutamide (3 mg/kg bw/day) plus TP (0.4 mg/kg bw/day).

Dosing was for 10 days, after which androgen-sensitive tissues (seminal vesicles; ventral prostate; levator ani-bulbocavernosus (LABC) muscle; Cowper's gland; and glans penis) and liver were removed and weighed.

In the agonist and antagonist assays, body weights, body weight gains, and food consumption in the flutolanil treatment groups and were comparable to the vehicle controls.

There were no treatment-related effects on weights of accessory sex organs or liver for any flutolanil treatment group. The positive control groups performed as expected.

Flutolanil was negative for androgenicity and anti-androgenicity in the Hershberger assay.

Materials and methods

Oral gavage was chosen for administration of Flutolanil, as oral intake is considered the most relevant exposure route of agricultural chemicals for human and the preferable procedure for this compound, that is insoluble in water or water based vehicle. Flutolanil was prepared in suspension using 0.5% methyl cellulose solution (MC) as a vehicle. In dose finding study conducted in 3 castrated male rats each by consecutive 10-days dosing at 1000 mg/kg/day (limit dose) and 500 mg/kg/day (its half dose),

no effect of treatment was found in body weight, food consumption, clinical signs and necropsy findings. Therefore, for main assay, 1000 and 500 mg/kg/day were employed for high and low dose group, respectively. Main assays for androgen and antiandrogen were conducted by using groups of castrated male rats (6 animals/group, 15 and 16 days after castration respectively) with repeated treatments for consecutive 10-days as follows. In androgen assay, vehicle control group and androgenic positive control group were given daily oral gavage dosing of MC at 10 mL/kg/day, while groups for low and high dose level of Flutolanil were dosed with Flutolanil at respective dose levels. Immediately after the dosing, subcutaneous injection of corn oil at 0.5 mL/kg/day was performed except for positive control group that was given subcutaneous injection of testosterone propionate (TP) in corn oil at 0.4 mg/kg/day. In antiandrogen assay, TP control group was given oral gavage of MC at 10 mL/kg/day, a positive antiandrogenic control group was given oral gavage dosing of Flutamide at 3 mg/kg/day using this vehicle, and groups for low and high dose level of Flutolanil were dosed with Flutolanil at respective dose levels. All of four experimental groups were then given subcutaneous injection of TP in corn oil at 0.4 mg/kg/day immediately after oral dosing. Then, 24-hours after final dosing, liver (target organ of Flutolanil) and five androgen dependent tissues/organs including glans penis, ventral prostates, combined seminal vesicles plus coagulating glands, combined levator ani muscle plus bulbocavernous muscle complex and Cowper's glands were weighed for evaluation.

Results

In Flutolanil treated groups, up to the limit dose of 1000 mg/kg/day, no toxicological effect was observed in any assay by means of body weight, body weight gain, food consumption, clinical sign, necropsy finding and liver weight. Also, no treatment related effects on the weight of five androgen dependent tissues/organs were observed in comparison with respective control groups. In both assays, technical performance and sensitivity of assays were verified as follows. Variation of organ weight (i.e. coefficient of variation) in each study group satisfied acceptable performance criteria for all of five androgen dependent tissues/organs. In positive control groups (i.e. TP group in androgen assay and Flutamide group in antiandrogen assay), expected statistically significant increase or decrease of the weight of five androgen-dependent tissues/organs was observed.

Conclusion

As a conclusion of this Hershberger bioassay, Flutolanil is not androgenic, antiandrogenic or inhibitor for 5 alpha-reductase up to the limit dose at 1000 mg/kg/day.

B.6.8.3.9 Studies on endocrine disruption, effects on androgen/ anti-androgenic pathway, study 2

Male Pubertal assay

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study was evaluated as part of USEPA's endocrine disruptor screening program. The USEPA concluded that the study was performed according to test guideline OPPTS 890.1500 (male rats) and OPPTS

	890.1450 (female rats). For these pubertal assays no OECD test guidance is available. Since the studies were already evaluated by the USEPA and an OECD guideline is not available, the RMS did not re-evaluate the studies. Based on the pubertal assays in male and female rats it can be concluded that flutolanil has no effects on thyroid parameters tested (including thyroid weight, histopathological findings an hormone levels), that there was no androgen-related response and no evidence of an estrogenic response (females).

reference	:	[redacted] (2011)	exposure	:	Via oral gavage
Report number	:	LSCR-T12-068A (T-3223)	doses	:	0, 500, or 1000 mg/kg bw/day
test substance	:	flutalonil	GLP	:	Yes
species	:	Sprague Dawley rats	guideline	:	OPPTS 890.1450 and OPPTS 890.1500
group size	:	15/ sex/ group	acceptability	:	yes

Executive summary

Male rats:

Flutolanil was tested in a Male Pubertal assay (USEPA TG OCSPP 890.1500). Male Sprague Dawley rats (n=15 per group) were treated daily via oral gavage, at doses of 0 (vehicle; 0.5% methyl cellulose), 500, or 1000 mg/kg bw/day from post-natal day (PND) 23 to 53. Animals were examined for preputial separation (PPS) and age and weight at day of attainment were recorded. Following sacrifice on PND 53, blood clinical chemistry and total testosterone levels were determined by EIA, total T4 levels were determined with ELISA, and TSH levels were determined by radioimmunoassay (RIA). Adrenals, liver, pituitary, thyroid, and urogenital organ weights were weighed and microscopic examinations of the testes, epididymides, thyroid, and kidneys were performed. Body weights, body weight gains, and food consumption in the flutolanil treatment groups were comparable to the vehicle controls. There were no treatment-related effects on age at PPS, body weight at PPS, organ weights (except liver), clinical chemistry, or hormone levels (serum T4, TSH, or testosterone). There were no treatment-related histopathological findings in the thyroid, testes, epididymides, or kidneys. Relative liver weights were increased in both dose groups. Flutolanil had no effects on endpoints diagnostic of interaction with the androgen and thyroid hormonal systems in this male pubertal assay.

Female rats:

Flutolanil was tested in a Female Pubertal assay. Female Sprague Dawley rats (n=15 per group) were treated daily via oral gavage, at doses of 0 (vehicle; 0.5% methyl cellulose), 500, or 1000 mg/kg bw/day from post-natal day (PND) 22 to 42. Animals were examined for vaginal opening (VO) and age and weight at day of attainment were recorded. Following sacrifice on PND 42, blood clinical chemistry and total T4 levels were determined with ELISA, and TSH levels were determined by radioimmunoassay (RIA). Adrenals, liver, pituitary, thyroid, and urogenital organ weights were weighed and microscopic examinations of the uterus, ovaries, thyroid, and kidneys were performed. Body weights, body weight gains, and food consumption in the flutolanil treatment groups were comparable to the vehicle controls. There were no treatment-related effects on age at VO, body weight at VO, organ weights (except liver), clinical chemistry, or hormone levels (serum T4 or TSH).

There were no treatment-related histopathological findings in the thyroid, uterus, ovaries or kidneys. Relative liver weights were increased in both dose groups. Flutolanil had no effects on endpoints diagnostic of interaction with the estrogen and thyroid hormonal systems in this female pubertal assay.

Materials and methods

Totally 45 male and 45 female pups of Sprague-Dawley rats (Crl:CD) were selected for assignment to 3 experimental groups (15 male and 15 females each for two dose levels of flutolanil and vehicle control) at their weaning on postnatal day (PND) 21. They were given daily oral gavage of 0.5% (w/v) methylcellulose solution or suspension of flutolanil in this vehicle at 500 and 1000 mg/kg/day during their postnatal day 23-53 and 22-42 for males and females, respectively. Clinical observation and measure of body weight were conducted daily and pubertal development (preputial separation and vaginal opening) were observed daily from PND 30 and 22 for males and females, respectively. After vaginal opening, their estrus cycling was examined daily by vaginal smears. Animals were subjected to necropsy and their blood was collected for analysis of serum hormone level (T4 & TSH for both sexes and testosterone for male) and the standard panel of blood chemistry. Kidneys, pituitary, adrenals, thyroid and male/female reproductive organs were collected for weigh as required by the testing guideline, and the liver as a target organ of flutolanil additionally. Histopathological examination was performed on the testis, epididymis, ovary, uterus, thyroid and kidney as required.

Results

Administration of flutolanil to male and female rats at any dose did not affect their body weight, sexual maturation, estrus cycling, hormone concentrations, blood chemical parameters and organ weight, except for the liver on which slight but clear increase was found in the absolute and adjusted weight in males at 1000 mg/kg/day, and the relative weight in males and females at and above 500 mg/kg/day. Effects of flutolanil treatment were not found in the findings of necropsy or histopathology.

Conclusion

As a conclusion of this study, flutolanil did not show any evidence for disruption of thyroid function, of the androgen or estrogen systems, or of sexual maturation in pubertal male and female rats.

B.6.8.3.10 Studies on endocrine disruption, effects on androgen/ anti-androgenic pathway, study 3

AR-binding assay

Previous evaluation	Newly submitted for the purpose of the renewal.
Evaluation of the RMS	The study was evaluated as part of USEPA's endocrine disruptor screening program. The USEPA concluded that the study was performed according to test guideline OSCPP 890.1150. For this AR binding assay no OECD test guidance is available. Since the study was already evaluated by the USEPA and an OECD guideline is not available, the RMS did not re-evaluate the studies. The study is performed under GLP. Based on the results it can be conclude that flutalonil resulted in a reduced binding in the first experiment (HV 10). However, the inbibition was not

	found to be dose-related nor was it observed in the following two experiments (HV 11 and HV 12). Taking into account the negative results of the AR transactivation assay and the in vivo Hersberger assay, it was concluded based on a weight-of-evidence approach, that the effect of flutalonil on AR binding is negative based on the experimental conditions applied.

reference	:	[redacted] (2011)	vehicle	:	EtOH
Report number	:	T-3207	doses	:	10^{-9} to 10^{-4} M
test substance		Flutalonil	GLP	:	Yes
test system	:	prostate cytosol from Sprague Dawley rats	guideline	:	OSCPP 890.1150
			acceptability	:	yes

Executive summary

Flutolanil was tested in an Androgen Receptor (AR) binding assay using prostate cytosol from Sprague Dawley rats. The range of flutolanil concentrations tested was 10^{-9} to 10^{-4} M. In the saturation binding experiment, the dissociation constant (Kd) and maximum binding capacity (Bmax) for [3H]-R1881 demonstrated that the receptor preparation was adequate for use. In 3 runs of the competitive binding experiment, flutolanil reduced the binding of the radiolabeled ligand in the first run but results were variable and not dose specific. In the second and third runs, flutolanil did not reduce the binding of the radiolabeled ligand at any concentration. Based on the results from the three assay runs, flutolanil was classified as Equivocal in the AR Binding Assay according to the data interpretation procedure in the TG. The overall conclusion however is that flutolanil does not bind to the AR.

Materials and methods

Test Item:	Flutolanil technical grade
Internal Test Item Number:	S 12538 11
Identity:	Flutolanil technical grade
Name for report:	Flutolanil
Batch-No.:	9AE0011P
Molecular Weight:	323.3 g/mol
Purity:	98.7 %, dose calculation will be adjusted to purity
Storage:	At room temperature, light protected
Expiration Date:	July 11, 2013

Strong positive control:	R1881
Internal Substance Number:	M 12300 11
Identity:	R1881
Batch-No.:	060M46381V
Molecular Weight:	284.39 g/mol
Purity:	98.0 % (HPLC), dose calculation will be adjusted to purity
Storage:	at 2 – 8°C
Expiration Date:	19.08.2015

Weak positive control:	Dexamethasone
Internal Substance Number:	M 12243 11
Identity:	Dexamethasone
Batch-No.:	1419230 &
Molecular Weight:	392.46 g/mol
Purity:	98.9 % (HPLC), dose calculation will be adjusted to purity
Storage:	at 2 – 8°C with light protection
Expiration Date:	11/2013
Internal Substance Number:	M 12243 22
Identity:	Dexamethasone
Batch-No.:	BCBF2909V
Molecular Weight:	392.46 g/mol
Purity:	99.4 % (HPLC), dose calculation will be adjusted to purity
Storage:	at 2 – 8°C with light protection
Expiration Date:	02/2016

The assay described in this study measured the ability of a radiolabeled ligand (radiolabeled R1881, [3H]-R1881) to interact with the androgen receptor (AR) in the presence of increasing concentrations of the test item Flutolanil. Rat prostate cytosol containing AR was incubated in test tubes with increasing concentrations of the test item and an aliquot of radiolabeled [3H]-R1881. If a test item interacted with the receptors hormone-binding domain (HBD), less radioligand was bound, so an active competitor produced a descending doseresponse plot. Similarly, compounds that did not displace radiolabeled R1881 from AR would be presumed to be devoid of androgen-binding activity. As test system rat prostate cytosol of castrated Sprague-Dawley rats was used (60-90 days of age at time of kill and castrated 24 h prior to kill). The assay was performed to evaluate the potential of the test item Flutolanil to inhibit the androgen receptor in the prepared cytosol and to interact with the binding of the strong substrate [3H]-R1881 to the receptor. In the competitive binding assay three replicates at each concentration of test item were prepared. R1881 (positive) and Dexamethasone (weak positive) were used as positive controls. The corresponding solvent, EtOH, was used as negative control. Each set of concentrations constituted a run. Three adequate independent runs constituted an experiment.

Results

The assay was performed to evaluate the potential of the test item Flutolanil to inhibit the androgen receptor in the prepared cytosol and to interact with the binding of the strong substrate [3H]-R1881 to the receptor. It can be clearly stated that up to 100 µM Flutolanil showed no potential to inhibit the androgen receptor binding of the substrate. In none of the tested batches of cytosol and runs (12 in total, nine not reported) any inhibition could be detected for the test item. Table B.6.8.3.10-01 presents the percent binding for the competitive inhibition with flutolanil. The validity of the assay could be shown by the strong positive control R1881 which inhibited the binding of the radiolabeled substrate completely with EC50 values of 0.97 nM to 2.79 nM. This validity was supported by the inhibition

detected with the weak inhibitor control dexamethasone, which showed EC50 values of 26.1 µM and 43.2 µM (about 10000 times lower than for the strong positive control). The batch of cytosol preparation used, showed a Kd of 3.02 nM in the saturation experiment with the expected saturation kinetics visible in the curve. The characterized cytosol had a Kd value slightly above the preferred range of 0.685 to 1.57 nM and the radioactivity bound (0.25 nM) was about 13 %, but this did not affect the acceptability of the batch and the following competitive inhibition experiments because saturation was detectable and the binding curve fitted very well to a one site binding using Hill Slope calculation. For the competitive binding assay, the optimal amount of cytosolic protein added contained enough receptor to bind approx. 10-15 % of the radiolabeled [3H]-R1881 that had been added to the sample. For all three runs this criterion was met with values between 11.6 % and 14.2 %. The competitive binding assay for R-1881 and Dexamethasone met all the requested criteria except for bottom level criteria for the weak inhibitor dexamethasone with the amount of bound radioactivity of about 12-15 %. Additionally the top level plateau values for the lowest concentrations added sometimes differed more than the preferred values of 82 – 114 % for the strong inhibitor R1881 and 87 – 106 % for the weak inhibitor dexamethasone. However, the EC50 values for each control were in the expected range and the overall curve shapes were as expected for receptor binding. The deviations could not be related to sensitivity of the assay system to detect weak inhibitors.

Table B.6.8.3.10-01: Percent binding for the competitive inhibition with flutolanil

[µM] Flutolanil applied	Percent binding Flutolanil HV 10 [specific bound/ mean specific negative control]			Percent binding Flutolanil HV 11 [specific bound/ mean specific negative control]			Percent binding Flutolanil HV 12 [specific bound/ mean specific negative control]		
100.000	104.63	98.84	90.04	107.63	106.59	99.05	96.95	101.16	96.74
10.000	84.02	83.32	77.07	85.78	98.79	109.19	94.22	84.35	87.50
1.000	81.70	78.92	74.29	107.11	102.43	104.25	85.19	83.09	83.09
0.100	68.26	67.10	65.48	99.05	85.52	90.20	80.78	84.14	80.57
0.010	78.69	73.36	71.27	*155.77	97.75	97.48	86.45	88.76	69.02
0.001	72.43	73.59	72.90	100.87	*243.45	104.51	88.55	87.29	84.14

* outlier, not used for calculation

Conclusion

The study authors concluded flutolanil to have no potential to inhibit androgen receptor binding. However, based on the results from the three assay runs, the RMS considers flutolanil to be equivocal in the androgen receptor binding assay. This is in line with the conclusion of the EPA evaluation.

B.6.9 Medical data and information

B.6.9.1 Data collected on humans

No data have been collected, other than the health monitoring of workers (section B.6.9.5.1 below).

B.6.9.2 Direct observations

In the open literature, since 01 January 2006 there have been no reports on adverse health effects of flutolanil in humans, and no clinical cases of poisoning have been reported.

B.6.9.3 Epidemiological studies

Flutolanil has been registered since 1992 in France, and since 1993 in The Netherlands. In 2000, 60,000 kg were sold. There have been no reports of any cases of flutolanil intoxication.

B.6.9.4 Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical tests

Based on clinical findings in a long-term dog study, signs of poisoning might include emesis, salivation and diarrhoea. There are no specific clinical tests.

B.6.9.5 Medical surveillance on manufacturing plant personnel and monitoring studies

B.6.9.5.1 Medical surveillance on manufacturing plant personnel and monitoring studies, study 1

Previous evaluation	DAR B.6.9.1
Evaluation of the RMS	The statement on medical surveillance was also included in the DAR and is considered acceptable.

reference	:	Sumitaka, K and Nakayama, S (2002)	exposure	:	professional
Report number	:	H-3001	doses	:	n.a.
test substance	:	Flutalonil	GLP	:	n.a.
species	:	Human	guideline	:	n.a.
group size	:	40	acceptability	:	acceptable

It is stated that in general, 40 workers are involved in production of products containing flutolanil, with production occupying 130 h per year. Regular health examinations are carried out, including blood chemistry, haematology, urinalysis and a general health questionnaire. There have been no incidences or indications (including sensitization) related to flutolanil in any of the workers.

B.6.9.5.1 Medical surveillance on manufacturing plant personnel and monitoring studies, study 2

Previous evaluation	DAR B.6.9.1
Evaluation of the RMS	The statement on medical surveillance was also included in the DAR and is considered acceptable.

reference	:	Mitsumoto, S and Fuji, T (2002)	exposure	:	professional
Report number	:	H-3002	doses	:	n.a.
test substance	:	Flutalonil	GLP	:	n.a.
species	:	Human	guideline	:	n.a.
group size	:	25	acceptability	:	acceptable

It is stated that in general, 25 workers are involved in production of products containing flutolanil, with production occupying 200 h per year. Regular health examinations are carried out, including blood

chemistry, haematology, urinalysis and a general health questionnaire. There have been no incidences or indications (including sensitization) related to flutolanil in any of the workers.

B.6.9.6 Proposed treatment: first aid measures, antidotes, medical treatment

In case of contact with eyes, flush eyes with large amount of water. If irritation persists, get medical attention. In case of contact with skin, wash with large amount of water, use soap if available, and remove contaminated clothing and wash. If irritation persists, get medical attention. If swallowed, wash out mouth with water, keep patient at rest and obtain immediate medical aid (show product label or the Material Safety Data Sheet if possible). Do not induce vomiting. If inhaled, remove individual to fresh air and obtain medical aid if symptoms persist. There is no specific antidote available.

B.6.9.7 Expected effects of poisoning

The material is of generally low toxicity and no information can be given, beyond that stated in section B.6.9.4 above.

B.6.10 References relied on

Literature search

A multi-concept search of the open peer reviewed literature was conducted focussed on the data requirements of Regulations (EC) 283/2013 and 284/2013 using an STN database. In accordance with Article 8(5) of Regulation (EC) No. 1107/2009 it included only literature that was published within ten years preceding the date of submission of this dossier. The search was conducted for any references which included flutolanil and its relevant metabolites in conjunction with any of the key words set out in Table B.6.10. The findings of this search were assessed for relevance and reliability.

Table B.6.10: detailed search parameters

Search strategy
Active substance terms:
L1 S (FLUTOLANIL OR 66332-96-5)
L2 S ((BENZAMIDE(2W)N(2W)3(2W)1(1W)METHYLETHOXY(1W)PHENYL(2W)2(2W)TRIFLUOROMETHYL) OR ((2(W)TRIFLUOROMETHYL)(2W)BENZAMIDE))
L3 S ((MONCUT(W)40SC) OR (MONCUT(W)40(W)SC) OR MONCUT40SC OR MONCUT-40-SC OR (MONCUT(W)40-SC) OR (MONARCH NOT (BUTTERFL? OR KING# OR QUEEN#)) OR RIALTO OR (NNF(W)136) OR NNF-136 OR NNF136) AND (FUNGICIDE# OR PESTICIDE# OR FLUTOLANIL))
Metabolites terms:
L4 S ((TRIFLUORO(5W)TOLUANILIDE) AND (FLUTOLANIL OR FUNGICIDE# OR BENZANILIDE))
L5 S ((TRIFLUORO(5W)TOLUOYLAMINO) AND (FLUTOLANIL OR FUNGICIDE# OR BENZANILIDE))
L6 S (((2(W)TRIFLUOROMETHYL)(2W)BENZAMIDE) AND (FLUTOLANIL OR FUNGICIDE# OR BENZANILIDE))
L7 S (((2(W)TRIFLUOROMETHYL)(2W)BENZONIC(W)ACID) AND (FLUTOLANIL OR FUNGICIDE# OR BENZANILIDE))
L8 S ((TRIFLUORO-4-HYDROXY-3-ISOPROPOXY-O-TOLUANILIDE) AND (FLUTOLANIL OR FUNGICIDE# OR BENZANILIDE))
L9 S ((TRIFLUORO(1W)3(3W)2(1W) HYDROXY(1W)1(1W)METHYLETHOXY(2W)O(1W)TOLUANILIDE) AND (FLUTOLANIL OR FUNGICIDE# OR BENZANILIDE))
L10 S ((TRIFLUORO-3-HYDROXY-O-TOLUANILIDE) AND (FLUTOLANIL OR FUNGICIDE# OR BENZANILIDE))
L11 S ((TRIFLUORO-3,4-DIHYDROXY-O-TOLUANILIDE) AND (FLUTOLANIL OR FUNGICIDE# OR BENZANILIDE))
L12 S ((TRIFLUORO-3-METHOXY-OTOLUANILIDE) AND (FLUTOLANIL OR FUNGICIDE# OR BENZANILIDE))

L13 S ((TRIFLUORO-4-HYDROXY-3-METHOXY-O-TOLUANILIDE) AND (FLUTOLANIL OR FUNGICIDE# OR BENZANILIDE))
L14 S ((3-ISOPROPOXYANILINE) AND (FLUTOLANIL OR FUNGICIDE# OR BENZANILIDE))
L15 S ((3-AMINOPHENOL) AND (FLUTOLANIL OR FUNGICIDE# OR BENZANILIDE))
L16 S ((TRIFLUORO(1W) O(1W) TOLUOYLAMINO(1W) PHENOXY(1W) PROPIONIC(1W)ACID) AND (FLUTOLANIL OR FUNGICIDE# OR BENZANILIDE))
L17 S ((2(2W)TRIFLUOROMETHYL(2W)BENZAMIDE) AND (FLUTOLANIL OR FUNGICIDE# OR BENZANILIDE))
L18 S ((2(2W)TRIFLUOROMETHYL(2W)BENZONIC(1W)ACID) AND (FLUTOLANIL OR FUNGICIDE# OR BENZANILIDE))
Active substance + metabolites terms:
L19 S (L1-L18) AND (ENG/LA OR ENGLISH/LA) AND (AR/DT OR ARTICLE/DT OR J/DT OR JOURNAL/DT OR ARTICLE IN PRESS/DT) AND PY>2005 NOT (P/DT OR PAT/DT OR PATENT/DT)
SET DUPORDER PERM
L20 DUP REM L19
People exposure terms:
L21 S L20 AND (HUMAN# OR MAN OR MEN OR WOMAN OR WOMEN OR MALE# OR FEMALE# OR GIRL# OR BOY# OR CHILD? OR JUVENILE# OR OFFSPRING OR EMBRYO# OR BABY OR BABIES OR FETUS OR FOET?)
L22 S L20 AND (WORKER# OR OPERATOR# OR EMPLOYE? OR FARMER# OR APPLICATOR# OR
PERSONNEL? OR WORKFORCE? OR BYSTANDER# OR RESIDENT#)
L23 S L20 AND (PERCUTANE? OR DERMAL? OR ORAL? OR INTOXICAT? OR INGEST? OR HAND# OR ACCIDENT? OR POISON? OR ALLERG? OR EXPOS? OR OCCUPAT? OR EPIDEMIOL?
OR SENSITIZ? OR SENSITIS?)
L24 S L20 AND (DIETARY? OR CONSUM? OR CUMULAT? OR ADDITIVE? OR (AGGREGAT?(5A)RISK?) OR ENDOCRIN? OR INHALAT? OR IRRITAT? OR REPROTOX?)
L25 S ((L21-22) AND (L23-L24))
SET DUPORDER FILE
L26 DUP REM L25
Toxicology terms:
LINES 1-20 WERE RERUN
L21 S L20 AND ((PRIMARY OR SECONDARY OR HEALTH OR ADVERSE) AND (EFFECT# OR RISK#))
L22 S L20 AND ((ADAPTIVE(3A)RESPONSE#) OR IMMUNE OR IMMUNOTOX? OR (IMMUNOLOGICAL(1W)EFFECT#) OR (OSSIFICATION AND DELAY) OR CORROSIVE TOXICOKINETIC# OR EXTRACTAB? OR (RADIO(1W)LABEL?) OR SURVEILLANCE)
L23 S L20 AND (DEVELOPMENT? AND (VARIATION OR VARIANT OR DELAY? OR ABNORMAL? OR REPRODUCT? OR PERTURBAT? OR DISTURB?))

L24 S L20 AND (MUTAG? OR CANCER? OR TERATO? OR GENETOX? OR CARCINOGEN? OR ONCO? OR TUMOUR? OR TUMOR? OR CYTOTOX? OR PHOTOTOX? OR DISRUPTOR OR DISRUPTER OR DISRUPTION OR ACCUMULATION OR BIOACCUMULATION OR (BENCHMARK(1W)DOSE#) OR BMD)
L25 S L20 AND (NEUROTOX? OR LD50 OR IC50 OR (LD(1W)50) OR (IC(1W)50) OR STUNTED OR
STUNTING OR RETARD? OR (SYNERGISTIC(5A)(EFFECT OR ACTIVITY)))
L26 S L20 AND ((ACUTE OR CHRONIC OR REPRODUCTIV?) AND (EFFECT# OR TOXI?))
L27 S L20 AND (((LONG OR SHORT)(1W)TERM) AND (EFFECT? OR STUDY OR STUDIES OR TOXIC?))
L28 S L20 AND ((OCULAR OR ENDOCRINE OR HORMON?) AND (DISRUPT? OR TOXIC? OR EFFECT#))
L29 S L20 AND ((MEDICAL OR (FIRST(1W)AID)) AND (TOXIC?(3A)STUD? OR THERAP?))
L30 S (L21-L29)
SET DUPORDER FILE
L31 DUP REM L30

The overall search statistics are shown in the table below.

Table B.6.10-2: results of study selection process

Data requirement(s) captured in the search	Number (initial search)
Total number of summary records retrieved after all* searches of peerreviewed literature (excluding duplicates)	99
Number of summary records excluded from the search results after rapid assessment for relevance	98
Total number of full-text documents assessed in detail*	1
Number of studies excluded from further consideration after detailed assessment for relevance	1
Number of studies not excluded for relevance after detailed assessment (i.e. relevant studies and studies of unclear relevance)	0

*both from bibliographic databases and other sources of peer-reviewed literature

The table below provides a list of the studies excluded from the risk assessment after a detailed assessment of the full-text documents or not excluded after a detailed assessment but considered not reliable.

Table B.6.10-3: list of references excluded following the detailed review

Author(s)	Year	Title	Source	Reason for exclusion
Yang, Y., Qi, S., Chen, J., Liu, Y., Teng, M. and Wang, C.	2016	Toxic effects of bromothalonil and flutalonil on multiple developmental stages in zebrafish	Bull Environ Contam Toxicol 97:91-7	The information is superseded by existing mammalian developmental studies. The article was used to support the ecotoxicology section.

Reference list

Reference	Author	Year	Title Test facility Report No. GLP [Y/N] Published [Y/N]	Vertebrate Study [Y/N]	Data Protection claimed [Y/N]	Justification	Owner
CA 5.1.1/01	Aizawa, H Murakami *et al.*	1982 1983	Metabolism of flutolanil in rats Mitsubishi-Kasei Inst. of Tox & Env. Sci., Japan T-3021 N (Pre-GLP) Y	Y	N	-	Nihon Nohyaku Co., Ltd
CA 5.1.1/02	[redacted]	1992 1994	The metabolism of aniline-ring ^{14}C-flutolanil in rats [redacted] T-3068 Y N	Y	N	-	Nihon Nohyaku Co., Ltd
CA 5.1.1/03	[redacted]	2012	Distribution, metabolism and excretion of [phenyl-(U)-^{14}C] flutolanil, following a single oral administration to male and female rats [redacted] T-3233 Y N	Y	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd
CA 5.1.1/04	Yasunaga, R	2016	*In vitro* metabolism study of flutolanil Research Centre, Nihon Nohyaku Co. Ltd, Japan T-3243 Y N	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd

Reference	Author	Year	Title Test facility Report No. GLP [Y/N] Published [Y/N]	Vertebrate Study [Y/N]	Data Protection claimed [Y/N]	Justification	Owner
CA 5.2.1/01	[redacted]	1982 a	NNF-136: Acute toxicity study in rats [redacted] T-3001 N (Pre-GLP) N	Y	N	-	Nihon Nohyaku Co., Ltd
CA 5.2.1/02	[redacted]	1982 b	NNF-136: Acute toxicity study in mice [redacted] T-3002 N (Pre-GLP) N	Y	N	-	Nihon Nohyaku Co., Ltd
CA 5.2.1/03	[redacted]	2009	Acute oral toxicity of flutolanil in rats [redacted] T-3182 Y N	Y	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd
CA 5.2.2/01	[redacted]	2009	Acute dermal toxicity of flutolanil in rats [redacted] T-3183 Y N	Y	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd
CA 5.2.3/01	[redacted]	1984	Acute inhalation toxicity of flutolanil (Moncut®) to rats [redacted] T-3003 Pre-GLP N	Y	N	-	Nihon Nohyaku Co., Ltd

Reference	Author	Year	Title Test facility Report No. GLP [Y/N] Published [Y/N]	Vertebrate Study [Y/N]	Data Protection claimed [Y/N]	Justification	Owner
CA 5.2.3/02	[redacted]	2012	Flutolanil technical: Acute inhalation toxicity study in rats [redacted] T-3230 Y N	Y	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd
CA 5.2.4/01	[redacted]	1986 a	Flutolanil: Acute dermal irritation/corrosion test in the rabbit [redacted] T-3207 Y N	Y	N	-	Nihon Nohyaku Co., Ltd
CA 5.2.5/01	[redacted]	1986 b	Flutolanil: Acute eye irritation/corrosion test in the rabbit [redacted] T-3206 Y N	Y	N	-	Nihon Nohyaku Co., Ltd
CA 5.2.6/01	[redacted]	1986 c	Flutolanil: Delayed contact hypersensitivity study in guinea-pigs [redacted] T-3208 Y N	Y	N	-	Nihon Nohyaku Co., Ltd
CA 5.2.7/01	Valin, M	2016	Flutolanil technical: 3T3 NRU phototoxicity test CiToxLAB, France T-3242 Y N	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd

Reference	Author	Year	Title Test facility Report No. GLP [Y/N] Published [Y/N]	Vertebrate Study [Y/N]	Data Protection claimed [Y/N]	Justification	Owner
CA 5.3.1/01	[redacted]	1977	One-month subacute toxicity study on flutolanil in rats [redacted] T-3011 N (Pre-GLP) N	Y	N	-	Nihon Nohyaku Co., Ltd
CA 5.3.2/01	[redacted]	1986 a	A three month oral toxicity study of flutolanil in rats [redacted] T-3024 Y N	Y	N	-	Nihon Nohyaku Co., Ltd
CA 5.3.2/02	[redacted]	1987	Flutolanil: Preliminary toxicity study by dietary administration to CD-1(ICR)BR mice for 13 weeks [redacted] T-3067 Y N	Y	N	-	Nihon Nohyaku Co., Ltd
CA 5.3.2/03	[redacted]	1986 b	A three month oral toxicity study of flutolanil in dogs [redacted] T-3025 Y N	Y	N	-	Nihon Nohyaku Co., Ltd
CA 5.3.3/01	[redacted]	1990	A 21-day dermal toxicity study in rats with flutolanil [redacted] T-3070 Y N	Y	N	-	Nihon Nohyaku Co., Ltd

Reference	Author	Year	Title Test facility Report No. GLP [Y/N] Published [Y/N]	Vertebrate Study [Y/N]	Data Protection claimed [Y/N]	Justification	Owner
CA 5.4.1/01	Moriya, M	1981	NNF-136: Microbial mutagenicity study Institute of Environmental Toxicology, Japan T-3016 N N	N	N	-	Nihon Nohyaku Co., Ltd
CA 5.4.1/02	Sutou, S	1986	Chromosomal aberration test of flutolanil with cultured mammalian cells NRI Life Science, Japan T-3044 Y N	N	N	-	Nihon Nohyaku Co., Ltd
CA 5.4.1/03	Jenkinson, P C	1990	Flutolanil: Metaphase analysis in human lymphocytes *in vitro* Safepharm Laboratories, UK T-3057 Y N	N	N	-	Nihon Nohyaku Co., Ltd
CA 5.4.1/04	Heidemann, A	1989	Cell mutation assay at the thymidine kinase (TK+/-) locus in mouse lymphoma L5178Y cells with flutolanil tech. CCR Cytotest Cell Research, Germany T-3077 Y N	N	N	-	Nihon Nohyaku Co., Ltd

Reference	Author	Year	Title Test facility Report No. GLP [Y/N] Published [Y/N]	Vertebrate Study [Y/N]	Data Protection claimed [Y/N]	Justification	Owner
CA 5.4.1/05	Fautz,	1989	Unscheduled DNA synthesis in primary hepatocytes of male rats *in vitro* with flutolanil tech. CCR Cytotest Cell Research, Germany T-3076 Y N	N	N	-	Nihon Nohyaku Co., Ltd
CA 5.4.2/01	Sasaki, Y F X	1983	Flutolanil: Micronucleus test Institute of Environmental Toxicology, Japan T-3019 Y N	Y	N	-	Nihon Nohyaku Co., Ltd
CA 5.4.2/02	[redacted]	2012	Flutolanil: Micronucleus test in the bone marrow of mice [redacted] Ltd, Japan T-3213 Y N	Y	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd
CA 5.5/01 Vol 1 CA 5.5/02 Vol 2 CA 5.5/03 Vol 3 CA 5.5/04 Vol 4	[redacted]	1982	Twenty-four month chronic toxicity study of NNF-136 in rats [redacted] T-3013 N (Pre-GLP) N	Y	N	-	Nihon Nohyaku Co., Ltd

Reference	Author	Year	Title Test facility Report No. GLP [Y/N] Published [Y/N]	Vertebrate Study [Y/N]	Data Protection claimed [Y/N]	Justification	Owner
CA 5.5/05	[redacted]	1990	Flutolanil: Oncogenicity study by dietary administration to CD-1 (ICR) BR mice for 78 weeks [redacted] T-3041 Y N	Y	N	-	Nihon Nohyaku Co., Ltd
CA 5.5/06	[redacted]	1982	Chronic toxicity study of NNF-136 administered orally to beagle dogs for 104 weeks [redacted] T-3014 N (Pre-GLP) N	Y	N	-	Nihon Nohyaku Co., Ltd
CA 5.6.1/01	[redacted]	1991	Two generation reproduction study in rats with flutolanil [redacted] T-3069 Y N	Y	N	-	Nihon Nohyaku Co., Ltd
CA 5.6.2/01	[redacted]	1992	Flutolanil: Teratology study in the rat [redacted] T-3043 Y N	Y	N	-	Nihon Nohyaku Co., Ltd

Reference	Author	Year	Title Test facility Report No. GLP [Y/N] Published [Y/N]	Vertebrate Study [Y/N]	Data Protection claimed [Y/N]	Justification	Owner
CA 5.6.2/02	[redacted]	1987	Teratogenicity study of flutolanil in the rabbit Imamichi [redacted] T-3042 Y N	Y	N	-	Nihon Nohyaku Co., Ltd
CA 5.6.2/03	[redacted]	2012	Study for effects on embryo-fetal development in rabbits treated orally with flutolanil [redacted] T-3229 Y N	Y	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd
CA 5.7.1/01	[redacted]	2011	Flutolanil: Single dose oral (gavage) administration neurotoxicity study in the rat [redacted] T-3201 Y N	Y	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd
CA 5.7.1/02	[redacted]	2012	Flutolanil: 13 Week oral (dietary) administration neurotoxicity study in the rat [redacted] T-3214 Y N	Y	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd

Reference	Author	Year	Title Test facility Report No. GLP [Y/N] Published [Y/N]	Vertebrate Study [Y/N]	Data Protection claimed [Y/N]	Justification	Owner
CA 5.8.1/01	Bayer CropScience AG, Germany	2016	Letter of Access, supporting European re-approval of flutolanil (the active substance and the representative formulation) Bayer CropScience AG, Germany - - N	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd
CA 5.8.1/02	[redacted]	2011	Acute oral toxicity study of 2-(trifluoromethyl)-benzamide in rats [redacted] T-3232 Y N	Y	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd
CA 5.8.1/03	[redacted]	2012	28 day repeated oral toxicity study of 2-(trifluoromethyl)-benzamide in rats [redacted] T-3231 Y N	Y	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd

Reference	Author	Year	Title Test facility Report No. GLP [Y/N] Published [Y/N]	Vertebrate Study [Y/N]	Data Protection claimed [Y/N]	Justification	Owner
CA 5.8.1/04	Inagaki, K	2011	Bacterial reverse mutation test of 2-(trifluoromethyl)-benzamide Research Center, Nihon Nohyaku Co., Ltd, Japan T-3200 Y N	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd
CA 5.8.1/05	Lloyd, M	2016 a	M-101: *In vitro* L5178Y Gene Mutation Assay at the *tk* locus Covance Laboratories Ltd, UK T-3250 Y N	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd
CA 5.8.1/06	Lloyd, M	2016 b	M-101: *In vitro* human lymphocyte chromosome aberration assay Covance Laboratories, UK T-3246 Y N	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd
CA 5.8.1/07	[redacted]	2016	Acute oral toxicity study of 2-(trifluoromethyl)-benzoic acid in rats [redacted] T-3244 Y N	Y	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd

Reference	Author	Year	Title Test facility Report No. GLP [Y/N] Published [Y/N]	Vertebrate Study [Y/N]	Data Protection claimed [Y/N]	Justification	Owner
CA 5.8.1/08	[redacted]	2010	28 day repeated oral toxicity study of 2-(trifluoromethyl)-benzoic acid in rats [redacted] T-3184 Y N	Y	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd
CA 5.8.1/09	Lloyd, M	2016c	M-102: Bacterial reverse mutation assay Covance Laboratories Ltd, UK T-3245 Y N	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd
CA 5.8.1/10	Lloyd, M	2016d	M-102: *In vitro* L5178Y Gene Mutation Assay at the *tk* locus Covance Laboratories, UK T-3248 Y N	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd
CA 5.8.1/11	Lloyd, M	2016e	M-102: *In vitro* L5178Y Gene Mutation Assay at the *tk* locus (further investigation under modified experimental conditions) Covance Laboratories, UK T-3249 Y N	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd

Reference	Author	Year	Title Test facility Report No. GLP [Y/N] Published [Y/N]	Vertebrate Study [Y/N]	Data Protection claimed [Y/N]	Justification	Owner
CA 5.8.1/12	Lloyd, M	2016 f	M-102: *In vitro* human lymphocyte chromosome aberration assay Covance Laboratories, UK T-3247 Y N	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd
CA 5.8.2/01	[redacted]	2011	4-week dietary T cell-dependent antibody assay with flutolanil TGAI in rats [redacted] T-3203 Y N	Y	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd
CA 5.8.3/01	Odum, J *et al.*	2016	Assessment of flutolanil and its potential for endocrine disruption RSA Associates NIC002_001 N (not relevant) N	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon Nohyaku Co., Ltd
CA 5.9.1/01	Sumitaka, K and Nakayama, S	2002	Statement of medical surveillance on manufacturing plant personnel Nihon Nohyaku Co., Ltd., Japan H-3001 N (not relevant) N	N	N	-	Nihon Nohyaku Co., Ltd
CA 5.9.1/02	Mitsumoto, S and Fuji, T	2002	Statement of medical surveillance on manufacturing plant personnel Nihon Nohyaku Co., Ltd., Japan H-3002 N (not relevant) N	N	N	-	Nihon Nohyaku Co., Ltd