Annex I to the CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2

International Chemical Identification:

Captan (ISO) 1,2,3,6-tetrahydro-N-(trichloromethylthio)phthalimide

EC Number: **205-087-0**

CAS Number: **133-06-2**

Index Number: **613-044-00-6**

Contact details for dossier submitter:

Austrian Agency for Health and Food Safety
Institute for Plant Protection Products Evaluation and Authorisation
Spargelfeldstraße 191, 1220 Vienna
Austria
on behalf of the
Federal Ministry for Climate Action, Environment, Energy, Mobility, Innovation and Technology
Radetzkystraße 2, 1030 Vienna
Austria

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1 PHYSICAL HAZARDS

These hazard classes are not assessed in this dossier.

2 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

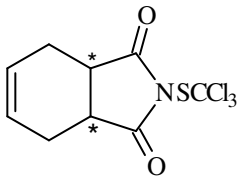
2.1 Study 1 (1990)

Reference:	Captan: Excretion and tissue retention of a single oral dose (10 mg/kg) in the rat
Author(s), year:	Anonymous (1990a)
Report/Doc. number:	ICI Americas Inc., unpublished report No. CTL/P/2820 (Company file: R-5829/TMN-0385)
Guideline(s):	USEPA 85-1 ; The study met the essential criteria of Directive 87/302/EEC Part B.
GLP:	Yes
Deviations:	No
Acceptability:	Yes

Material and Methods

A metabolism study was conducted in Sprague-Dawley rats (5 animals/sex/dose group) with [^{14}C]-cyclohexene ring-labelled captan (radiochemical purity > 95%; specific activity 769.6 MBq/mmol). The position of the radiolabelling is shown in Figure 2.1.1-1.

Figure 2.1.1-1 [^{14}C]-cyclohexene ring-labelled captan



Five males and five females each received by gavage a single oral dose of 10 mg/kg body weight (bw) (vehicle of 0.7% carboxymethyl cellulose (CMC) in 0.5% Tween 80, dosing volume 4 mL/kg bw). Each dose was equivalent to 2 Mbq/kg bw. Urine and faeces were collected separately at 6, 12, 24, 36 and 48 hours and then at 24 hourly intervals up to 7 days after dosing. Exhaled carbon dioxide was collected from one female at 24 and 48 hours after dosing. Animals were sacrificed 7 days after dosing and blood and tissues collected for analysis.

Findings

The mean total recovery of radioactivity was 91.8% (ranging from 73.6 to 107.3%) and 90.8% (ranging from 67.4 to 118.2%) in male and female rats, respectively. The variation in recovery was attributed to the innate difficulties involved in producing a completely homogenous dosing suspension.

The excretion profiles for male and female rats were found to be similar with the majority of radioactivity excreted in the urine after 24 hours. Over the first 24 hours after dosing, 77.2 and 7.4% of the administered radioactivity was excreted via the urine and faeces, respectively, in males and 71% and 6.2% of the administered dose was excreted in the urine and faeces, respectively, in females. The mean recovery of radioactivity excreted in urine and faeces is summarised in Table 2.1-1.

Table 2.1-1 Summary of urinary and faecal excretion in male and female rats following a single oral administration of [^{14}C]-captan (10 mg/kg)

Time post dose (hours)	Percentage administered radioactivity							
	Male				Female			
	Urine	Faeces	Cage wash	Total	Urine	Faeces	Cage wash	Total
0 - 6	17.1 ± 4.7	0.4 ± 0.3	-	-	6.2 ± 4.6	NS	-	-
6 - 12	28.2 ± 3.7	2.4 ± 0.7	-	-	33.8 ± 11.6	2.1 ± 1.5	-	-
12 - 24	31.9 ± 7.9	4.6 ± 0.7	-	-	31.0 ± 10.6	4.1 ± 1.0	-	-
24 - 36	3.4 ± 1.3	1.4 ± 0.5	-	-	5.1 ± 3.3	1.3 ± 0.6	-	-
36 - 48	0.6 ± 0.2	0.3 ± <0.1	-	-	5.0 ± 8.9	0.5 ± 0.5	-	-
48 - 72	0.3 ± 0.1	0.1 ± <0.1	-	-	0.6 ± 0.7	0.3 ± 0.3	-	-
72 - 96	0.1 ± <0.1	0.1 ± <0.1	-	-	0.2 ± 0.1	0.2 ± 0.2	-	-
96 - 120	<0.1	<0.1	-	-	0.1 ± <0.1	<0.1	-	-
120 - 144	<0.1	<0.1	-	-	<0.1	<0.1	-	-
144 - 168	<0.1	<0.1	-	-	<0.1	<0.1	-	-
0 - 168	81.9 ± 13.0	9.5 ± 1.7	<0.1	91.4 ± 14.6	82.0 ± 19.6	8.5 ± 1.8	0.2 ± 0.2	90.7 ± 21.0

NS = no sample.

The total radioactivity excreted in exhaled carbon dioxide in the one female examined was < 0.14% of the administered dose.

The levels of radioactivity in tissues 7 days following the administration of [^{14}C]-ring labelled-captan were negligible in most tissues and were either at or below the limit of detection. The highest concentration of radioactivity was present in the kidneys where mean levels were equivalent to 0.079 µg equivalent /g captan and 0.099 µg equivalent/g captan in males and females, respectively. This is consistent with the role of this organ in the excretion of captan. The levels present in the kidneys correspond to only 0.008 and 0.009% of the administered dose, respectively. In both male and female rats the total percentage of administered radioactivity remaining in the tissues was < 0.06% and in the carcass was < 0.45%.
The levels of radioactivity in tissues of animals sacrificed 7 days following dosing are summarised in Table 2.1-2.

Table 2.1-2 Summary of tissue concentration of radioactivity in male and female rats seven days following a single oral administration of [^{14}C]-ring - labelled-captan (10 mg/kg bw)

Tissue	Male		Female	
	% Dose	µg equivalents of captan/g	% Dose	µg equivalents of captan/g
brain	<0.001	<0.018	<0.001	<0.020
gonads	0.001 ± <0.001	<0.018	0.000	<0.015
heart	<0.001	<0.029	<0.001	<0.029
kidneys	0.008 ± 0.002	0.079 ± 0.014	0.009 ± 0.002	0.099 ± 0.024
liver	0.009 ± 0.002	<0.020	0.006 ± 0.003	<0.020
lungs	0.001 ± <0.001	<0.024	0.002 ± 0.001	<0.029
spleen	<0.001	<0.032	<0.001	<0.043
uterus	-	-	<0.001	<0.051
stomach*	0.011 ± 0.003	0.034 ± 0.020	0.010 ± 0.005	0.038 ± 0.012
small intestine*	0.018 ± 0.003	<0.028	0.014 ± 0.004	<0.030
large intestine*	0.005 ± <0.001	<0.025	0.007 ± 0.003	<0.033
bone	-	<0.028	-	<0.030
fat	-	<0.023	-	<0.039
muscle	-	<0.032	-	<0.035
blood	-	0.054 ± 0.005	-	0.050 ± 0.009
plasma	-	<0.023	-	<0.024
carcass	0.337 ± 0.088	0.036 ± 0.009	0.448 ± 0.294	0.055 ± 0.039
TOTAL	0.391 ± 0.089		0.496 ± 0.296	

* These tissues include contents.

Conclusion

Following a single oral administration of 10 mg/kg bw [^{14}C]-cyclohexene ring-labelled captan, radioactivity was rapidly excreted. Urinary excretion predominated with approximately 82% of the administered radioactivity excreted in the urine over a period of 48 h post dosing. Elimination via the faeces accounted for 9% of the administered radioactivity over the same period. Tissue residues were negligible with the highest concentration found in the kidneys at <0.01% of the administered radioactivity. The excretion profiles of male and female rats were very similar.

2.2 Study 2 (1990)

Reference:	Captan: Excretion and tissue retention of a single oral dose (500 mg/kg) in the rat
Author(s), year:	Anonymous (1990b)
Report/Doc. number:	ICI Central Toxicology Laboratory, unpublished report No. CTL/P/2862 (Company file: R-5830/TMN-0386)
Guideline(s):	USEPA 85-1
GLP:	Yes
Deviations:	No
Acceptability:	Yes

Material and Methods

A metabolism study was conducted in Sprague-Dawley strain rats with [^{14}C]-cyclohexene ring-labelled captan (radiochemical purity > 95%; specific activity 769.6 MBq/mmol). Five males and five females each received a single oral dose of 500 mg/kg bw delivered by oral gavage in a dosing vehicle of 0.7% carboxymethyl cellulose (CMC) in 0.5% Tween 80 (dosing volume 4 mL/kg bw). Each dose was equivalent to 2 Mbq/kg bw. Urine and faeces were collected separately at 6, 12, 24, 36 and 48 hours and then at 24 hourly intervals up to 7 days after dosing. Exhaled carbon dioxide was collected from one male at 24 and 48 hours after dosing. Animals were sacrificed 7 days after dosing and blood and tissues collected for analysis.

Findings

The mean total recovery of radioactivity was 93.4% (ranging from 83.5 to 96.8%) in males and 98.9% (ranging from 95.7 to 104.4%) in female rats, respectively.

The excretion profiles of male and female rats were found to be similar. In males 68.8% and 23.1% of the administered radioactivity was excreted via the urine and faeces, respectively, over a period of 96 hours, and in female rats 73.4% and 25.0% of the administered dose was excreted via the urine and faeces, respectively, over the same period of time. In both sexes excretion was virtually complete within this period. Urinary excretion was slightly prolonged when compared to a single oral dose of 10 mg/kg bw (Trivedi, 1990a) where elimination was essentially complete within 48 hours. This slightly protracted excretion profile is reflected by the amounts remaining in the blood after 7 days.
The mean recovery of radioactivity excreted in urine and faeces are summarised in Table 2.2-1.

Table 2.2-1 Summary of urinary and faecal excretion in male and female rats following a single oral administration of [^{14}C]-ring-labelled-captan (500 mg/kg bw)

Time post dose (hours)	Percentage administered radioactivity							
	Male				Female			
	Urine	Faeces	Cage wash	Total	Urine	Faeces	Cage wash	Total
0 - 6	1.3 ± 0.4	NS	-	-	1.4 ± 0.4	NS	-	-
6 - 12	1.8 ± 0.5	1.9 ± 3.1	-	-	2.2 ± 0.4	2.4 ± 2.3	-	-
12 - 24	7.4 ± 3.5	2.0 ± 0.9	-	-	8.3 ± 2.0	3.2 ± 2.6	-	-
24 - 36	16.8 ± 6.8	3.0 ± 1.8	-	-	17.1 ± 3.7	3.1 ± 2.5	-	-
36 - 48	21.4 ± 11.9	10.3 ± 4.9	-	-	21.2 ± 2.0	12.8 ± 4.4	-	-
48 - 72	19.0 ± 10.4	5.3 ± 2.3	-	-	22.7 ± 5.5	3.2 ± 0.6	-	-
72 - 96	1.2 ± 1.7	0.5 ± 0.3	-	-	0.5 ± 0.1	0.3 ± 0.1	-	-
96 - 120	0.3 ± 0.3	0.5 ± 0.9	-	-	0.1 ± <0.1	<0.1	-	-
120 - 144	0.1 ± 0.2	0.1 ± 0.2	-	-	0.1 ± <0.1	<0.1	-	-
144 - 168	<0.1	<0.1	-	-	0.1 ± <0.1	<0.1	-	-
0 - 168	69.3 ± 5.0	23.7 ± 2.9	0.1 ± 0.2	93.1 ± 5.6	73.4 ± 3.8	25.0 ± 5.4	0.1 ± <0.1	98.6 ± 3.6

NS = no sample.

The total radioactivity excreted in exhaled carbon dioxide in the one male examined was 0.06% of the administered dose.

The levels of radioactivity in tissues 7 days following the administration of [^{14}C]-ring-labelled-captan were negligible in most tissues and were either at or below the limit of detection. The highest concentration of radioactivity was present in the blood where mean levels were 2.649 µg equivalents captan/g and 1.974 µg equivalents captan/g in males and females, respectively. In both male and female rats the total percentage of administered radioactivity remaining in the tissues was < 0.04%. Amounts remaining in the carcass were 1.764 and 1.577 µg equivalents captan/g in male and female rats, respectively, which corresponded to 0.327% and 0.284% of the administered dose, respectively.

The levels of radioactivity in tissues of animals sacrificed 7 days following dosing are summarised inTable 2.2-2.

Table 2.2-2 Summary of tissue concentration of radioactivity in male and female rats seven days following a single oral administration of [^{14}C]-ring-labelled-captan (500 mg/kg bw)

Tissue	Male		Female	
	% Dose	µg equivalents of captan/g	% Dose	µg equivalents of captan/g
Brain	0.001 ± 0.001	<1.231	0.002 ± 0.002	<1.282
Gonads	0.001 ± <0.001	<0.720	<0.001	<0.633
Heart	0.001 ± <0.001	<1.264	0.001 ± 0.001	<1.374
Kidneys	0.002 ± 0.001	<1.223	0.002 ± 0.002	<0.939
Liver	0.012 ± 0.007	<0.978	0.009 ± 0.010	<0.985
Lungs	<0.001	<1.046	0.001 ± 0.001	<0.897
Spleen	<0.001	<2.224	<0.001	<2.298
Uterus	-	-	<0.001	<2.473
stomach*	0.010 ± 0.004	<1.378	0.004 ± 0.004	<0.892
small intestine*	0.010 ± 0.001	<1.015	0.009 ± 0.010	<1.093
large intestine*	0.050 ± 0.002	<1.075	0.004 ± 0.005	<1.037
Bone	-	<1.176	-	<0.968
Fat	-	<2.861	-	<2.454
Muscle	-	<1.719	-	<1.757
Blood	-	2.649 ± 0.823	-	1.974 ± 0.244
Plasma	-	<1.201	-	<1.058
Carcass	0.327 ± 0.045	1.764 ± 0.312	0.284 ± 0.061	1.577 ± 0.358
TOTAL	0.371 ± 0.046		0.316 ± 0.067	

* These tissues include contents

Conclusions

Following a single oral dose of [^{14}C]-cyclohexene ring-labelled-captan (500 mg/kg bw), radioactivity was principally eliminated in the urine with virtually no retention in the tissues or metabolism to exhaled carbon dioxide. There were no sex differences in the rate or route of elimination of captan with elimination essentially complete by 96 hours. Elimination was slightly prolonged when compared to a single oral dose of 10 mg/kg captan, where elimination was complete within 48 hours.

2.3 Study 3 (1990)

Reference:	Captan: Repeat dose study (10 mg/kg) in the rat
Author(s), year:	Anonymous (1990)
Report/Doc. number:	ICI Central Toxicology Laboratory, unpublished report No. CTL/P/2958 (Company file: R-5832/TMN-0387)
Guideline(s):	Not stated; study meets the essential criteria of USEPA 85-1
GLP:	Yes
Deviations:	No
Acceptability:	Yes

Material and Methods

A metabolism study was conducted in which eight male and eight female Sprague-Dawley strain rats were given single daily doses of unlabelled captan (purity 99.9%) at a dose level of 10 mg/kg bw over a period of 14 days. Twenty-four hours after the final unlabelled dose, five male and five female rats were given a single oral dose of [^{14}C]-cyclohexene ring-labelled captan (radiochemical purity 99.6% w/w; specific activity 769.6 Mbq/mmol) at a dose level of 10 mg/kg bw. Doses were delivered by oral gavage in a dosing vehicle of 0.7% w/v carboxymethyl cellulose (CMC) in 0.5% Tween 80. The dosing volumes of unlabelled and labelled test substance were 2 mL/kg bw and 4 mL/kg bw, respectively. The radiolabelled dose was equivalent to 2 Mbq/kg bw. Urine and faeces were collected separately at 6, 12, 24, 36 and 48 hours and then at 24 hourly intervals up to 7 days after dosing with the [^{14}C]-ring-labelled-captan. Animals were sacrificed 7 days after dosing and blood and tissues collected for analysis.

Findings

The excretion profiles for male and female rats were found to be similar. In the first 48 hours after administration of [^{14}C]-ring-labelled-captan, 87.7% and 8.6% of the administered radioactivity was excreted via the urine and faeces, respectively, in males, and 90.5% and 6.8% of the administered dose was excreted via the urine and faeces, respectively, in females. In both sexes, excretion was virtually complete within this period. During the 7-day period following administration of [^{14}C]-ring-labelled-captan, males and females had excreted 97.6 and 98.1% of the dose, respectively.
The mean recovery of radioactivity excreted in urine and faeces are summarised in Table 2.3-1.

Table 2.3-1 Summary of urinary and faecal excretion in male and female rats following a single oral administration of [^{14}C]-ring-labelled-captan (10 mg/kg bw) - repeat dose study

Time post dose (hours)	Percentage administered radioactivity					
	Male			Female		
	Urine	Faeces	Total	Urine	Faeces	Total
0 - 6	16.9 ± 6.1	<0.1		18.2 ± 8.0	0.3 ± 0.2	
6 - 12	23.5 ± 4.5	1.2 ± 0.4		37.9 ± 10.9	1.9 ± 0.6	
12 - 24	34.8 ± 3.8	4.0 ± 0.4		29.5 ± 6.5	3.0 ± 1.0	
24 - 36	10.2 ± 4.2	2.2 ± 0.4		4.3 ± 2.5	1.3 ± 0.9	
36 - 48	2.3 ± 2.7	1.2 ± 0.4		0.6 ± 0.3	0.3 ± 0.1	
48 - 72	0.4 ± 0.6	0.3 ± 0.2		0.2 ± 0.2	0.1 ± <0.1	
72 - 96	<0.1	<0.1		<0.1	<0.1	
96 - 120	<0.1	<0.1		<0.1	<0.1	
120 - 144	<0.1	<0.1		<0.1	<0.1	
144 - 168	<0.1	0.3 ± 0.2		<0.1	0.3 ± 0.1	
0 - 168	88.3 ± 2.3	9.3 ± 0.73	97.6 ± 1.9	90.8 ± 2.6	7.3 ± 2.1	98.1 ± 2.1

NS = no sample.

The levels of radioactivity in tissues 7 days following the administration of [^{14}C]-ring-labelled-captan were negligible in most tissues and were either at or below the limit of detection. The highest concentration of radioactivity was found in the kidney where values were 0.043 and 0.039 µg/g in males and females, respectively. In both male and female rats the total percentage of administered radioactivity remaining in the tissues was < 0.02%. Amounts remaining in the carcass were 0.054 and 0.033 µg equivalents captan/g, which corresponded to 0.43 and 0.25% of the administered dose.

The levels of radioactivity in tissues of animals sacrificed 7 days following dosing with [^{14}C]-ring-labelled-captan are summarised in Table 2.3-2.

Table 2.3-2 Summary of tissue concentration of radioactivity in male and female rats seven days following a single oral administration of [^{14}C]-ring-labelled-captan (10 mg/kg bw) - repeat dose study

Tissue	Male		Female	
	% Dose	µg equivalent captan/g	% Dose	µg equivalent captan/g
Brain	<0.001	<0.011	<0.001	<0.011
Gonads	0.002 ± <0.001	0.017 ± 0.002	<0.001	<0.021
Heart	<0.001	0.023 ± 0.005	<0.001	<0.015
Kidneys	0.004 ± <0.001	0.043 ± 0.008	0.003 ± <0.001	0.039 ± 0.003
Liver	<0.006	<0.014	<0.004	<0.011
Lungs	0.002 ± <0.001	0.037 ± 0.006	0.002 ± <0.001	0.034 ± 0.005
Spleen	<0.001	0.034 ± 0.005	<0.001	<0.033
Uterus			<0.001	<0.012
stomach*	<0.002	<0.011	<0.001	<0.011
small intestine*	<0.004	0.014 ± 0.002	<0.005	<0.016
large intestine*	<0.003	<0.016	<0.003	<0.021
Bone	-	0.019 ± 0.003	-	0.020 ± 0.002
Fat	-	<0.011	-	<0.011
Muscle	-	0.018 ± 0.002	-	0.016 ± 0.002
Blood	-	0.036 ± 0.010	-	0.033 ± 0.005
Plasma	-	<0.011	-	<0.011
Carcass	0.427 ± 0.068	0.054 ± 0.012	0.251 ± 0.090	0.033 ± 0.012
TOTAL	0.441 ± 0.069	-	0.262 ± 0.095	-

* These tissues include contents.

Conclusions

Repeat oral administration of unlabelled captan (10 mg/kg bw) over a 14 day period followed by a single oral dose of [^{14}C]-ring-labelled-captan (10 mg/kg bw), produced similar results to those obtained in a separate study in which [^{14}C]-ring-labelled-captan was administered without pre-administration of unlabelled captan (Anonymous, 1990a). Radioactivity was principally eliminated via urine with virtually no retention in the tissues. There were no sex differences in the rate or route of elimination of captan with elimination essentially complete within 48 hours after dosing. It can therefore be concluded that pre-dosing of rats with captan had no effect on the route or rate of elimination of a single oral dose of [^{14}C]-ring-labelled-captan.

2.4 Study 4 (1990)

Reference:	Captan: Biotransformation study in the rat
Author(s), year:	Anonymous (1990)
Report/Doc. number:	ICI Central Toxicology Laboratory, unpublished report No. CTL/P/2951 (Company file: R-5831/TMN-0384)
Guideline(s):	USEPA 85-1
GLP:	Yes
Deviations:	No
Acceptability:	Yes

Material and Methods

The metabolite profile of captan was examined in the urine and faeces collected from male and female Sprague Dawley rats which had been dosed with either a single oral dose of 10 mg/kg bw or 500 mg/kg bw [^{14}C]-cyclohexene ring-labelled captan, or 14 consecutive daily doses of 10 mg/kg bw/day unlabelled captan followed by a single oral dose of 10 mg/kg bw [^{14}C]-cyclohexene ring-labelled captan. Details of the dosing regimes and sampling times are outlined in the previous summaries [Anonymous (1990a and b), Anonymous (1990)]. For the purposes of the current study urine and faecal samples were pooled as summarised in Table 2.4-1. Samples from males and females were pooled separately.

Table 2.4-1 Summary of urine and faecal samples pooled for analysis

Dose group (mg/kg)	Urine		Faeces	
	Sample time (hours)	Pool	Sample time (hours)	Pool
10 (single)	6 - 36	U1	6 - 72	F1
10 (repeat)	6 - 36	U2	6 - 72	F2
500 (single)	6 - 36	U3	6 - 72	F3
	48 - 72	U4		
	6 - 72	U5 (equal volumes U3 and U4 combined)		

Pooled urine samples were extracted in chloroform and acetonitrile, and pooled faeces samples were extracted with acetone. Urine and faecal extracts were applied to silica gel plates and developed in either of the following solvent systems, (i) glacial acetic acid - acetonitrile (1:99) (v/v), (ii) ethyl acetate - acetone - glacial acetic acid (50:49:1) (v/v). Authentic standards were also developed in the solvent systems. Radioactive metabolites were quantified using a linear plate scanner, after which selected plates were examined by autoradiography. The identity of metabolites was confirmed by gas chromatography (GC) and mass spectroscopy (MS).

Findings

Urinary metabolites: Mostly, there were no significant differences between the proportions of the urinary metabolites for the three dosing regimes or between the male and female rats. However, for THPI there was a clear difference in quantity in males and females as well as for dose regimens (Table 2.1.4-2). Furthermore, solvent system (i) was found to produce a superior separation to that of system (ii). The following values were therefore calculated as an average of the values obtained for males and females for all three dosing regimes, measured in solvent system (i). A total of seven urinary metabolites out of nine were identified. The most abundant metabolites, as determined by thin layer chromatography (TLC) were 3-hydroxy-4,5-cyclohexene-1,2-dicarboximide (3OH-THPI) and 5-hydroxy-3,4-cyclohexene-1,2-dicarboximide (5OH-THPI). Measurements of the two alcohols by gas chromatography (they could not be separated by TLC with sufficient resolution) revealed that they represented 42.3% and 5.9% of the urinary radioactivity, respectively. The overall relative amounts were determined by comparison of the areas under the gas chromatography peaks appropriate to the semipurified metabolites obtained from urine pool U5. No further separate quantitation data for the metabolites 3OH-THPI and 5OH-THPI information is available on other urine pools. Furthermore, quantification of 5OH-THPI and 3OH-THPI was not conducted in faeces; therefore quantification of the individual metabolites in faeces is not possible.
The polar region accounted for 20.4% of the urinary radioactivity, among which 6-hydroxy-1-amido-2-carboxy-4,5-cyclohexene (3-OH THP-amic acid) was found to account for approximately 13.4% of urinary metabolites. The metabolites 4,5-cyclohexene-1,2-dicarboximide (THPI), 1-amido-2-carboxy-4,5-cyclohexene (THPAM), 4,5-dihydroxy-1,2-dicarboximide (4,5-diOH THPI) and 4,5-epoxy-1,2-dicarboximide (THPI-epoxide) were found to account for 10.6%, 7.2%, 5.5% and 4.8% of urinary metabolites, respectively. One 'metabolite' provisionally identified as unmetabolised captan was detected only in some sexes and dosing regimes and never exceeded 1.6% of the radioactivity in urine extracts. In addition, one other unidentified metabolite was detected and this represented 4.0% of the urinary radioactivity. Radioactivity remaining at the origin of TLC plates, which could not be matched to identified metabolites accounted for 7.0% of the urinary radioactivity. The levels of metabolites present in urine (using solvent system (i)) for each sex and dosing regime are presented in Table 2.4-2.

Faecal metabolites: As with the thin layer chromatography (TLC) data for urine, solvent system (i) produced a superior separation to solvent system (ii). Therefore values referred to below are based upon data obtained from solvent system (i). Significant differences in the quantities of certain metabolites in the faecal extracts were observed between the dosing regimes and sexes. Faecal extracts obtained from rats dosed with 500 mg captan/kg bw contained 42.5% of provisionally identified unmetabolised captan. In rats dosed with 10 mg captan/kg bw this metabolite accounted for only 7.0% of the faecal metabolites recovered. This result is consistent with saturation of absorption at the higher dose level. THPI was a major faecal metabolite and accounted for an average of 35% (ranging from 29.5 to 38.5%) of the faecal metabolites. THPAM accounted for 3.5% (ranging from 0 - 8.5%) of faecal metabolites. There were

no significant differences between sexes and dosing regimes in the quantities of these two metabolites. The metabolite 4,5-diOH THPI was not detected in faecal extracts from rats dosed with 500 mg captan/kg bw and accounted for only 3.9% of the faecal metabolites found in samples obtained from rats dosed with 10 mg captan/kg bw, with no significant sex difference. Polar metabolites were present in relatively small amounts. The alcohols 3OH-THPI and 5OH-THPI were major metabolites, the quantities of which were dependent on the dosing regime and sex of the animals. In faecal extracts from high and low dose regimes these metabolites accounted for 10.6% and 26.8% of the metabolites. The repeat dose data showed an apparent sex difference with alcohols accounting for 14.9% and 30.6% of metabolites in faecal extracts from males and females, respectively. The metabolite THPI-epoxide was not detected in faecal extracts. Many of the urinary metabolites were also found in the faeces and this may indicate excretion from liver via bile. The levels of metabolites present in faecal extracts (using solvent system (i)) for each sex and dosing regime are presented in Table 2.4-3.

Table 2.4-2 Quantitation data (solvent system i) for captan metabolites in urine (% urinary radioactivity)

Metabolites	Low dose 6 - 36 hours		Repeat dose 6 - 36 hours		High dose 6 - 36 hours		High dose 48 - 72 hours	
	Male	Female	Male	Female	Male	Female	Male	Female
THPI	15.4	6.97	12.4	6.1	5.7	7.0	11.5	19.8
3OH-THPI 5OH-THPI	36.1	50.5	51.4	51.7	53.5	52.3	46.0	43.7
THPI-epoxide	4.6	4.7	4.6	3.4	4.3	4.3	4.2	8.2
4,5-diOH THPI	7.1	4.7	5.2	5.7	5.9	5.1	6.0	4.4
THPAM	7.4	6.2	10.4	5.3	5.8	6.4	6.2	9.5
(polar region) 3-OH THP-amic acid	26.7	23.6	14.4	24.2	20.7	20.9	21.3	11.0
Unknown (captan)	ND	ND	ND	0.7	1.3	1.3	1.6	1.4

ND = not detected.

Table 2.4-3 Quantitation data (solvent system i) for captan metabolites in faecal extracts (% faecal radioactivity)

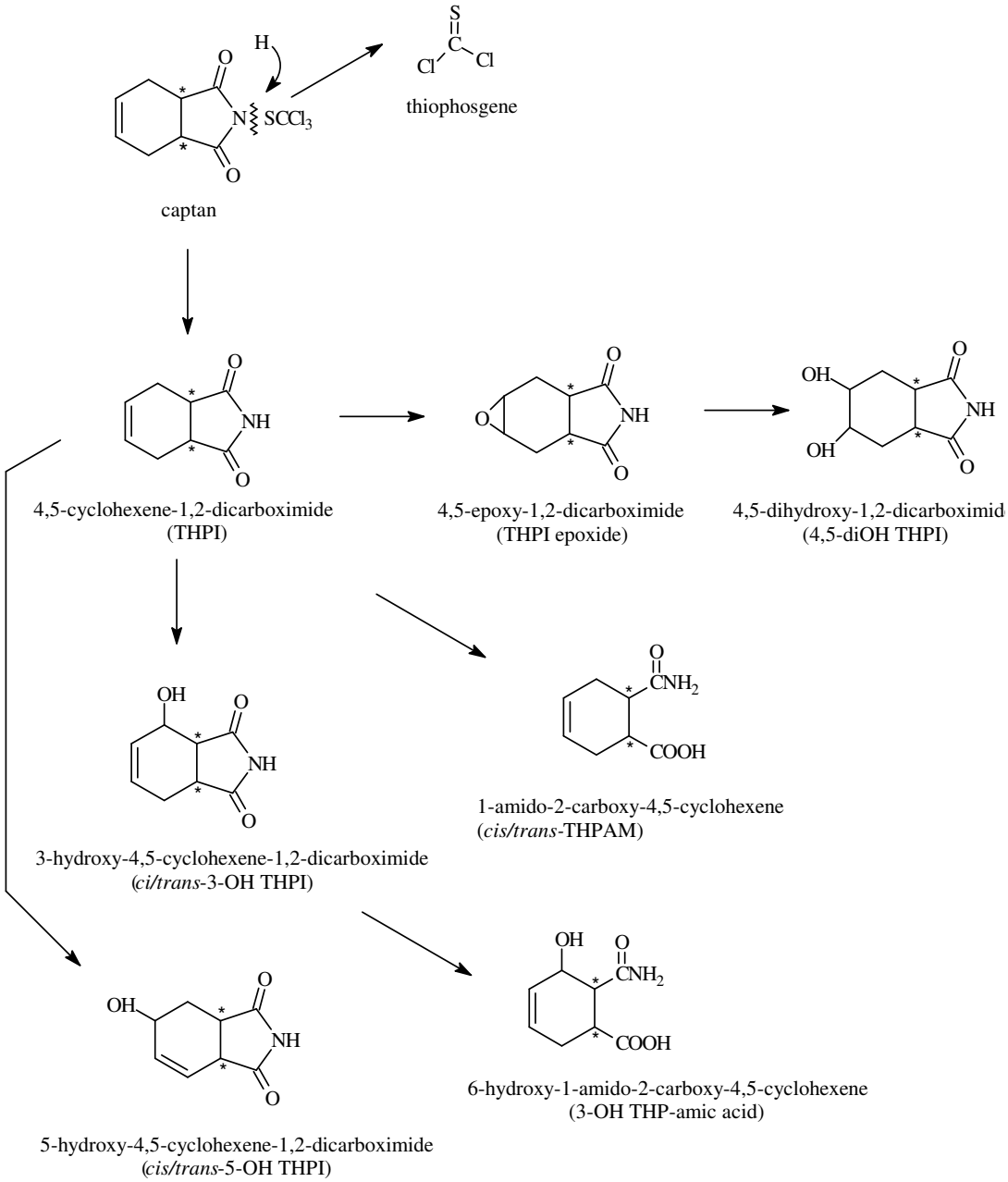
Metabolites	Low dose 6 - 36 hours		Repeat dose 6 - 36 hours		High dose 6 - 36 hours	
	Male	Female	Male	Female	Male	Female
THPI	29.5	38.5	37.6	35.7	30.0	38.4
3OH-THPI 5OH-THPI	36.2	25.5	14.9	30.6	11.8	9.4
THPI-epoxide	NS	NS	NS	NS	NS	NS
4,5-diOH THPI	2.7	1.3	7.7	3.9	ND	ND
THPAM	8.5	3.9	2.6	5.7	ND	ND
(polar region) 3-OH THP-amic acid	10.8	3.5	31.0	11.4	6.7	2.9
Unknown (captan)	6.5	16.8	ND	4.6	44.1	40.9

ND = not detected.
NS = not separated.

The metabolism of captan involves the cleavage of the trichloromethylthio moiety resulting in the formation of THPI. The exact site where cleavage of the trichloromethylthio moiety occurs could not be ascertained from the current study. However, further metabolism of THPI probably occurs in the liver. THPI is metabolised by epoxidation to the double bond of the cyclohexene ring to form THPI-epoxide. The epoxide is then further metabolised to the corresponding diol (4,5-diOH THPI). The epoxide and diol formation is a typical hepatic process. The metabolite THPI also undergoes hydroxylation at both the 3 and 5 positions on the cyclohexene ring (3OH-THPI and 5OH-THPI). Although no further metabolism of the 5-OH metabolite is apparent, the 3-OH metabolite is further metabolised with the opening of the dicarboximide ring to form the hydroxylated acid amide, 3-OH THP-amic acid.

Metabolism of THPI also occurs with the cyclohexene ring undergoing direct opening with the formation of the THPAM. The proposed route of metabolism in the rat is given in Figure 2.4-1.

Figure 2.4-1 Proposed metabolic pathway of [^{14}C]-cyclohexene ring-labelled captan in the rat



Conclusions

The metabolism of [^{14}C]-cyclohexene ring-labelled captan was studied in rats.
No significant differences in the quantities of most of the urinary metabolites were noted between both sexes or dosing regimes. However, for THPI there was a clear difference in quantity in males and females as well as for dose regimens. The main metabolites identified in the urine are 4,5-cyclohexene-1,2-dicarboximide (THPI, 11%), 3-hydroxy-4,5-cyclohexene-1,2-dicarboximide (3OH-THPI, 42%), 5-hydroxy-4,5-cyclohexene-1,2-dicarboximide (5OH-THPI, 6%), 4,5-epoxy-1,2-dicarboximide (THPI-epoxide, 5%), 4,5-dihydroxy-1,2 dicarboximide (4,5-diOH THPI, 6%), 1-amido-2-carboxy-4,5-cyclohexene (THPAM, 7%) and 6-hydroxy-1-amido-2-carboxy-4,5-cyclohexene (3-OH THP-amic acid,

13%). Two further unidentified metabolites were present and accounted for 4% and 2% of the urinary radioactivity.

Significant differences in the quantities of certain metabolites in the faecal extracts were observed between the dosing regimes and sexes. Faecal extracts from rats administered the low and high doses contained 7% and 43%, respectively of an unidentified metabolite (tentatively identified as Captan). This result is consistent with saturation of absorption at the higher dose level. Many of the metabolites that were detected in the urine were also found in the faecal extracts, indicating that excretion from the liver via the bile occurs. THPI accounted for 35% of the faecal metabolites, 3 OH-THPI and 5 OH-THPI were major urine metabolites. The amount of these metabolites in the faeces was dependant on the sex and the dosing regime and ranged from 11% to 27% at high and low dose, respectively. 4.5-diOH THPI (3.9%), THPAM (5.2%) and polar metabolites were only present in small amounts.

The metabolism of [^{14}C]-cyclohexene labelled captan involves the cleavage of the trichloromethylthio moiety resulting in the formation of THPI. Although the exact site where cleavage of the trichloromethylthio moiety occurs was not ascertained, further metabolism of THPI probably occurs in the liver, where metabolism to the epoxide, THPI-epoxide and then to the diol, 4,5-diOH THPI occurs. The epoxide and diol formations are typical hepatic processes. THPI also undergoes hydroxylation on the cyclohexene ring to 3OH-THPI and 5OH-THPI. The 3-OH metabolite is further metabolised with the opening of the dicarboximide ring to form the hydroxylated acid amide (3-OH THP-amic acid). THPI is also metabolised to the THPAM.

2.5 Study 5 (1988)

Reference:	Evaluation of the in vivo metabolism of captan in rats
Author(s), year:	Anonymous (1988)
Report/Doc. number:	Hazleton Laboratories America, Inc., unpublished report No. HLA 6183-107 (Company file: R-4988/TMN-0388)
Guideline(s):	USEPA 85-1
GLP:	Yes
Deviations:	No
Acceptability:	Yes

Material and Methods

A metabolism study was conducted in Crl:CD(SD)BR strain rats with [^{14}C]-trichloromethyl-labelled captan (radiochemical purity 98%; specific activity 38 mCi/mmol). Three test groups were studied and these are summarised below in Table 2.5-1. The position of the radiolabelling in captan is shown in Figure 2.5-1.

Figure 2.5-1 [^{14}C]-trichloromethyl-labelled captan

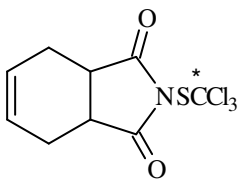


Table 2.5-1 Test groups used in the metabolism study with [^{14}C]-trichloromethyl labelled captan

Test group	Dose level (mg/kg bw)	Number of animals	
		males	females
A (low dose)	10	5	5
B (repeat dosing)	10	10	10
D (high dose)	500	5	5

Because of test material preparation problem (inability to maintain homogeneity of the dose solution) for the high dose group (group C at 1000 mg/kg bw, not mentioned on the table), a new high group was added at 500 mg/kg bw, group D. Doses were administered in a vehicle of 0.7% carboxymethyl cellulose (CMC) in 0.5% Tween 80 by oral gavage at a dose volume of 8 mL/kg bw. In test groups A and D, animals received a single dose of the [^{14}C]-trichloromethyl-labelled captan. In the repeat dose group, animals received daily doses of non-labelled captan (purity 99%) for 14 days. On day 15, five males and five females from the same group were administered [^{14}C]-trichloromethyl-labelled captan. Urine and faeces from dose groups A and B were collected separately during the time intervals 0 - 6, 6 - 12, 12 - 24, 24 - 36, 36 - 48, 48 - 72 and 72 - 96 hours after dosing. For group D, urine and faeces samples were also collected during an additional time interval, 96 - 120 hours after dosing. Exhaled carbon dioxide and volatiles were collected from group A and B animals for the time intervals 0 - 6, 6 - 12, 12 - 24, 24 - 36 and 36 - 48 hours after dosing with [^{14}C]-captan, this period was extended to 72 hours post-dose for group D animals. After the final collection interval, the animals were sacrificed and blood and tissues collected for analysis. Samples were analysed for radioactivity by liquid scintillation counting (LSC). Metabolites in urine and faecal were extracted and analysed using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

Findings

Radioanalysis data from rats receiving a single oral dose of 10 mg/kg bw (group A) were almost identical to those from rats receiving repeat oral doses of 10 mg/kg bw captan (group B), indicating that repeat dosing did not alter the metabolism of captan. Between 75 and 85% of the administered dose was excreted during the first 24 hours after the administration of [^{14}C]-captan. The major route of elimination was via the urine with between 41 and 50% excreted in the urine by 96 hours. Other important routes of excretion were via the faeces and as expired carbon dioxide with 14 to 22% and 22 to 26% excreted during the 96 hours post-dosing. Organic volatiles represented less than 1% of the administered dose. At sacrifice, the levels of radioactivity remaining in the tissues were low with tissues containing between 1.61 and 1.75% of the administered dose, the majority of radioactivity being found in the carcass. The overall recoveries from group A and group B animals were between 91 and 92% of the administered dose.

Results from the high dose group (500 mg/kg bw) were different to those observed in the single oral and repeat oral dose groups receiving 10 mg/kg bw captan. During the first 24 hours, only 21 - 24% of the administered dose had been excreted. In contrast to the lower dose groups, the major route of excretion by the high dose group was via the faeces, with 33 - 40% of the administered radioactivity excreted in the faeces during the 120 hours post-dosing. Urinary excretion accounted for 23 - 27% of the administered radioactivity, and excretion as carbon dioxide for 14 - 15%. Although volatiles accounted for less than 1% of the administered dose in low dose animals, they accounted for 4 to 7% in high dose group animals. At sacrifice, 120 hours after dosing, tissues contained less than 1% of the administered dose, mainly detected in the carcass. The mean overall recovery for males and females was 83%. The reduced levels of recovery are attributed to the presence of organic volatiles which were trapped at an unknown efficiency.
The mean material balance for each test group is summarised in Table 2.5-2.

Table 2.5-2 Material balance (%) of total dose recovered following administration of [^{14}C]-trichloromethyl-labelled captan

	single dose (10 mg/kg bw)		**repeat dosing (10 mg/kg bw)**		**single (500 mg/kg bw)**	
	males	**females**	**males**	**females**	**males**	**females**
CO_2 traps	22.01 ± 3.12	24.51 ± 2.09	23.54 ± 0.91	26.31 ± 1.42	14.45 ± 1.38	14.88 ± 0.72
faeces	21.51 ± 3.91	13.59 ± 6.56	17.58 ± 1.37	19.47 ± 5.24	40.14 ± 4.56	32.92 ± 2.76
tissues	1.73 ± 0.11	1.75 ± 0.14	1.63 ± 0.10	1.61 ± 0.24	0.90 ± 0.05	0.86 ± 0.09
urine	44.56 ± 5.79	50.20 ± 6.40	47.50 ± 3.50	40.87 ± 5.90	22.73 ± 5.47	27.25 ± 1.23
volatiles	0.13 ± 0.03	0.14 ± 0.09	0.53 ± 0.37	0.26 ± 0.11	3.59 ± 0.64	6.66 ± 0.59
cagewash	0.43 ± 0.24	0.40 ± 0.12	0.72 ± 0.46	1.86 ± 0.89	1.34 ± 0.85	0.66 ± 0.54
cage wipe	0.17 ± 0.14	0.07 ± 0.06	0.32 ± 0.15	0.78 ± 0.48	0.14 ± 0.09	0.13 ± 0.16
total	90.54 ± 2.89	90.66 ± 9.27	91.80 ± 1.50	91.17 ± 1.15	83.28 ± 1.96	83.37 ± 2.84

Total amounts of radioactivity in the faeces, urine, carbon dioxide and volatiles are summarised in Table 2.5-3.

Table 2.5-3 Total amounts of radioactivity (expressed as percentage of the total dose) in faeces, urine, carbon dioxide and volatiles

Collection time (hour)	Faeces		Urine		Carbon dioxide		Volatiles	
	male	female	male	female	male	female	male	female
single oral dose of [^{14}C]-trichloromethyl labelled captan (10 mg/kg bw)								
0 - 6	1.96 ± 2.88	0.15 ± 0.33	26.25 ± 15.66	29.54 ± 11.49	19.46 ± 2.99	21.76 ± 2.10	0.09 ± 0.02	0.09 ± 0.06
6 - 12	5.25 ± 3.77	6.47 ± 3.46	12.52 ± 16.85	15.56 ± 12.09	1.90 ± 0.17	2.14 ± 0.51	0.03 ± 0.01	0.03 ± 0.02
12 - 24	6.07 ± 3.20	4.47 ± 2.55	3.48 ± 1.14	3.74 ± 1.48	0.37 ± 0.13	0.33 ± 0.06	0.02 ± 0.01	0.02 ± 0.02
24 - 36	1.65 ± 1.48	0.70 ± 0.38	1.14 ± 0.44	0.69 ± 0.29	0.18 ± 0.04	0.16 ± 0.02	<0.01	<0.01
36 - 48	6.03 ± 5.11	1.56 ± 1.69	0.58 ± 0.32	0.26 ± 0.08	0.09 ± 0.03	0.12 ± 0.01	<0.01	<0.01
48 - 72	0.38 ± 0.33	0.14 ± 0.04	0.41 ± 0.26	0.25 ± 0.11	-	-	-	-
72 - 96	0.16 ± 0.09	0.11 ± 0.08	0.18 ± 0.07	0.16 ± 0.07	-	-	-	-
total	21.51 ± 3.91	13.59 ± 6.56	44.56 ± 5.79	50.20 ± 6.40	22.01 ± 3.12	24.51 ± 2.09	0.13 ± 0.03	0.14 ± 0.09
repeat oral dose [^{14}C]-trichloromethyl labelled captan (10 mg/kg bw)								
0 - 6	NS	NS	32.39 ± 3.23	19.74 ± 12.03	18.03 ± 0.77	21.17 ± 0.74	0.28 ± 0.26	0.13 ± 0.05
6 - 12	0.93 ± 2.06	7.14 ± 9.08	7.25 ± 2.20	11.50 ± 10.82	4.47 ± 0.58	4.03 ± 0.53	0.18 ± 0.14	0.10 ± 0.07
12 - 24	12.67 ± 2.12	6.65 ± 4.29	4.89 ± 1.59	5.39 ± 2.64	0.71 ± 0.14	0.70 ± 0.16	0.08 ± 0.04	0.02 ± 0.01
24 - 36	0.85 ± 0.44	1.17 ± 1.34	0.93 ± 0.24	1.31 ± 0.50	0.19 ± 0.08	0.30 ± 0.13	<0.01	<0.01
36 - 48	2.61 ± 1.31	3.03 ± 3.22	1.07 ± 0.42	1.68 ± 1.18	0.10 ± 0.04	0.12 ± 0.04	<0.01	<0.01
48 - 72	0.31 ± 0.21	1.11 ± 1.16	0.78 ± 0.30	0.87 ± 0.63	-	-	-	-
72 - 96	0.21 ± 0.08	0.38 ± 0.24	0.19 ± 0.07	0.39 ± 0.16	-	-	-	-
total	17.58 ± 1.37	19.47 ± 5.24	47.50 ± 3.50	40.87 ± 5.90	23.54 ± 0.91*	26.31 ± 1.42	0.53 ± 0.37	0.26 ± 0.108
single oral dose of [^{14}C]-trichloromethyl labelled captan (500 mg/kg bw)								
0 - 6	0.47 ± 0.81	0.29 ± 0.53	1.69 ± 0.69	2.48 ± 0.41	3.38 ± 0.39	3.50 ± 0.31	0.64 ± 0.29	0.89 ± 0.48
6 - 12	1.39 ± 1.77	NS	1.48 ± 1.60	0.33 ± 0.20	2.02 ± 0.77	1.22 ± 0.13	0.81 ± 0.40	0.94 ± 0.33
12 - 24	2.19 ± 1.58	2.27 ± 0.90	4.66 ± 2.33	3.48 ± 2.49	4.18 ± 0.68	4.13 ± 0.54	0.78 ± 0.19	1.52 ± 0.16
24 - 36	4.54 ± 2.60	2.86 ± 3.56	9.20 ± 2.55	9.00 ± 1.13	3.31 ± 0.43	3.29 ± 0.25	0.98 ± 0.33	1.52 ± 0.39
36 - 48	5.87 ± 4.09	6.37 ± 5.17	3.41 ± 1.49	4.50 ± 1.08	1.23 ± 0.44	1.89 ± 0.54	0.38 ± 0.17	1.19 ± 0.65
48 - 72	21.56 ± 11.29	16.72 ± 2.99	1.86 ± 1.30	6.11 ± 2.27	0.18 ± 0.05	0.86 ± 0.53	0.06 ± 0.03	0.60 ± 0.50
72 - 96	1.40 ± 1.06	2.69 ± 1.45	0.28 ± 0.05	1.04 ± 0.42	-	-	-	-
96 - 120	2.70 ± 2.89	1.72 ± 2.06	0.14 ± 0.02	0.32 ± 0.11	-	-	-	-
total	40.14 ± 4.56	32.92 ± 2.76	22.73 ± 5.47	27.25 ± 1.23	14.45 ± 1.38**	14.88 ± 0.72	3.59 ± 0.64	6.66 ± 0.59

NS = not sampled * and ** include an additional 0.04 ± 0.09% and 0.15 ± 0.21% radioactivity collected in a backup trap over the 0 - 48 and 0 - 72 hour post-dose periods, respectively.

The concentrations of radioactivity in tissues following dosing with [^{14}C]-captan are summarised in Table 2.5-4, Table 2.5-5 and Table 2.5-6.

Table 2.5-4 Radioactivity in tissues following a single oral dose of [^{14}C] trichloromethyl labelled captan (10 mg/kg bw)

Tissue	males		females	
	µg equivalents captan/g tissue	% total dose	µg equivalents captan/g tissue	% total dose
blood	0.139 ± 0.003	-	0.135 ± 0.003	-
bone	0.136 ± 0.006	<0.01	0.147 ± 0.014	<0.01
brain	0.062 ± 0.005	<0.01	0.076 ± 0.011	<0.01
carcass	0.138 ± 0.010	1.23 ± 0.106	0.146 ± 0.009	1.23 ± 0.077
fat	0.059 ± 0.010	<0.01	0.063 ± 0.006	<0.01
heart	0.124 ± 0.005	<0.01	0.130 ± 0.015	<0.01
kidney	0.610 ± 0.031	0.06 ± 0.007	0.614 ± 0.080	0.06 ± 0.008
large intestine*	0.100 ± 0.031	0.04 ± 0.012	0.112 ± 0.015	0.04 ± 0.005
liver	0.484 ± 0.069	0.29 ± 0.038	0.513 ± 0.088	0.28 ± 0.058
lungs	0.183 ± 0.008	<0.01	0.224 ± 0.006	0.01 ± 0.000
muscle	0.112 ± 0.006	<0.01	0.116 ± 0.004	<0.01
ovaries	-	-	0.173 ± 0.054	<0.01
small intestine*	0.110 ± 0.005	0.06 ± 0.005	0.136 ± 0.013	0.07 ± 0.013
spleen	0.182 ± 0.040	<0.01	0.260 ± 0027	<0.01
stomach*	0.169 ± 0.043	0.04 ± 0.016	0.327 ± 0.065	0.05 ± 0.012
testes	0.106 ± 0.005	0.01 ± 0.000	-	-
uterus			0.251 ± 0.076	<0.01
total	-	1.73 ± 0.114	-	1.75 ± 0.137

* These tissues include contents

Table 2.5-5 Radioactivity in tissues following repeat oral doses of [^{14}C] trichloromethyl labelled captan (10 mg/kg bw)

Tissue	males		females	
	µg equivalents captan/g tissue	% total dose	µg equivalents captan/g tissue	% total dose
blood	0.165 ± 0.017	-	0.139 ± 0.178	-
bone	0.131 ± 0.024	<0.01	0.132 ± 0.022	<0.01
brain	0.086 ± 0.009	<0.01	0.080 ± 0.008	<0.01
carcass	0.145 ± 0.019	1.24 ± 0.124	0.142 ± 0.021	1.20 ± 0.199
fat	0.050 ± 0.012	<0.01	0.069 ± 0.027	<0.01
heart	0.156 ± 0.009	<0.01	0.173 ± 0.025	<0.01
kidney	0.725 ± 0.094	0.06 ± 0.012	0.627 ± 0.064	0.05 ± 0.008
large intestine*	0.167 ± 0.087	0.04 ± 0.011	0.140 ± 0.056	0.05 ± 0.017
liver	0.400 ± 0.091	0.17 ± 0.048	0.399 ± 0.128	0.18 ± 0.057
lungs	0.227 ± 0.028	0.01 ± 0.004	0.244 ± 0.050	0.01 ± 0.005
muscle	0.094 ± 0.003	<0.01	0.090 ± 0.010	<0.01
ovaries	-	-	0.221 ± 0.036	<0.01
small intestine*	0.164 ± 0.051	0.05 ± 0.004	0.150 ± 0.028	0.06 ± 0.013
spleen	0.295 ± 0.037	<0.01	0.297 ± 0.053	<0.01
stomach*	0.359 ± 0.189	0.04 ± 0.008	0.437 ± 0.109	0.05 ± 0.008
testes	0.146 ± 0.015	0.02 ± 0.005	-	-
uterus	-	-	0.200 ± 0.064	<0.01
total	-	1.63 ± 0.095	-	1.61 ± 0.240

* These tissues include contents.

Table 2.5-6 Radioactivity in tissues following a single oral dose of [^{14}C] trichloromethyl labelled captan (500 mg/kg bw)

Tissue	males		females	
	µg equivalents captan/g tissue	% total dose	µg equivalents captan/g tissue	% total dose
blood	4.36 ± 0.282	-	6.45 ± 0.752	-
bone	2.93 ± 0.970	<0.01	3.23 ± 0.864	<0.01
brain	1.87 ± 0.106	<0.01	2.43 ± 0.405	<0.01
carcass	3.81 ± 0.238	0.67 ± 0.027	3.67 ± 0.424	0.61 ± 0.057
fat	2.27 ± 0.542	<0.01	1.93 ± 0.259	<0.01
heart	4.71 ± 0.191	<0.01	6.63 ± 0.844	<0.01
kidney	11.20 ± 1.030	0.02 ± 0.004	16.20 ± 2.67	0.03 ± 0.005
large intestine*	2.57 ± 0.276	0.02 ± 0.000	3.74 ± 0.897	0.03 ± 0.008
liver	9.90 ± 0.601	0.11 ± 0.013	11.40 ± 1.730	0.10 ± 0.015
lungs	4.92 ± 0.257	<0.01	7.03 ± 0.944	<0.01
muscle	2.88 ± 0.322	<0.01	2.71 ± 0.166	<0.01
ovaries	-	-	7.67 ± 2.077	<0.01
small intestine*	2.53 ± 0.310	0.03 ± 0.005	3.80 ± 0.754	0.04 ± 0.005
spleen	4.83 ± 0.280	<0.01	6.62 ± 1.293	<0.01
stomach*	11.10 ± 5.58	0.05 ± 0.028	19.4 ± 8.07	0.06 ± 0.016
testes	2.81 ± 0.190	<0.01	-	
uterus	-	-	6.21 ± 1.526	<0.01
total	-	0.90 ± 0.052	-	0.86 ± 0.088

* These tissues include contents.

Three major metabolites were observed in urine by HPLC. Thiazolidine-2-thione-4-carboxylic acid, (TTC) accounted for 22.7% of the urinary radioactivity and dithio-bis-methanesulphonic acid (DMS) and its monsulphoxide (DMS-O), which appeared to coelute under HPLC, accounted for 65.2% (combined) of the urinary radioactivity. Two minor unidentified metabolites accounted for 2.6% and 2.3% of the radioactivity. TLC confirmed the presence of the thiazolidine metabolite and resolved the mixture of sulphonic acids to be approximately. DMS and DMS-O were present in approximately equal quantities in males, whereas in females the former predominated. A summary of the metabolites detected in the urine is presented in Table 2.5-7.

Table 2.5-7 Summary of metabolites detected in urine from single oral dose animals (10 and 500 mg/kg bw)

Collection time	sex	Recovery (%)	SF[a]	18-19 minutes	DMS + DMS-O[b]	TTC[c]
single oral dose (10 mg/kg bw)						
0 - 6	M	98.3	3.6	3.0	65.8	22.6
	F	102.0	1.6	4.4	69.4	21.9
single oral dose (500 mg/kg bw)						
0 - 6	M	95.2	2.6	1.3	64.7	22.1
	F	92.9	3.1	1.1	61.6	23.1
24 - 36	M	93.4	2.3	0.8	62.7	22.8
	F	100.0	2.5	1.4	67.2	23.8
Mean ± sd		97.0 ± 3.38	2.6 ± 0.63	2.3 ± 1.05	65.2 ± 2.63	22.7 ± 0.63

[a] eluting at solvent front; [b] dithio-bis-methane sulphonic acid and it's disulphide monoxide derivative; [c] thiazolidine-2-thione-4-carboxylic acid.

In the single oral dose group (10 mg/kg bw) evidence for the presence of TTC and DMS in the faeces was equivocal according to HPLC analysis, but this could not be confirmed by TLC. The majority of radioactivity was incorporated into natural products or covalently bound to them. This may have occurred by acylation of macromolecules (proteins and polysaccharides) by thiophosgene, which is thought to be generated from captan in the gastrointestinal tract, and exchange of [^{14}C] (from $^{14}CO_2$) with macromolecules and polysaccharides.

Faeces from the high dose group animals (500 mg/kg bw) contained unmetabolised [^{14}C]-trichloromethyl-labelled captan and this accounted for approximately 2.4% and 24.2% of the radioactivity in males and females, respectively. This observation tends to indicate that there is lack of

absorption if the dose level is high enough to overcome the detoxification capacities of the gastrointestinal tract. The metabolite TTC was found to represent 10.8% and 3.3% of the radioactivity in males and females, respectively. Equivocal evidence only was found for the presence of DMS and DMS-O. The majority of radioactivity in faeces was either covalently bound or incorporated into natural products.

Attempts to identify the metabolites in the organic volatiles were inconclusive.

The proposed route of metabolism for [^{14}C]-trichloromethyl labelled captan in rats is presented in Figure 2.5-2.

Conclusions

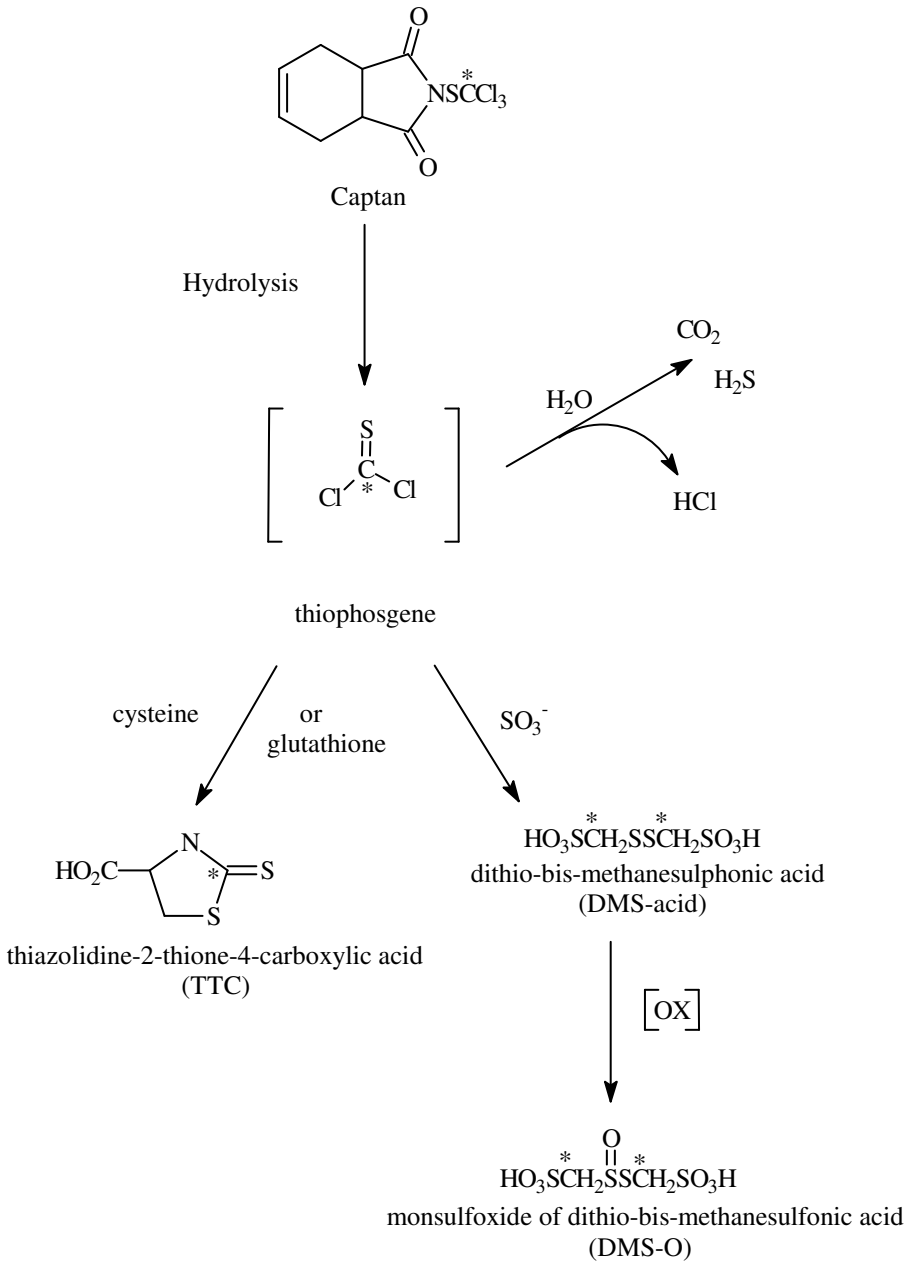
Following a single oral administration of 10 mg/kg bw [^{14}C]-trichloromethyl labelled captan, the major route of excretion was via the urine, with up to 50% of the administered dose excreted in the urine and about 15% excreted in the faeces over 96 hours. Approximately 23% of the administered radioactivity was eliminated via the pulmonary route as CO_2. Radioactivity was rapidly excreted in males and female rats with the majority (about 80%) excreted within 24 h. Tissue residues at sacrifice were less than 2% of the administered radioactivity.
Repeated oral administration of 10 mg captan/kg bw for 14 days prior to the administration of 10 mg/kg bw [^{14}C]-trichloromethyl labelled captan had negligible effect on the routes and rates of elimination.
Excretion of radioactivity following a single oral administration of 500 mg/kg bw [^{14}C]-trichloromethyl labelled captan was slower with only around 24% of the administered dose excreted in 24 hours. In contrast with the low dose, faeces were the major route of excretion with up to 40% of the administered dose excreted through this route and around 25% excreted via the urine and 15% in expired CO_2. Although excretion as volatile organics only accounted for less than 1% of the administered radioactivity in low dose animals, it accounted for 4 to 7% in high dose group animals.
The distribution in the tissues was very similar to that following administration of the low dose.

The metabolites thiazolidine-2-thione-4-carboxylic acid (TTC) and dithio-bis-methanesulfonic acid and its disulfide monoxide derivative (DMS and DMS-O) were identified in the urine, accounting for 22.7% and 65% (combined DMS and DMS-O) of the urinary radioactivity, respectively.

TTC was detected in the faeces of high dose group animals but its presence in the low dose animals was equivocal. The presence of DMS and DMS-O in faeces was equivocal in both, the low dose and the high dose group. The majority of radioactivity in faeces was incorporated into natural products or covalently bound to them. This may have occurred by acylation of macromolecules (proteins and polysaccharides) by thiophosgene or exchange of ^{14}C from $^{14}CO_2$, which is thought to be generated from captan in the gastrointestinal tract. There was evidence that the percentage absorption of the administered dose was reduced at high doses of [^{14}C]-trichloromethyl captan, as unmetabolised captan was detected in the faecal extracts from 500 mg/kg bw animals.

Attempts to characterise the volatile radioactivity were inconclusive.

Figure 2.5-2 Proposed metabolic pathway of [^{14}C] trichloromethyl labelled captan in rats



2.6 Study 6 (1985)

Reference:	The comparative metabolism of captan in the rat and mouse
Author(s), year:	Anonymous (1985)
Report/Doc. number:	Chevron Chemical Company, unpublished report No. not given (Company file: TMN-0383)
Guideline(s):	In house method
GLP:	Yes
Deviations:	Not applicable
Acceptability:	Yes

Material and Methods

A study was conducted to investigate the metabolism and gastro-intestinal fate of captan in Sprague Dawley rats and CD-1 mice (ICR derived) with [^{14}C]-trichloromethyl labelled captan (purity > 99%, specific activity 56 Ci/mole).

1. A preliminary study was carried out to determine the time-point at which peak or elevated concentrations of [^{14}C]-captan were achieved in the duodenum. Unfasted male Sprague Dawley rats and CD-1 mice (ICR derived) were administered single doses of 250 mg/kg bw by oral gavage. Animals were sacrificed at 30 minutes, 1 hour, 1.5 hours and 4 hours after dosing and the levels of radioactivity were measured.

2. Groups of 2 rats and 6 mice were maintained on normal diet (non-pretreated) and then orally intubated with 250 mg/kg bw [^{14}C]-captan. Animals were sacrificed two hours after dosing and the distribution of radioactivity determined.

3. In a separate study where animals were not pretreated, the stomach, duodenum and distal small intestine were removed from rats and mice sacrificed two hours after dosing with 250 mg/kg bw [^{14}C]-captan. Tissues were extracted with ethyl acetate and methanol:water (1:1).

4. Gastrointestinal fate study: In the main study groups of 20 rats and 20 mice of each sex were maintained on a diet containing 5000 ppm for 90 or 148 days. A single dose of [^{14}C]-captan (in a vehicle of 1% CMC) was administered by oral gavage at 250 mg/kg bw on day 90 or 5 mg/kg bw on day 148. Animals were sacrificed approximately 2 hours after the administration of [^{14}C]-captan. Tissues were collected for analysis.

5. Metabolism study: Three unfasted males of each species were administered 250 mg/kg bw [^{14}C]-captan in 1% CMC (10 mL/kg bw dosed) by oral gavage. Urine and faeces were collected 5, 12, 24, 48, 72 and 96 hours after dosing. Carbon dioxide was collected 3, 5, 12, 24, 48, 72 and 96 hours after dosing. Animals were sacrificed 96 hours after dosing, necropsied and tissues collected for analysis.

6. Determining pH of stomach and duodenum: Rats and mice were fed diets containing either 0, 500 or 5000 ppm captan technical (purity 90%) for 21 weeks. The stomach and duodenum were isolated immediately after sacrifice and the pH of the mucosal surface measured.

Findings

1. In the preliminary study to determine the appropriate termination time for the determination of the fate of captan at the duodenum, there was no obvious peak distribution of radioactivity in the duodenum of the mouse or rat (Table 2.6-1). Radioactivity tended to distribute in greater proportions in the lower gastrointestinal tract over time, although the stomach at 4 hours after dosing retained over 83% of the radioactivity found in the gastrointestinal tract. The mouse was found to retain higher levels of radioactivity in the duodenum than the rat.

Table 2.6-1 Summary of percentage distribution of radioactivity in the gastrointestinal tract of the mouse and rat following a single oral administration of [^{14}C] trichloromethyl captan (250 mg/kg bw) – no pre-conditioning

Gastrointestinal section	% radioactivity*			
	0.5 h	1.0 h	1.5 h	4.0 h
Mouse				
Stomach	81**, 77	35	-	83.5
Duodenum	8**, 4	5	-	1.5
Distal Small Intestine	10**, 18	54	-	5.0
Large Intestine	1**, 0	5	-	10.0
Rat				
Stomach	91	75	65	87.5
Duodenum	2	1	2	0
Distal Small Intestine	7	24	32	3.5
Large Intestine	0.5	0	0	9.0

* One male rat or mouse except where indicated.
** Combined sections from five mice.

2. In a separate study, where mice and rats (not preconditioned by feeding a diet containing captan) were administered a single oral dose of 250 mg/kg bw [^{14}C]-captan and sacrificed 2 hours later, the mouse appeared to eliminate the administered radioactivity faster than the rat, with 8.9% excreted in the mouse compared to 2.2% in the rat after 2 hours. A summary of the percentage recovery of radioactivity from the mouse and rat is given in Table 2.6-2. Also, the mouse showed substantially higher levels of [^{14}C] than the rat in the duodenum (6.8% compared to 0.6%) and the large intestine (28.2% compared to 0.1%). In contrast, higher levels of radioactivity were detected in the distal small intestine of the rat (39.5%) compared to the mouse (7.1%). The results suggest that there is more active gastrointestinal passage of [^{14}C]-captan or metabolites from the stomach to the large intestine in the mouse.

Table 2.6-2 Summary of percentage recovery of radioactivity following a single oral administration of [^{14}C] trichloromethyl captan (250 mg/kg bw) no pre conditioning

	% radioactivity*	
	Mouse**	Rat***
Expired air	6.2	2.1
Cage washing	0.1	0.1
Faeces	0.1	0.1
Urine	2.7	-
Carcass	ND	ND
Stomach	48.9	57.6
Duodenum	6.8	0.6
Distal small intestine	7.1	39.5
Large Intestine	28.2	0.1

* Collected 2 hours after dosing.
** Results combined from six mice.
*** Results combined from two rats.
ND Not determined.

3. A profile of [^{14}C] extracts recovered from the gastrointestinal tracts of the mouse and rat is summarised in Table 2.6-3. The findings indicated that captan remains stable in the acidic conditions of the stomach, but is degraded or metabolised in the duodenum. The percentage of radioactivity in the duodenum identified as captan was higher in the mouse (65.7%) than in the rat (33.0%).

Table 2.6-3 TLC Profile of [^{14}C] extracts recovered from the gastrointestinal tract of the mouse and rat following a single oral administration of [^{14}C] trichloromethyl captan (250 mg/kg bw) – no pre conditioning

TLC position	% radioactivity*					
	Stomach		Duodenum		Small intestine	
	mouse**	rat***	mouse**	rat***	mouse**	rat***
Non-polar	0.3	0.1	2.2	8.7	8.2	0.4
Captan	98.2	99.0	65.7	33.0	55.3	92.8
Origin polar	1.5	0.8	32.1	58.2	36.4	6.8

* Collected 2 hours after dosing.
** Results combined from six mice.
*** Results combined from two rats.

4. The percentage recovery of radioactivity from preconditioned mice and rats following a single oral administration of either 5 or 250 mg/kg bw [^{14}C]-captan is summarised in Table 2.6-4. Absorption was highest in the low dose group versus the high dose group (increased elimination and higher residual carcass levels). Levels of radioactivity found in the duodenum of high dose mice were proportionately higher than those found in the low dose mice (5.3% compared to 1.1%). In contrast, the proportion of radioactivity in the duodenum of rats was similar for both doses (0.7% and 1.7%). While the high dose showed higher levels of label in mouse duodenum versus the rat, the low dose showed comparable levels of label in the duodenum of both species. The rat had higher levels of [^{14}C] in the stomach and distal small intestine than the mouse at both dose levels and the mouse had the greatest proportion of radioactivity in the large intestine. At the low dose, absorption of [^{14}C]-captan was greater in the mouse compared to the rat, as indicated by the higher levels of radioactivity in the urine. Again, excretion via the urine and expired air was higher in the mouse than the rat. Consequently, it appears that the relative absorption rate of captan is faster in the mouse than the rat. The tissue distribution and elimination of patterns from mice and rats from the oral administration of [^{14}C]-captan were comparable between animals that had not been preconditioned and those that had been preconditioned.

Table 2.6-4 Summary of percentage recovery of radioactivity following a single oral administration of [^{14}C] captan (5 or 250 mg/kg bw) – pre conditioned

	% radioactivity			
	250 mg/kg bw		5 mg/kg bw	
	mouse	rat	mouse	rat
Expired air	7.1	1.6	12.9	6.6
Cage washing	2.8	0.1	0.3	0.6
Faeces	0.2	0.0	0.0	0.0
Urine	11.4	1.2	23.1	7.6
Carcass	4.1	1.9	13.8	15.7
Stomach	10.5	66.6	6.3	21.1
Duodenum	5.3	0.7	1.1	1.7
Distal small intestine	13.0	27.8	8.5	46.4
Large Intestine	45.4	0.1	33.9	0.3

Analysis of the metabolic profile in the gastrointestinal tract of the low dose group mice and rats revealed that most of the radioactivity in the stomach was captan (Table 2.6-5). In the small intestine, captan accounted for 15 to 28% of the radioactivity, unidentified polar metabolites (origin) for 17 to 40%, TTC for 6 to 13% and non-polar unidentified metabolites, 1 to 2%. The proportion of TTC and polar metabolites increased markedly in the small intestine, indicating either effective degradation/metabolism of captan in the small intestine or active biliary excretion of captan metabolites. Although the high-dose study revealed higher levels of captan and [^{14}C] radioactivity in the duodenum of the mouse compared to the rat, levels of radioactivity and captan in the duodenum of low dose were more comparable. Levels of TTC and polar metabolites were similar between the mouse and rat in the low dose study.

Table 2.6-5 Profile of [^{14}C] extracts recovered from the gastrointestinal tract of the mouse and rat following a single oral administration of [^{14}C] captan (5 mg/kg bw) – pre conditioned

TLC position	% radioactivity*					
	Stomach		Duodenum		Distal small intestine	
	mouse**	rat***	mouse	rat	mouse	rat
Captan and Non-polar	73	83	15	25	34	18
(Captan)	(65)	(78)	(6)	(12)	(28)	(15)
Unidentified	2	1	2	2	1	2
TTC****	2	1	14	11	6	13
Origin	5	3	28	21	17	40

* Collected 2 hours after dosing.
** Results combined from six mice.
*** Results combined from two rats.
**** Thiazolidine-2-thione-4-carboxylic acid.

5. Metabolism study: Overall excretion of [^{14}C]-captan recovered after 96 hours from the urine, faeces, and carbon dioxide were similar for the rat and mouse, with the majority of radioactivity excreted via the urine, 44 ± 13.8% and 42.2 ± 4.2% in the mouse and rat, respectively. Other important routes of excretion were via the faeces, with 22.0 ± 9.2% and 15.6 ± 3.8% excreted by the mouse and rat, respectively, and in carbon dioxide, with 18.8 ± 4.3% and 24.2 ± 3.6% of the administered dose excreted by the mouse and rat, respectively. However, it was evident from the levels of radioactivity recovered after 12 hours (58.6% and 23% in the mouse and rat, respectively) that the elimination rate was faster in the mouse compared to the rat. A summary of the recovery of radioactivity in excreta from the mouse and rat is given in Table 2.6-6.

Table 2.6-6 Summary of the recovery from male mice and rats following a single oral administration of [^{14}C] captan (250 mg/kg bw) – no pre conditioning

Hours post-dose	Percentage administered dose*							
	Urine		Faeces		Carbon dioxide		Other**	
	mouse	rat	mouse	rat	mouse	rat	mouse	rat
12	31.1 ± 10.7	12.0 ± 2.6	9.8 ± 7.3	0.0 ± 0.0	17.7 ± 4.5	11.0 ± 1.0	-	-
24	8.0 ± 1.3	19.6 ± 1.5	8.7 ± 1.6	7.5 ± 3.3	0.4 ± 0.1	10.0 ± 3.2	-	-
48	3.3 ± 2.4	9.4 ± 2.5	0.9 ± 1.0	4.5 ± 3.9	0.3 ± 0.2	2.6 ± 0.6	-	-
72	1.3 ± 0.4	0.9 ± 0.5	0.2 ± 0.1	3.0 ± 2.7	0.2 ± 0.0	0.5 ± 0.3	-	-
96	0.4 ± 0.2	0.3 ± 0.1	2.3 ± 3.6	0.6 ± 0.7	0.1 ± 0.0	0.2 ± 0.1	0.7 ± 0.1	1.2 ± 0.3
Total***	44.0 ± 13.8	42.2 ± 4.2	22.0 ± 9.2	15.6 ± 3.8	18.8 ± 4.3	24.2 ± 3.6	0.7 ± 0.1	1.2 ± 0.3

* Mean ± SD for three male rats or mice.
** Includes cage wash and tissues.
*** Mean total calculated from individual totals.

6. Measurements of the stomach and duodenal mucosal pH are presented in Table 2.6-7. There were no dose-related trends in either male or female mice or rats. Treated male rats had a lower pH than mice at the same treatment levels and the overall mean pH value of the rat stomach mucosa was significantly lower than that of the mouse in both males and females.

Binding of captan to histone proteins in the calf thymus has been shown to be pH dependant (Couch and Segal, 1977), with binding occurring at pH 9.0 but not at pH 7.5. As captan has been shown to be carcinogenic in the upper small intestine of the mouse, the stability or reactivity potential of captan in the gastric-pyloric region may be critical to the duodenum. The duodenal mucosal pH of male mice fed either 500 ppm or 5000 ppm was significantly lower than the controls. This suggests that prolonged exposure to dietary captan may lower the pH of the duodenal mucosa in male mice. Various cell types in the duodenum are highly dependent on the alkalinity provided by biliary excretion. In the presence of thiols the relative alkalinity of the duodenum favours the hydrolysis of captan to thiophosgene which may be further degraded to carbonyl sulphide and HCl producing localised acidic change in the duodenum. However, whether this is the cause in this case is uncertain.

Table 2.6-7 Stomach and duodenal pH in rats and mice preconditioned with 500 and 5000 ppm captan

	Mouse		Rat	
	mean ± sd	**N**	**mean ± sd**	**N**
Stomach mucosal pH:				
Male				
Control	3.58 ± 0.74	15	3.17 ± 0.85	10
500 ppm	4.01 ± 0.56[a]	7	3.02 ± 0.73[a]	5
5000 ppm	3.96 ± 0.53[a]	5	2.98 ± 0.87[a]	9
Overall Mean	3.76 ± 0.68[b]	-	3.07 ± 0.80[b]	-
Female				
Control	3.53 ± 0.79	14	3.09 ± 0.82	11
500 ppm	3.50 ± 1.03	8	2.91 ± 0.61	6
5000 ppm	3.58 ± 0.75	8	2.96 ± 0.59	5
Overall Mean	3.54 ± 0.82[a]	-	3.01 ± 0.69[a]	-
Duodenal mucosal pH:				
Male				
Control	6.33 ± 0.18	15	6.10 ± 0.29[c]	10
500 ppm	6.09 ± 0.19[d]	7	6.18 ± 0.28	5
5000 ppm	6.08 ± 0.21[d]	5	6.19 ± 0.28	9
Overall Mean	6.22 ± 0.22	-	6.15 ± 0.27	-
Female				
Control	6.15 ± 0.29	14	6.07 ± 0.22	11
500 ppm	6.11 ± 0.14	8	6.11 ± 0.22	6
5000 ppm	6.13 ± 0.24	8	6.27 ± 0.34	5
Overall Mean	6.13 ± 0.23	-	6.13 ± 0.23	-

[a] Mean stomach pH of rat significantly lower ($p \leq 0.05$) than mouse (Student's t-test).
[b] Overall mean stomach pH of rats significantly lower ($p \leq 0.01$) than that of the mouse (Student's t-test).
[c] Mean duodenal pH of control rat is significantly lower ($p \leq 0.05$) than that of the mouse (Student's t-test).
[d] Mean duodenal pH is significantly lower ($p \leq 0.05$) than the control (two tail analysis of variance).

Conclusions

The gastrointestinal fate of captan in rats and mice was compared in a study in which animals were administered single oral doses of 5 or 250 mg/kg bw of [^{14}C]-trichloromethyl-labelled Captan following pre-treatment with dietary exposures to 5000 ppm of captan during 90 or 148 days. Elimination of [^{14}C]-trichloromethyl-labelled captan following oral gavage was greater in the mouse compared to the rat. Gastric retention of captan was longer and the gastric mucosal pH was lower in the rat than in the mouse. In the high dose group, markedly higher duodenal levels of captan were found in the mouse compared to the rat. The duodenal pH of the male mice preconditioned with 500 or 5000 ppm dietary captan was significantly lower than the pH in control mice. It was suggested that these findings may be important in explaining the observed species differences for duodenal carcinogenicity in the mouse and rat.
Results from these studies indicate that the absorption of captan in rats is dose dependent. Biotransformation of captan occurs in the gastrointestinal tract. At low doses a high proportion is extensively metabolised prior absorption, whilst at higher doses captan is incompletely metabolised and a proportion is excreted unchanged. Captan was not detected in the tissues and was only detected in the faeces. Captan undergoes metabolic cleavage of the nitrogen-sulphur bond, which probably occurs rapidly since no metabolites with an intact nitrogen-sulphur bond have been detected in the excreta, although elimination of both radiolabelled forms were rapid. There was no evidence for dose-related changes in the relative proportions of metabolites formed from either the tetrahydrophthalamide or trichloromethyl moieties. Although biotransformation and excretion were similar in the rat and mouse, Captan is absorbed more rapidly in mice at higher doses and higher concentrations of captan and/or its metabolites were retained in the mouse duodenum.

2.7 Berthet, A., Bouchard, M., Danuser, B. (2012)

Reference:	Toxicokinetics of captan and folpet biomarkers in orally exposed volunteers
Author(s), year:	Berthet, A., Bouchard, M., Danuser, B. (2012b)
Report/Doc. number:	Journal of Applied Toxicology, Vol. 32, 194-201 (Company file: R-31005)
Guideline(s):	None
GLP:	No
Deviations:	Not applicable
Acceptability:	Supplemental data only

Material and Methods

Test material:

Identification: Captan
Lot/Batch #: Not reported
Purity: 99.1-99.4%
Vehicle: Orange juice

Test subjects:

Participants: Volunteers were healthy, non-smokers and did not take medication or drugs. They underwent a medical examination by an occupational physician prior to enrolment. They had never been exposed to captan, except perhaps through their diet. During the study period and 2 days prior to dosing, they were asked not to eat fruit and vegetables in order to limit the ingestion of contaminated food. They were also requested to avoid consumption of alcohol during this period.
Age: 20 - 30 years
Sex: Male
Number of volunteers: 10
Weight: 60 - 85 kg

Test method:

Study design: A controlled kinetic time course study in accessible biological matrices of healthy subjects was conducted following an acute oral exposure to captan. Captan metabolites were measured in urine and blood samples collected prior to treatment to obtain pre-test values, and then at predetermined time points post-dosing (amounting to a total of 9 samples for plasma and 11 samples for urine).
Dosing: A single dose was weighted in a labelled plastic cup and adjusted to the weight of each volunteer. The fungicide dose was mixed with 25 mL of vehicle and administered to the participants. The cup used for dosing was then rinsed with another 25 mL of vehicle and also administered to the participants.
Dose level: 1 mg/kg bw
Volunteers per group: 5 (the other volunteers received captan)

Examinations:

Urine samples: Samples were taken at 0, 3, 6, 9, 12, 24, 36, 48, 60, 72, 84 and 96 h post-dosing
Blood samples: Samples were taken at 0, 2, 4, 6, 8, 10, 24, 48 and 72 h post-dosing

Analysis:

Detection of THPI: A liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry (LC/APCI-MS/MS) method was applied to analyse THPI (tetrahydrophthalimide) in urine and plasma following solid-phase extraction.

Findings

In control samples taken prior to captan ingestion (t_0), concentrations of THPI were below the analytical limit of detection. Following ingestion, plasma levels of THPI increased progressively with peak levels at time 10 h post-dosing. Subsequently, elimination phase from plasma appeared monophasic with a mean apparent elimination half-life of 15.7 hours.

The time course of THPI excretion rate in the urine of volunteers over a 96 h period following ingestion of 1 mg/kg bw of captan showed peak levels on average 9 h post-dosing. Following peak excretion, the mean elimination half-life was calculated to be 11.7 hours. Overall, 3.5% of the ingested dose of captan was excreted as THPI. Elemination of THPI in urine was almost complete 96 hours post-dosing.
The relatively small volume of distribution (V_d = 3.4 L) indicated that accumulation and distribution to organs is minimal.

Table 2.7-1 Toxicokinetic parameters for THPI in human volunteers following single oral administration of 1 mg/kg bw of captan

	THPI
Route of Captan administration	**Oral**
T_{max} (h)	10 (plasma)
C_{max} (nmol)	~10000 nmol
$T_{1/2}$ (h)	15.7 (plasma) 11.7 (urine)
AUC (nmol × h/L/kg)	1652 ± 289
CL (L/h)	0.18 ± 0.03
V_d (L)	3.4 ± 0.6
Recovery (% of ingested dose in urine)	3.5

Conclusions

The results of this toxicokinetic study with human volunteers following oral administration may be regarded as supplemental data despite the fact that, for ethical reasons, toxicological studies carried out on humans are not accepted according to the preamble (13) of Regulation (EC) 1107/2009.
No relevant guideline was followed and the study was not conducted according to GLP.
The study supports the *in vitro* data that the major pathways of metabolism are similar in experimental animals and in humans. Also toxicokinetics appear to be comparable between species.
THPI is considered to be a biomarker for captan in conjunction with a clearly defined exposure to captan. Validated analytical method is available to detect THPI (and captan) in body fluids (urine and blood). Please refer to Schulz (2015c), S12-04648, R-34762 (reported in the captan dRAR Volume 3-B.5.2.6) that addresses the analytical method validation.

2.8 Study 8 (1999)

Reference:	The Stability of Captan and Folpet in Whole Blood
Author(s), year:	Anonymous (1999)
Report/Doc. number:	Horizon Laboratories, unpublished report 10238 (Company file R-11143/TMN-0392A)
Guideline(s):	None
GLP:	Yes
Deviations:	Not applicable
Acceptability:	Yes

Material and Methods

1,2-^{14}C-captan (0.936 µg/mL, 0.11 µCi) and benzene ring-U-^{14}C-folpet (1.056 µg/mL, 0.074 µCi) were incubated in vitro in heparinised whole human blood at 37°C for time intervals ranging from zero to ca. 31 seconds. All blood came from the same volunteer, considered representative of all human genotypes and phenotypes. Samples were taken following overnight fasting. After incubation with test material,

the reaction was rapidly terminated by injection of the samples into phosphoric acid and acetone. Sample clean-up was by silica gel/Florisil column chromatography, and fractionation by HPLC/UV chromatography to separate radiolabelled parent materials from degradates. Fractions were assayed by scintillation to quantifiy captan, folpet and their purported degradates 1,2,3,6-tetrahydrophthalimide (THPI, from captan) and phthalimide (PI, from folpet). These degradates were identified in earlier rat studies. Control samples of captan and folpet were incubated in saline and analysed, to determine if the method of extraction, clean-up and analysis resulted in conversion of either molecule to the purported degradates.

Findings

Degradation of captan in human blood was time-dependent and exceptionally rapid, with a calculated half-life of 0.97 seconds, modelled by a first-order equation (given as µg captan = 0.643(e-0.718t)). Loss of captan was accompanied by a time-dependent appearance of THPI. All captan was converted to THPI by the end of the 31 second incubation period. Incubation of radiolabelled captan in normal saline for 60.4 seconds at 37°C was quantitatively recovered as parent material, demonstrating that whole blood was required for conversion.

For captan, radiolabel in final extracts was quantitatively recovered in eluted fractions, indicating that no sample radiolabel was retained by the HPLC column, and that no significant peaks other than parent and/or the named degradate were noted. These findings indicate that degradation of captan to THPI is direct, i.e. that no intermediate compounds are formed. THPI is not degraded to other compounds in the short time intervals of this study. The degradates in human blood were those also seen in rat metabolism studies.

Conclusions

Captan degrades rapidly in whole human blood at 37°C to THPI with a half-life of 0.97 seconds. No other short term degradates or intermediates were present. There was no significant degradation of captan in saline at 37°C, demonstrating that whole blood was required for conversion. This study demonstrated that any systemically available captan will degrade rapidly in whole human blood, to degradates also seen in rat studies. Dermal risk assessment models require absorption and assume systemic transport to other sites. This study shows that any captan absorbed dermally will degrade rapidly in the blood, such that effective exposure of other organs or tissues to the molecule is zero.

2.9 Study 9 (2004)

Reference:	Measurement of the half-life of thiophosgene in human blood
Author(s), year:	Anonymous (2004)
Report/Doc. number:	PTRL West, Inc., unpublished report number 1146W-1 (Company file: R-17121)
Guideline(s):	In house method
GLP:	Yes
Deviations:	Not applicable
Acceptability:	Yes

Material and Methods

Thiophosgene is an important degradate of captan. Data indicate that thiophosgene is produced if folpet or its analogue captan is present in the blood. Thiophosgene itself reacts rapidly with blood nucleophiles, but the reaction is quenched with phosphoric acid in acetone.
A method of detecting thiophosgene in the blood was developed. Thiophosgene reacts with cysteine to produce 2-thioxo-4-thiazolidinecarboxylic acid (TTCA), which is capable of detection with UV light (maximum absorbency at 271 nm) and quantification by HPLC at that wavelength.

Human blood was collected under heparin from a single male volunteer (33 years old), stored overnight at 10°C and brought back to 37°C before use. The experimental method involved adding 10 µL of 10 mg/mL thiophosgene (batch number 22123BO, purity 99.4%) to duplicate 1 mL samples of human whole

blood at 37°C, incubating for various times (< 3, 3, 7.5, 15 and 30 seconds), quenching the reaction with chilled 1.5% phosphoric acid in acetone, and then adding cysteine in a 0.2M phosphate/ 1mM EDTA buffer to produce TTCA, which was then quantified using HPLC. TTCA was shown to be produced in a linear relationship to the amount of thiophosgene added.

Positive and negative control samples were also prepared. The negative controls were blood samples that were not fortified with thiophosgene. Positive controls were blood samples that were quenched and chilled (to prevent thiophosgene reaction with blood nucleophiles) before addition of thiophosgene, such that the thiophosgene was fully able to react with the cysteine without competition from the blood nucleophiles. Positive control samples were prepared with 10, 30 or 100 µL of 10 mg/mL thiophosgene added to the quenched, chilled blood.
An exponential decline equation of the form y = a + b*exp(-kt) was generated by plotting the reaction time (in seconds) versus normalised thiophosgene recovery data from <3 to 7.5 second timepoints. The half life was determined by inserting 50% as y and solving for t: where t = {Ln[50-a) ÷ -k.

Findings

The positive controls showed an average recovery of 42% ± 8.6% SD (n = 6 from the three duplicate positive control samples at 10, 30 and 100 µL) calculated from the mass of thiophosgene added to quenched chilled blood samples and the mass of thiophosgene detected, calculated as thiophosgene equivalents from a TTCA standard curve. The recovery was very consistent; all kinetics sample values were corrected for 42% recovery.

Table 2.9-1 Positive control sample recovery

Repetition	Mass thiophosgene added (µg)	Mass thiophosgene detected (µg)	% Recovery
1	10	2.8	28
2	10	5	50
1	30	14.7	49
2	30	10.5	35
1	100	44.8	45
2	100	43	43
		Mean Recovery	42

No TTCA was detected in the negative control samples.

Thiophosgene rapidly disappeared from the whole blood samples. Samples from > 7.5 seconds showed a low, consistent, residual level of TTCA. This was considered to be the result of saturation of the active sites in the blood that react with thiophosgene. The sample recovery data were normalised for this residual level by subtracting the average corrected recovery from the 15 and 30 second samples from each of the corrected recovery kinetics samples. The resulting normalised % thiophosgene recovery data were fitted to the equation and a half-life of 0.6 seconds was derived.

Table 2.9-2 Kinetics sample summary

Kinetics sample identification	Actual reaction time (s)	Mass thiophosgene added (µg)	Mass thiophosgene detected (µg)	Corrected % recovery[1]	Normalised % recovery[2]
30 second rep.1	31.1	100	0.38	0.9	-0.2
30 second rep.2	30.7	100	0.46	1.1	0.1
15 second rep.1	16.3	100	0.40	1.0	0.0
15 second rep.2	15.8	100	0.50	1.2	0.2
7.5 second rep.1	7.3	100	0.54	1.2	0.2
7.5 second rep.2	7.3	100	0.51	1.2	0.2
3 second rep.1	4.4	100	0.74	1.7	0.7
3 second rep.2	3.7	100	2.55	6.2	5.2
<3 second rep.1	1.9	100	4.08	9.8	8.8
<3 second rep.2	2.4	100	5.58	13.3	12.3

[1] Corrected % Recovery = recovery based on TTCA standard curve divided by the average recovery value (42%) of positive control samples from initial blood test analysis
[2] Normalised % Recovery = Corrected % Recovery – average corrected recovery value (1.05%) of 15 and 30 second (nominal) samples.

Conclusions

Thiophosgene disappears rapidly when added in excess (100 µg/mL) to human whole blood *in vitro*. The half-life was calculated to be 0.6 seconds.

2.10 Study 10 (2015)

Reference:	Comparative in vitro metabolism of [^{14}C]-Captan using rat and human liver microsomes
Author(s), year:	Anonymous (2015)
Report/Doc. number:	TNO Triskelion, unpublished report R-34966 (Study code 20538)
Guideline(s):	None
GLP:	Yes
Deviations:	Not applicable
Acceptability:	Yes

Executive Summary:

The present study was designed to compare the *in vitro* metabolism of carboximide labelled [^{14}C]-captan by liver microsomes of Wistar rats and humans. Metabolic activity of the microsomes towards a standard substrate (testosterone) was determined and found appropriate.
The average recovery of radioactivity was 90.3%, of which 87.3% was present in the supernatant and 3.0% in the pellet.
The final incubations with rat and human liver microsomes were conducted for 5 and 240 minutes at 37ºC, with 10 µM [^{14}C]-Captan and 1.0 mg/mL microsomal protein. The reactions were terminated by the addition of acetonitrile. [^{14}C]-Captan and metabolite peaks were detected using HPLC with on-line radioactivity detection.
In conclusion, observed transformations of [^{14}C]-Captan in the test system were dominated by chemical reactions (N-S cleavage and protein reactivity). Biological metabolism (THPI hydroxylation) was less significant (≤ 25%) even at the longest incubation time compatible with functionality of the test system. Reactions in rat and human liver microsomes were similar and no unique human metabolite was observed.

Material and Methods

1. Test Material

Chemical name:	Captan
Radiolabelled material:	Carboximide-^{14}C-Captan
Batch no.:	XXI/58/A/1
Specific activity:	5.11 MBq/mg
Radiochemical purity:	95.2%

2. Vehicle and reference substances

Vehicle:	Acetonitrile
Positive control:	[4-^{14}C] -Testosterone, 6β-hydroxytestosterone
Reference substances	THPI (cis-4-cyclohexene-1,2-dicarboximide), 3-OH-THPI (3-hydroxy-4,5-cyclohexene-1,2-dicarboximide), 5-OH-THPI (5-hydroxy-3,4-cyclohexene-1,2-dicarboximide), THPAM [3-cyclohexene-1-carboxylic acid-6-(aminocarbonyl)], 3-OH-THP-amic acid (6-hydroxy-1-amido-2-carboxy-4,5-cyclohexene)

3. Rat liver microsomes

Species:	Rat
Strain:	Wistar
Source:	XenoTech, LLC, Lenexa, Kansas 66219, USA: Pool of untreated females (n=100), lot number 0610168
Protein concentration:	20 mg/mL

Storage temperature: -70°C

4. Human liver microsomes

Source: XenoTech, LLC, Lenexa, Kansas 66219, USA: Pool of males and females (n=50), lot number 1110189

Protein concentration: 26 mg/mL

Storage temperature: -70°C

5. Metabolic capacity of microsomes

No expiry date was given by the supplier of the microsomes. Metabolic capacity was investigated in the study itself.

Study design:

1. In life dates: 18. November – 23.December 2014 and 8 – 26. June 2015 at TNO Triskelion B.V., Zeist, The Netherlands

2. Incubations with [^{14}C]-captan:

Incubations with [^{14}C]-captan (dissolved in acetonitrile) were performed at 37°C in a shaking water bath in 200 µL incubation mixtures containing 0.1 M potassium phosphate buffer pH 7.4 and 3 mM NADPH. Blanks were incubated without NADPH, in the absence and presence of microsomes. The enzymatic reactions were started by addition of [^{14}C]-captan and appropriate mixing of the incubation samples. The reactions were terminated by the addition of 100 µL ice-cold acetonitrile and, immediately after, the samples were placed on ice. After centrifugation (10 minutes, approximately 2000xg, room temperature), the supernatant was analysed by HPLC using on-line radioactivity and UV detection. Samples of the dose solutions were counted for radioactivity in order to determine homogeneity.

3. HPLC analysis with radiodetection:

HPLC analysis was conducted with mobile phases of demineralised water + 0.1% formic acid and acetonitrile + 0.1% formic acid, with a Prodigy ODS-3 column (250 x 4.6 mm, 5 µm) at a flow rate of 1 mL/min. Detection was achieved with a LabLogic β-ram model 4 detector fitted with a 0.5 mL flow cell and a flow rate of 3.0 mL/min of scintillation fluid (Ultima-Flo M).

4. Preliminary experiment:

In preliminary (range-finding) experiments, [^{14}C]-captan was first incubated with rat and human liver microsomes (0.2 and 1 mg/mL) for 30 minutes at two concentrations (10 and 50 µM). Incubations were performed in duplicate. In addition, a recovery experiment was conducted at 10 µM using the same conditions. Total counts of the incubation mixtures were taken at t = 0. After the incubations, acetonitrile was added and the samples centrifuged. Next, the supernatant and the remaining pellet were counted for radioactivity.

Since significant chemical degradation of [^{14}C]-captan was observed, a second experiment was conducted in buffer only. After incubation for 30 minutes with a [^{14}C]-captan concentration of 10 µM, the incubation sample was spiked with the captan reference substances for co-elution.

5. Main experiment:

Based on the results of the preliminary experiments, the final incubations were conducted with 10 µM [^{14}C]-captan for 5 and 240 minutes and a microsomal protein concentration of 1.0 mg/mL. The aim of the main experiment was to determine the fate of captan after a short and extended incubation time. The time points were chosen to investigate the effect of microsomes on the ongoing chemical degradation. Incubations were performed in duplicate.

Since addition of NADPH showed an additional metabolite peak at 3.4 minutes, co-elution was performed with 3-OH-THPI and 5-OH-THPI.

6. Characterization of the metabolic activity of the microsomal preparations:

To determine if and to which extent the microsomal preparations were active, CYP3A activity (6β-hydroxylation of testosterone as a standard substrate) was measured according to the following method. Briefly: Incubations were performed with rat and human liver microsomes (microsomal protein concentration 0.15 mg/mL) at 37°C for 15 minutes in 200 µL incubation mixtures containing 0.1 M potassium phosphate buffer pH 7.4, 3 mM NADPH and 200 µM [^{14}C]-testosterone. The reactions were terminated by the addition of 100 µl acetonitrile and, immediately after, the samples were placed on ice.

After centrifugation (10 minutes, 3000 rpm, room temperature), the supernatant was analysed by HPLC using on-line radioactivity detection. Blanks were incubated without NADPH. HPLC analysis was performed at 48°C using a 250 x 4.6 mm Inertsil ODS-3 column, methanol/water (65:35, v/v) as HPLC eluent, and a flow-rate of 1.0 mL/min.

Findings

Metabolic activity of the microsomes towards a standard substrate (testosterone) was determined and found appropriate. The homogenicity of the dose solutions was checked. The results show that the dose solutions were homogeneous. The average [^{14}C]-captan concentration in the dose solutions was 1.08 mM, giving an incubation concentration of 10.8 µM.

Preliminary experiment:
The average recovery of radioactivity in the recovery experiment was 90.3%, of which 87.3% was present in the supernatant and 3.0% in the pellet.
It was demonstrated that [^{14}C]-captan (eluting at 21.6 min) degrades very quickly in buffer into metabolites eluting at 7.0 and 14.4 minutes. Addition of microsomal protein increased this process: after 30 minutes, no parent compound was present anymore at both protein concentrations. Addition of NADPH showed an additional metabolite peak at 3.3 minutes. Increasing the protein concentration from 0.2 to 1.0 mg/mL showed a slight increase of the peak eluting at 3.3 minutes, and the complete disappearance of the peak at 14.4 minutes.
Based on these results, it was decided to incubate [^{14}C]-captan at 10 µM in buffer only, and to spike this sample with the reference metabolites of captan. The reference compounds dissolved in acetonitrile (THPI, 3-OH-THPI, 5-OH-THPI) or acetonitrile:water = 1:1 (v/v) (THPAM, 3-OH-THP-amic acid) were also injected on HPLC without incubation sample. THPAM and 3-OH-THP-amic acid could not be observed clearly in the spiked incubation samples. 3-OH-THP-amic acid eluted at the same position as the injection signal at UV220, while for THPAM only a very small signal was observed.
Incubation in buffer showed radioactive peaks at 7.0 minutes, 14.4 minutes and 21.9 minutes (captan). Spiking the sample with THPI showed that the radioactive peak at 7.0 minutes co-eluted with THPI. The radioactive peak eluting at 14.4 minutes did not co-elute with any of the reference metabolites.

Main experiment:
Based on the results of the preliminary and co-elution experiments, it was decided to conduct the main study with 10 µM [^{14}C]-captan and 1.0 mg/mL microsomal protein. Samples were incubated for 5 and 240 minutes.
The results of the main experiment showed that after 5 minutes in buffer about 20% of [^{14}C]-captan had been degraded in about equal amounts of THPI (7.0 minutes) and the peak eluting around 14.4 minutes. After 4 hours in buffer, [^{14}C]-captan had been degraded completely into THPI (55%), a cluster of radioactivity around 9.4 minutes (21%) and the peak at 14.4 minutes (24%).
Addition of microsomes showed complete degradation of [^{14}C]-captan into THPI after 5 minutes. Incubation for 240 minutes showed similar results.
Addition of NADPH showed an additional peak at 3.4 minutes. Co-elution with 3-OH-THPI and 5-OH-THPI showed that this peak co-eluted with 3-OH-THPI. Co-elution with THPAM and 3-OH-THP-amic acid was not performed due to interference with the UV signal at 220 nm. Injection of these compounds as standard solution showed that THPAM and 3-OH-THP-amic acid eluted at 4.3 and 3.1 minutes. In addition, the formation of hydroxy metabolites of THPI in microsomal incubations in the presence of NADPH is more likely than the formation of THPAM and 3-OH-THP-amic acid.
In rat liver microsomes, about 24% was present as 3-OH-THPI after 5 minutes, and 76% as THPI. In human liver microsomes these percentages were 8.4% and 92%, respectively.
After 4 hours, about 25% was present as 3-OH-THPI in rat liver microsomes, and 75% as THPI. In human liver microsomes these percentages were 15% and 85%, respectively.

Table 2.10-1 [^{14}C]-Captan metabolite formation in rat and human liver microsomes

Incubation time	NADPH 3 mM	Microsomes 1 mg/mL	% Peak area (rounded)									
			3-OH-THPI		THPI		Unknown		Unknown		Captan	
			RT 3.4 min		RT 7.0 min		RT 9.4 min		RT 14.4 min		RT 21.65 min	
			Rat	Human	Rat	Human	Rat	Human	Rat	Human	Rat	Human
5 min	-	-	-	-	9.6	9.6	-	-	8.9	8.9	81.5	81.5
	-	+	-	-	100	100	-	-	-	-	-	-
	+	+	23.6	8.4	76.4	91.7	-	-	-	-	-	-
240 min	-	-	-	-	54.8	54.8	20.7	20.7	24.5	24.5	-	-
	-	+	-	-	100	100	-	-	-	-	-	-
	+	+	25.1	14.9	74.9	85.1	-	-	-	-	-	-

Incubation time = Incubation time 10 µM [14C]-Captan
Unknown = identity not confirmed
RT = retention time (HPLC)
- = not detected

Conclusions

In conclusion, observed transformations of [^{14}C]-captan in the test system were dominated by chemical reactions (N-S cleavage and protein reactivity). Biological metabolism (THPI hydroxylation) was less significant (≤ 25%) even at the longest incubation time compatible with functionality of the test system for both human and rat microsomes.
Reactions in rat and human liver microsomes were similar and no unique human metabolite was observed.

2.11 Study 7 (2014)

Reference:	Captan - Waiver for an intravenous study on oral bioavailability
Author(s), year:	Anonymous (2014)
Report/Doc. number:	GAB Consulting GmbH, Report no. 961562-CA-050101-1
Guideline(s):	None
GLP:	Not applicable
Deviations:	Not applicable
Acceptability:	Yes

Executive Summary

Based on the available data on the toxicokinetics of captan, it is concluded that captan is rapidly and almost quantitatively absorbed following oral administration in the rat. In accordance with the conclusions in the EFSA report absorption from a low oral dose is estimated to be at least 81% of the oral dose judged by the proportion that was excreted via the urinary route. Radioactivity excreted with faeces (8% of the oral dose) was characterised mostly as metabolites suggesting that this fraction had been absorbed and excreted via the bile. Thus, the proportion of absorbed captan following oral administration was in fact exceeding 85%.
This approach in estimating bioavailability based on excretion data is in accordance with the current guidance on setting of the AOEL where it is also stated that for a value of 80% and above the external dose and the systemic dose are considered equal.
In light of these available data, it is not considered necessary to conduct a new vertebrate study to investigate the oral bioavailability of captan.

RMS AT: The waiver is acceptable to the RMS. Conducting a new vertebrate study with intravenous dosing is considered to be not justified for the active substance captan.

2.12 Berthet, A., Heredia-Ortiz, R., Vernez, D., Danuser, B., Bouchard, M. (2012)

Reference:	A detailed urinary excretion time course study of Captan and Folpet biomarkers in workers for the estimation of dose, main route-of-entry and most appropriate sampling and analysis strategies
Author(s), year:	Berthet, A., Heredia-Ortiz, R., Vernez, D., Danuser, B., Bouchard, M. (2012a)
Report/Doc. number:	Ann. Occup. Hyg., Vol. 56(7), 815-828
Guideline(s):	None
GLP:	No
Deviations:	Not applicable
Acceptability:	Supplementary information only

Abstract

Captan and folpet are two fungicides largely used in agriculture, but biomonitoring data are mostly limited to measurements of captan metabolite concentrations in spot urine samples of workers, which complicate interpretation of results in terms of internal dose estimation, daily variations according to tasks performed, and most plausible routes of exposure. This study aimed at performing repeated biological measurements of exposure to captan and folpet in field workers (i) to better assess internal dose along with main routes-of-entry according to tasks and (ii) to establish most appropriate sampling and analysis strategies. The detailed urinary excretion time courses of specific and non-specific biomarkers of exposure to captan and folpet were established in tree farmers (n = 2) and grape growers (n = 3) over a typical workweek (seven consecutive days), including spraying and harvest activities. The impact of the expression of urinary measurements [excretion rate values adjusted or not for creatinine or cumulative amounts over given time periods (8, 12, and 24 h)] was evaluated. Absorbed doses and main routes-of-entry were then estimated from the 24-h cumulative urinary amounts through the use of a kinetic model. The time courses showed that exposure levels were higher during spraying than harvest activities. Model simulations also suggest a limited absorption in the studied workers and an exposure mostly through the dermal route. It further pointed out the advantage of expressing biomarker values in terms of body weight-adjusted amounts in repeated 24-h urine collections as compared to concentrations or excretion rates in spot samples, without the necessity for creatinine corrections.

Material and Methods

The detailed time profiles of key biomarkers of exposure to captan were characterized in the urine of two agricultural workers (tree farmers) subjected to different exposure scenarios, (i) preparing/mixing/loading/spraying activities, and (ii) harvest activities following the 48 h required delay of re-entry. At least 2 weeks separated the two exposure scenarios. Captan ring-metabolites were quantified in pre-seasonal urines and, for each exposure scenario, in all urines voided over seven consecutive days.
All participants are described as male, aged 35-55 years, body weight of 74-115 kg, healthy non-smokers that underwent a medical examination by an occupational physician prior to enrolment. Distinct questionaires were elaborated for the two exposure scenarios (e.g. work habits such as safety equipments and hand washing, other pestices used during the study period, medication intake).

THPI was isolated by solid phase extraction, eluated in dichloromethane, and analysed by liquid chromatography – atmospheric pressure chemical ionisation-tandem mass spectrometry (LC-APCI-MS/MS). THPI was adjusted by creatinine contents. Multi compartment toxicokinetic modelling was used to reconstruct the absorbed dose and to obtain an indication of the predominant route of exposure for these workers. The rates of change in the amounts of compounds or its metabolites in the different compartments were represented by a set of linear first-order ordinary differential equations. For oral absorption the considered compartments were the parent compound and its almost instantaneously generated metabolite in the GI-tract, the body burden of metabolites in blood and in tissues in dynamical equilibrium with blood, both monitored and non-monitored, and the different excretion compartments representing cumulative amounts of monitored metabolites in urine and faeces. Dermal exposure was simulated with epidermis and dermis representing distinct compartments. Inhalation exposure was modelled with direct inputs to the blood compartment.

Findings

Workers exposed to captan conducted field spraying by using tractors with a cabin. They did not wear masks or coveralls during spraying or harvest activities, and only one wore gloves during preparation, mixing, loading and cleaning tasks but not during harvest activities. In the following table, the major parameters for exposure and exposure results for the two individuals (orchard workers) exposed to captan are summarised.

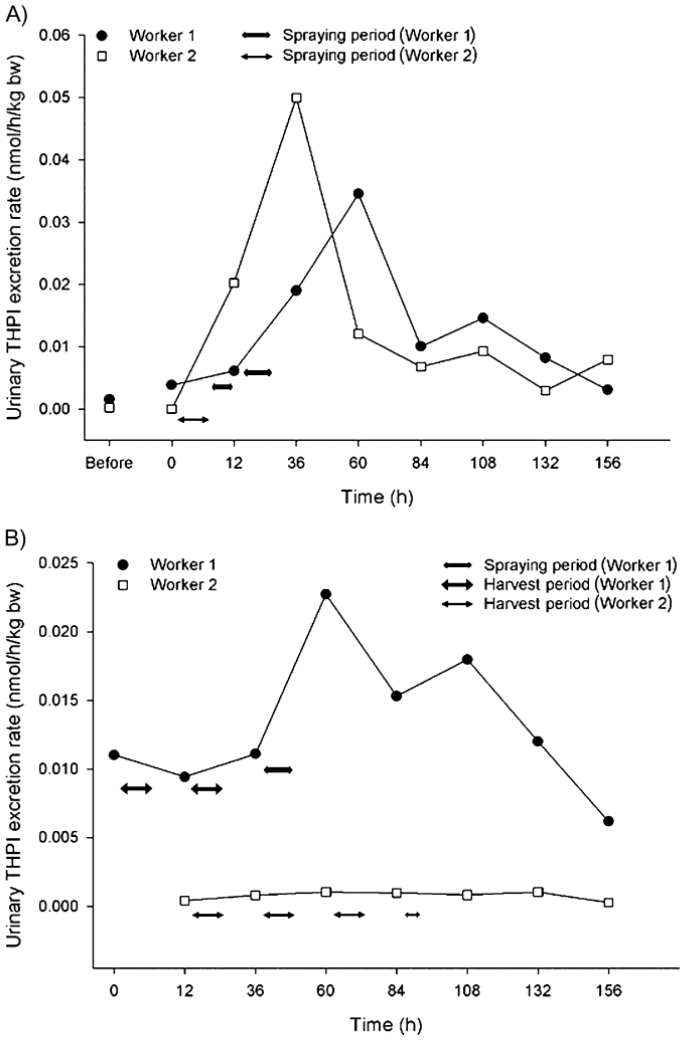
Table 2.12-1 Characteristics of captan exposure for each worker following fungicide treatment and harvest activities

	Captan exposure	
	Worker 1	Worker 2
Application activities		
Active ingredient, %	80% Captan	80% Captan
Amounts (kg)	4.5 (or 1 kg ha^{-1})	NR
Water volume	400 L ha^{-1}	500 L ha^{-1}
Treated area (ha)	2.5	5
Spraying date	07–08/05/2009	08/08/2009
Total spraying duration, h	2	8
Spraying technique	Airblast pulled by a tractor with an opened cabin	Airblast pulled by a tractor with a closed cabin
Safety equipment worn during application	Leather shoes	Rubber boots
	Waterproof gloves	Cap
Symptoms	No	No
Harvest activities		
Active ingredient, %	80% Captan	80% Captan
Harvesting date	15/06/2009	17–20/06/2009
Total harvesting duration, h	5	30
Symptoms	No	No

NR, not reported.
Workers exposed to captan wore the same safety equipment during preparation and cleaning as described for application. No personal protective equipment was worn during harvesting period. Hands were washed after spraying and harvesting, and the clothes were removed at home at the end of the workday for both activities. For Worker 1, preparation was conducted inside and gloves only were decontaminated with water post-spraying. Conversely, for Worker 2, preparation was conducted outside and no decontamination of equipment was performed post-spraying.
All workers performed mixing, loading, and material cleaning activities in addition to spraying.

Exposure to captan was found to be higher during spraying than during harvest activities, particularly for worker 2 (Figure 2.12-1).
Creatinine adjustment of urinary excretion of THPI had litte effect on the time courses of the biomarker in spot or pooled samples.
Exposure route simulations of the time course of THPI in workers were conducted for both spraying and harvest scenarios, showing that simulations of a dermal exposure scenario provided the closest description of the observed time course as compared to oral and inhalation scenarios.

Figure 2.12-1 Time courses of THPI urinary excretion rate (expressed as nanomoles per hour per kilogram of body weight) over a 168-h period in two workers exposed to captan following spraying activities (A) and harvest activities in a captan-treated area (B). Arrows represent treatment period or harvesting period (for the two workers).



Conclusions

Only two individuals exposed as operators during mixing/loading, spray application and cleaning and as re-entry workers during harvest were included in the study.
Exposure to captan was more important during spraying period than harvest activities.
Modelling simulation of exposure considering the various possible routes (inhalation, dermal or oral) indicated that dermal exposure is the main route for both operators and re-entry workers.

2.13 Berthet, A., Bouchard, M., Vernez, D. (2011)

Reference:	Toxicokinetics of captan and folpet biomarkers in dermally exposed volunteers
Author(s), year:	Berthet, A., Bouchard, M., Vernez, D. (2011)
Report/Doc. number:	Journal of Applied Toxicology, 32, 202-209 (Company file: R-29191)
Guideline(s):	None
GLP:	No
Deviations:	Not applicable
Acceptability:	Supplementary information only

Abstract

To better assess biomonitoring data in workers exposed to captan and folpet, the kinetics of ring metabolites [tetrahydrophthalimide (THPI), phthalimide (PI) and phthalic acid] were determined in urine and plasma of dermally exposed volunteers. A 10 mg kg-1 dose of each fungicide was applied on 80 cm^2 of the forearm and left without occlusion or washing for 24 h. Blood samples were withdrawn at fixed time periods over the 72 h following application and complete urine voids were collected over 96 h post-dosing, for metabolite analysis. In the hours following treatment, a progressive increase in plasma levels of THPI and PI was observed, with peak levels being reached at 24 h for THPI and 10 h for PI. The ensuing elimination phase appeared monophasic with a mean elimination half-life (t½) of 24.7 and 29.7 h for THPI and PI, respectively. In urine, time courses PI and phthalic acid excretion rate rapidly evolved in parallel, and a mean elimination t½ of 28.8 and 29.6 h, respectively, was calculated from these curves. THPI was eliminated slightly faster, with a mean t½ of 18.7 h. Over the 96 h period post-application, metabolites were almost completely excreted, and on average 0.02% of captan dose was recovered in urine as THPI while 1.8% of the folpet dose was excreted as phthalic acid and 0.002% as PI, suggesting a low dermal absorption fraction for both fungicides. This study showed the potential use of THPI, PI and phthalic acid as key biomarkers of exposure to captan and folpet.

Material and Methods

Test material:

Identification:	Captan
Lot/Batch #:	Not reported
Purity:	99.1 - 99.4%
Vehicle:	Acetone

Test subjects:

Participants:	Volunteers were healthy, non-smokers and did not take medication or drugs. They underwent a medical examination by an occupational physician prior to enrolment. They had never been exposed to folpet, except for an acute oral dose of 1 mg/kg bw 2 weeks previously, and perhaps through their diet. During the study period and 2 days prior to dosing, they were asked not to eat fruit and vegetables in order to limit the ingestion of contaminated food. They were also requested to avoid consumption of alcohol during this period.
Age:	20 - 30 years
Sex:	Male
Number of volunteers:	9
Weight:	60 - 85 kg

Test method:

Study design:	A controlled kinetic time course study in accessible biological matrices of healthy subjects was conducted following dermal exposure to captan. Captan metabolites were measured in urine and blood samples collected prior to treatment to obtain pre-test values, and then at predetermined time points post-dosing (amounting to a total of 9 samples for plasma and 11 samples for urine).
Dosing:	A single dose was weighted in a labelled glass container and adjusted to the weight of each volunteer. The fungicide dose was dissolved in 30 mL of acetone, mixed and dermally applied on 80 cm^2 of one forearm of each

volunteer. The participants washed their arm only 24 h following application. The exposed area was not occluded to avoid influencing absorption, except that some of the participants wore long sleeve shirts.

Dose level: 10 mg/kg bw

Volunteers per group: 5 (the other volunteers received folpet)

Examinations:

Urine samples: Samples were taken at 0, 3, 6, 9, 12, 24, 36, 48, 60, 72, 84 and 96 h post-dosing

Blood samples: Samples were taken at 0, 2, 4, 6, 8, 10, 24, 48 and 72 h post-dosing

Analysis:

Detection of THPI: A liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry (LC/APCI-MS/MS) method was applied to analyse THPI in urine and plasma following solid-phase extraction.

Findings

Following dermal application of captan, a progressive increase in plasma levels of THPI was observed, with peak levels being reached 24 h post-dosing. The subsequent elimination phase from plasma over the 24 - 72 h period post-dosing appeared monophasic with an apparent elimination half-life of 24.7 hours.

THPI in plasma had a relatively small volume of distribution (V_d). Mean residence time (MRT) of THPI was 34 hours.

The time course of THPI excretion rate in the urine of volunteers over a 96 h period following application of 10 mg/kg bw of captan showed that, following peak excretion, THPI was excreted with an elimination half-life of 18.7 hours. It was calculated that 0.02% of the applied captan dose was recovered in urine as THPI.

Table 2.13-1 Toxicokinetic parameters in human volunteers following dermal administration

	THPI
Route of captan administration	**dermal**
T_{max} (h)	24 (plasma)
C_{max} (nmol)	> 500 nmol
$T_{1/2}$ (h)	24.7 (plasma) 18.7 (urine)
AUC (nmol × h/L/kg)	137.3 ± 43.38
CL (L/h)	0.24 ± 0.07
V_d (L)	7.44 ± 2.14
Recovery (% of applied dose) in urine	0.02

Conclusions

The results of this toxicokinetic study with human volunteers following dermal administration may be regarded as supplemental data despite the fact that, for ethical reasons, toxicological studies carried out on humans are not accepted according to the preamble (13) of Regulation (EC) 1107/2009.

No relevant guideline was followed and the study was not conducted according to GLP.

Toxicokinetic parameters (T_{max}, $T_{1/2}$, V_d) in humans for THPI were different upon oral and dermal application. The study supports the *in vitro* data that the major pathways of metabolism are similar in experimental animals and in humans. Also toxicokinetics appear to be comparable between species. Dermal absorption was low despite the fact that captan was dissolved in acetone and applied on a rather large skin surface area.

2.14 Heredia-Ortiz, R., Bouchard, M. (2012)

Reference:	Toxicokinetic modelling of captan fungicide and its tetrahydrophthalimide biomarker of exposure in humans
Author(s), year:	Heredia-Ortiz, R., Bouchard, M. (2012)
Report/Doc. number:	Toxicology Letters, Vol. 213(1), 27-34
Guideline(s):	None
GLP:	No
Deviations:	Not applicable
Acceptability:	Yes

Abstract

Measurement of tetrahydrophthalimide (THPI) in urine has been used for the biomonitoring of exposure to the widely used captan fungicide in workers. To allow a better understanding of the toxicokinetics of captan and its key biomarker of exposure, a human multi-compartment model was built to simulate the transformation of captan into THPI and its subsequent excretion while accounting for other non-monitored metabolites. The mathematical parameters of the model were determined from best-fits to the time courses of THPI in blood and urine of five volunteers administered orally 1mg/kg and dermally 10 mg/kg of captan. In the case of oral administration, the mean elimination half-life of THPI from the body (either through faeces, urine or metabolism) was found to be 13.43 h. In the case of dermal application, mean THPI elimination half-life was estimated to be 21.27 h and was governed by the dermal absorption rate. The average final fractions of administered dose recovered in urine as THPI were 3.6% and 0.02%, for oral and dermal administration, respectively. Furthermore, according to the model, after oral exposure, only 8.6% of the THPI formed in the GI reaches the bloodstream. As for the dermal absorption fraction of captan, it was estimated to be 0.09%. Finally, the average blood clearance rate of THPI calculated from the oral and dermal data was 0.18 ± 0.03 ml/h and 0.24 ± 0.6 ml/h while the predicted volume of distribution was 3.5 ± 0.6l and 7.5 ± 1.9l, respectively. Our mathematical model is in complete accordance with both independent measurements of THPI levels in blood (R^2 = 0.996 for oral and R^2 = 0.908 for dermal) and urine (R^2 = 0.979 for oral and R^2 = 0.982 for dermal) as well as previous experimental data published in the literature.

Evaluation

The development of a toxicokinetic model for captan and its THPI metabolite in humans, which is in agreement with experimental data available for captan, was based on human time-course data of the reports Berthet et. al. (2012b) and Berthet et al. (2011).
According to model simulations, the first biological process governing the kinetics of captan is the almost instantaneous non-enzymatic breakdown of captan into THPI and thiophosgene. This was modelled to occur in the gastrointestinal tract following oral exposure and at first contact with blood in the dermis following dermal exposure. Once in blood, THPI elimination was found to be relatively rapid (half-ilfe of 13.4 h), and was modelled to occur through metabolism to other ring-derivatives mainly and to some extent by renal clearance and faecal excretion.
Levels of THPI in non-monitored tissues (such as liver, kidney, lung) were taken to rapidly reach a dynamical equilibrium with blood and were theoretically assumed to evolve in parallel. Therefore, blood and these tissues were lumped under a single compartment defined as the blood compartment for simplicity. THPI metabolism to derivatives is expected to occur in large part in the liver given that derivatives identified in rat urine were mostly oxidative products (hydroxylation and epoxidation), which formation requires cytochrome P450 activity (Anonymous, 1990).

Conclusions

The toxicokinetic model for captan and its metabolite THPI in humans developed in this study provides a very good approximation of the experimental time course data of Berthet et al. (2012 b and 2011) in volunteers orally and dermally exposed to captan under controlled conditions.
The overall conclusion is that metabolism and toxicokinetics of captan are conserved and similar for experimental animals and humans.

3 HEALTH HAZARDS

Acute toxicity

3.1 Acute toxicity - oral route

3.1.1 Animal data

3.1.1.1 Study 1 (1991)

Reference:	Captan technical: acute oral toxicity (limit test) in the rat.
Author(s), year:	Anonymous (1991a)
Report/Doc. number:	Safepharm Laboratories Ltd., Rep. R-6290
Guideline(s):	OECD 401 (1987) and 84/449/EEC B1
GLP:	Yes
Deviations from OECD 401 (1987):	No
Acceptability:	Yes

Material and Methods

Following a range-finding study, captan, suspended in distilled water, was administered orally by gavage to five male and five female Sprague-Dawley rats, at a level of 2000 mg/kg bw. Signs of toxicity and body weights were recorded up to 14 days after dosing. Gross pathological examinations were performed on all main study animals.

Findings

No mortalities and no clinical observations were recorded during the study. All animals showed expected body weight gain throughout the study. No abnormalities were detected in any animal at necropsy.

Conclusion

The acute oral median lethal dose (LD_{50}) of captan in rats was greater than 2000 mg/kg body weight. Based on the acute oral LD_{50} value, captan does not require classification and labelling according to Regulation (EC) No. 1272/2008.

3.1.1.2 Study 2 (1984)

Reference:	Acute oral toxicology
Author(s), year:	Anonymous (1984a)
Report/Doc. number:	Stauffer Chemical Company, Report No. T-11474 (Company file R-4367/TMN-0706)
Guideline(s):	OECD guidelines (1981), USEPA Fed. Reg. 43:163, 37336-37402 (1978)
GLP:	No
Deviations from OECD 401 (1987):	No; the essential criteria of OECD 401 are met
Acceptability:	Yes

Material and Methods

Captan, suspended in corn oil was administered orally, by gavage, to ten male and ten female Sprague-Dawley rats, at a dose level of 5000 mg/kg bw. Animals were observed for clinical signs of toxicity up to 14 days after dosing. Body weights were recorded at the beginning and at the end of the study. Gross pathological examinations were performed at the end of the observation period.

Findings

Two males died on days 1 and 12 and one female died on day 4. Transient clinical signs of toxicity were observed, but disappeared by days 4 and 6 in surviving males and females, respectively, except for alopecia in 3 males and 3 females which persisted throughout the observation period.
Observations in the male rats that died were salivation, bloated appearance, reddened lungs, dark edged and/or dark liver, bloated gastrointestinal tract, test material like fluid in the stomach and small darkened kidneys. The female that died was cannibalized and was therefore not necropsied. Surviving animals appeared normal at necropsy except for alopecia.

Conclusion

The acute oral median lethal dose (LD_{50}) of captan in rats was greater than 5000 mg/kg body weight.
Based on the acute oral LD_{50} value, captan does not require classification and labelling according to Regulation (EC) No. 1272/2008.

3.1.1.3 Study 3 (1982)

Reference:	Acute oral toxicity of Captan technical in adult male and female rats
Author(s), year:	Anonymous (1982a)
Report/Doc. number:	Chevron Environmental Health Center Inc., report No. SOCAL 1906 (Company file: TMN-0705)
Guideline(s):	40 CFR 163.80-3
GLP:	No
Deviations from OECD 401 (1987):	Not evaluable; in the original DAR it is stated that the study met the essential criteria of USEPA
Acceptability:	Yes

Material and Methods

Captan, suspended in a vehicle of 0.7% carboxy methyl cellulose (CMC) and 1% Tween 80 in distilled water, was administered by oral gavage to Sprague Dawley rats. Dose levels in males in test 1 were 0, 5000, 6500, 8300, 10800, 14000 mg/kg bw (5 males/dose) and in test 2 were 0 and 7800 mg/kg bw (10 males/dose) in dose volumes of 2.7 to 7.5 mL/kg bw. Dose levels in females in test 1 were 0, 5000, 6500, 8300, 10800 and 14000 (5 females/dose) and in test 2 were 0 and 7200 mg/kg bw in dose volumes of 2.2 to 6.2 mL/kg bw (10 females/dose). Animals were observed for clinical signs of toxicity and mortality up to 14 days after dosing. Body weight was recorded prior to dosing and 7 and 14 days after dosing. Rats were necropsied and examined for gross pathological changes at the end of the observation period.

Findings

Mortality occurred at dose levels above 5000 mg/kg bw in males and females. The number and timing of mortalities is summarised in the following table.

Clinical findings: Symptoms observed were diarrhoea, reduced food consumption, decreased motor activity, ocular and nasal discharge, weakness, ataxia, tremors, bloody urine and collapse. The frequency of symptoms tended to increased with dose. Time to recovery increased with dose level, ranging from 2 hours at a dose of 5000 mg/kg bw to 13 days after treatment at 10800 mg/kg bw.

Autopsy: Stomach lesions, mottled livers, red lungs and kidneys, and skin lesions caused by self-mutilation were observed at autopsy. One animal was observed to have an enlarged spleen.

Histopathology: Multifocal acute gastric mucosal necrosis and multifocal acute ulcerative gastritis.

Table 3.1.1.3-1 Summary of acute toxicity of captan in mice

Test	Dose (mg/kg bw)	Toxicological results[1]	Time of death[2]	Calculated LD_{50} (mg/kg) [3]
Male				
1	0	0/5	-	7000 (5100 - 9600)
1	5000	0/5	-	
1	6500	1/5	5 days	
1	8300	4/5	2 - 5 days	
1	10800	4/5	3 - 7 days	
1	14000	5/5	3.5 hr - 4 days	
2	0	0/10	-	
2	7800	8/10	1 - 7 days	
Female				
1	0	0/5	-	6170 (3800 – 10010)
1	5000	0/5	-	
1	6500	2/5	5 days	
1	8300	5/5	2 - 6 days	
1	10800	4/5	3 - 5 days	
1	14000	5/5	1 - 3 days	
2	0	0/10	-	
2	7200	10/10	1 - 11 days	

[1] Number animals dying/number of animals dosed.
[2] Time (hr = hours) after dosing when symptoms/deaths occurred.
[3] 95% confidence limits in parenthesis.

Conclusion

The acute oral median lethal dose (LD_{50}) of captan in rats was 7000 mg/kg body weight in males and 6170 mg/kg body weight in females.
Based on the acute oral LD_{50} values, Captan does not require classification and labelling according to Regulation (EC) 1272/2008.

3.1.1.4 Study 4 (1983)

Reference:	Acute toxicological study of Captan after oral application to the mouse
Author(s), year:	Anonymous (1983)
Report/Doc. number:	Pharmatox GmbH; R-3585
Guideline(s):	OECD guidelines (1981)
GLP:	No
Deviations from OECD 401 (1987):	No; the essential criteria of OECD 401 are met
Acceptability:	Yes

Material and Methods

Captan (purity 92.7%), suspended in 1% methyl cellulose, was administered by oral gavage to CFI-strain mice at dosages of 1500, 1890, 2380 and 3000 mg/kg bw in a dose volume of 20 to 30 mL/kg bw (5 animals/sex/dose). Animals were observed for clinical signs of toxicity and mortality up to 14 days after dosing. Body weight was recorded at the beginning and end of the study. Mice were necropsied and examined for gross pathological changes at the end of the observation period.

Findings

The number and timing of mortalities is summarised in the following table.
The majority of mortalities occurred within the first 24 hours of dosing. Three animals died after 24 hours. In all dose groups clinical signs were essentially abdominal ache syndrome, reduced activity, reduced frequency of respiration, titubation, tremors. Surviving animals exhibited signs of sedation and reduced readiness for reflexing. There was a dose dependant reduction in body weight.

Macroscopic examination of the abdominal cavity revealed marked hyperaemia of the gastrointestinal tract at necropsy. Kidneys of some animals were too bright and livers of some animals were found to be swollen.

Table 3.1.1.4-1 Summary of acute toxicity of captan in mice

Dose (mg/kg bw/day)	Toxicological results[1]	Time of death[2]
Males		
1500	0/5	-
1890	1/5	1 day
2380	4/5	1 - 7 day
3000	5/5	1 day
Females		
1500	0/5	-
1890	1/5	2 days
2380	3/5	1 - 7 day
3000	5/5	1 day

[1] Number animals dying/number of animals dosed.
[2] Time after dosing when deaths occurred.

Conclusion

The acute oral median lethal dose (LD_{50}) of captan in mice calculated for males and females combined was 2110 mg/kg body weight.
Based on the acute oral LD_{50} value, captan does not require classification and labelling according to Regulation (EC) No. 1272/2008.

3.1.2 Human data

No relevant studies.

3.1.3 Other data

No relevant studies.

3.2 Acute toxicity - dermal route

3.2.1 Animal data

3.2.1.1 Study 1 (1984)

Reference:	Acute dermal toxicology
Author(s), year:	Anonymous (1984b)
Report/Doc. number:	Stauffer Chemical Company, Report No. T-11474 (Company file R-4378/TMN-0714)
Guideline(s):	OECD guidelines (1981), USEPA 81-2
GLP:	No
Deviations from OECD 402 (1987):	In half of the animals abrasion of stratum corneum was conducted in addition to shaving of fur before application of test substance; the essential criteria of OECD 402 are met
Acceptability:	Yes

Material and Methods

A single dose of captan was applied to the closely clipped abdominal skin of male and female Stauffland albino rabbits at a dose of 2000 mg/kg bw (5 animals/sex). The skin was abraded on half of the animals

and left intact on the others. The area was covered with a protective binder. The binder and test material was removed after 24 hours, the area was inspected and rewrapped in a gauze binder. After three days, the gauze binder was removed. A concurrent control (2 animals/sex) was also conducted. All animals were observed for clinical signs for at least 14 days after treatment and were necropsied at the end of the observation period.

Findings

There were no mortalities. All animals appeared normal throughout the observation period, except two rabbits which had wet areas around the eyes on days 4 and 5. These rabbits appeared normal by day 6. There were no signs of dermal irritation. No abnormalities were observed at necropsy.

Conclusion

The acute dermal median lethal dose (LD_{50}) of captan in rabbits was greater than 2000 mg/kg body weight.
Based on the acute dermal LD_{50} value, captan does not require classification and labelling according to Regulation (EC) 1272/2008.

3.2.1.2 Study 2 (1989)

Reference:	Acute dermal toxicity to rats of Captan technical
Author(s), year:	Anonymous (1989)
Report/Doc. number:	Huntingdon Research Centre Ltd, report No. 89683D/NBS 35/AC (Company file: R-5464)
Guideline(s):	OECD 402 and 79/831/EEC method B3
GLP:	Yes
Deviations from OECD 402 (1987):	No; the essential criteria of OECD 402 are met
Acceptability:	Yes

Material and Methods

A single dose of 2000 mg/kg bw of captan (72% w/v in distilled water; administration volume 2.75 mL), was applied to the intact, clipped lumbar skin (area clipped 10% total body surface area) of 5 male and 5 female CD strain rats (Crl: CD(SD) BR VAF plus). The treated area (approximately 50 x 50 mm) was covered with gauze held in place by an impermeable dressing. After 24 hours the dressing and test substance were removed and the area washed with warm water to remove any remaining test substance. Animals were observed for 14 days after treatment for clinical signs. The treated area was examined for signs of dermal irritation. Body weights were measured on days 1 (day of dosing), 8 and 15. On day 15 animals were sacrificed and subjected to macroscopic inspection of abdominal and thoracic cavities.

Findings

There were no mortalities. There were no signs of systemic toxicity. There were no signs of dermal irritation. All animals showed the expected gain in body weight during the study. No abnormalities were observed at necropsy.

Conclusion

The acute dermal median lethal dose (LD_{50}) of captan in rats was greater than 2000 mg/kg body weight in male and female rats.
Based on the acute dermal LD_{50} value, captan does not require classification and labelling according to Regulation (EC) 1272/2008.

3.2.1.3 Study 3 (1991)

Reference:	Captan Technical: Acute dermal toxicity (limit test) in the rat
Author(s), year:	Anonymous (1991b)
Report/Doc. number:	Safepharm Lab. Ltd., report No. 306/101 (Company file: R-6291)
Guideline(s):	OECD 402 (1981) and 84/449/EEC (1984)
GLP:	Yes
Deviations from OECD 401 (1987):	No; the essential criteria of OECD 401 are met
Acceptability:	Yes

Material and Methods

A single dose of 2000 mg/kg bw of captan was applied to intact, clipped skin (back and flanks clipped to expose an area of approximately 10% total body surface) of 5 male and 5 female Sprague Dawley strain rats. The skin was moistened with distilled water prior to the administration of test substance. The treated area (approximately 50 x 40 mm) was covered with gauze held in place by a semi-occlusive dressing. After 24 hours the dressing and test substance were removed and the area wiped with moistened cotton wool to remove any remaining test substance. Animals were observed for mortality and clinical signs at 0.5, 1, 2 and 4 hours after dosing and daily thereafter until 14 days after treatment. The treated area was examined for signs of dermal reaction. Body weights were recorded on day 0 (day of dosing) and on days 7 and 14. At the end of the study, animals were sacrificed and subjected to gross pathological examination.

Findings

There were no mortalities. There were no signs of systemic toxicity or dermal irritation. All animals showed the expected gain in body weight during the study. No abnormalities were observed at necropsy.

Conclusion

The acute dermal median lethal dose (LD_{50}) of captan in rats was greater than 2000 mg/kg body weight in male and female rats.
Based on the acute dermal LD_{50} value, captan does not require classification and labelling according to Regulation (EC) 1272/2008.

3.2.2 Human data

No relevant studies.

3.2.3 Other data

No relevant studies.

3.3 Acute toxicity - inhalation route

3.3.1 Animal data

3.3.1.1 Study 1 (1995)

Reference:	Merpan (Captan) Technical: Acute inhalation toxicity study in the rat
Author(s), year:	Anonymous (1995)
Report/Doc. number:	Pharmaco LSR. Ltd., report No. 94/MAK261/1087 (Company file: R-8072)
Guideline(s):	OECD 403 (1981), USEPA 81-3
GLP:	Yes
Deviations from OECD 403 (2009):	MMAD of 4.95 - 6.51 µm (recommended: 1-4 µm); the respirable fraction (< 4 µm) is therefore to low and consequently the acute inhalation toxicity is underestimated.
Acceptability:	Limited

Material and Methods

Captan technical was administered as a single 4-hour exposure (nose only) to CD (remote Sprague-Dawley) strain rats. Three groups of five males and five females were each exposed to nominal concentrations of 2.30, 4.45 and 6.23 mg/L captan, equivalent to achieved concentrations of 0.43, 0.82 and 1.36 mg/L captan, respectively. Test atmospheres were generated by the suspension of captan particulate in air using a Wright Dust Feed generator. The air flow rate was 17.9 L/min. Particle size was determined using a Sierra- Marple model 296 cascade impactor. Animals were observed for mortality and clinical signs 15 and 30 minutes after the beginning of exposure and at 30 minute intervals during the remainder of the exposure. Subsequently, animals were observed at 30 minute intervals during the first two hours after exposure and then twice daily until 14 days after exposure. Body weight was recorded daily. Rats were necropsied and observed for gross pathological changes at the end of the observation period. The weight of the lungs (with bronchi and trachea), liver and kidneys was recorded.

Findings

A summary of the nominal and achieved concentrations of captan and the particle size distribution is given in Table 3.3.1.1-1. The acute inhalation toxicity of captan in rats is presented in Table 3.3.1.1-2.

Table 3.3.1.1-1 Acute inhalation toxicity of captan in rats: particle size distribution

Concentration (mg/L)		Mass median	% <6 µm
Nominal	**Achieved[1]**	**EAD[2] (µm)**	**EAD[2] ± SD**
0	0	-	-
2.30	0.43 ± 0.01	4.95	58.6 ± 7.6
4.45	0.82 ± 0.05	5.59	54.1 ± 1.0
6.23	1.36 ± 0.22	6.51	47.5 ± 9.5

[1] Mean ± SD.
[2] Equivalent aerodynamic diameter.

Table 3.3.1.1-2 Acute inhalation toxicity of captan in rats: summary of toxicity

Concentration (mg/L)		Toxicological results[2]	Duration of signs[3]	Time of death[3]	LC_{50} mg/L (14 days)[6]
Nominal	Achieved[1]				
male					
0	0	0/5/5	2 - 3 h[4]	-	1.21 (0.75 - 1.66)
2.30	0.43 ± 0.01	0/5/5	1.5 h[4] - 3 d[5]	1 d	
4.45	0.82 ± 0.05	1/5/5	1 h[4] - 14 d[5]	1 d	
6.23	1.36 ± 0.22	3/5/5	15 min[4] – 8 d[5]	1 d	
female					
0	0	0/5/5	2 - 4 h[4]	-	1.05 (0.21 - 1.89)
2.30	0.43 ± 0.01	1/5/5	2 h[4] - 3 d[5]	1 d	
4.45	0.82 ± 0.05	2/5/5	2 h[4] - 4 d[5]	1 d	
6.23	1.36 ± 0.22	3/5/5	30 min[4] - 5 d[5]	1 d	

[1] Mean ± sd.
[2] Number of animals which died/number of animals with clinical signs/number of animals in dose group.
[3] Hours or days after exposure.
[4] During exposure.
[5] Post exposure.
[6] Acute inhalation median lethal concentration (95% confidence limits)

Mortality: All death as a result of the inhalation of the test substance occurred during the first 18 hours following exposure. The number of animals which died in the different dose groups is presented in Table 3.3.1.1-2.

Clinical signs: Clinical signs observed in animals exposed to captan were wet fur, hypothermia (cold to touch), pigmented staining of the snout, piloerection, underactivity, hunched posture, slow and deep respiration, gasping, rales, ungroomed appearance, pigmented orbital secretion, thin appearance, closed eyes and pallor of the skin. The signs were similar at all concentrations but were more persistent at the highest concentration (1.36 mg/L). Wet fur was also observed in control animals. Most animals that survived were normal in appearance by day 6, except rales and hunched posture in one male (0.82 mg/L) from day 8 to 14 and rales in another male (1.36 mg/L) on day 7 and 8.

Body weight: Animals that died overnight following exposure exhibited weight loss prior to death. Weight loss was recorded in all animals exposed to captan on day 2. Weight loss continued into day 3 for animals exposed to 1.36 mg/L and males exposed to 0.82 mg/L. Thereafter, body weight gain of most treated animals was similar to the controls except in two males (one exposed to 0.43 mg/L and one to 0.82 mg/L) whose body weight did of return to normal until day 9 and 12, respectively.

Autopsy and organ weights: Observations in animals that died were hepatisation of the lungs, incomplete lung collapse when the trachea was cut, pale or clear aerated fluid in the trachea, presence of pale brown material or fluid in the nasal cavity, soft pale amorphous material in the larynx, and clear fluid in the thoracic cavity. In animals that survived there were no treatment related necropsy findings.
Lung weights of animals that died as a result of treatment were high and liver and kidney weights of one male exposed to 1.36 mg/L captan were also higher than expected. Liver and kidney weights for most animals that died were higher than expected possibly due to pooling of blood post mortem.

Conclusion

The acute (4-hour) inhalation LC_{50} for rats exposed to captan was 1.21 mg/L and 1.05 mg/L in males and females, respectively. The combined sex LC_{50} for captan was 1.16 mg/L (95% confident limits 0.65 - 1.66 mg/L).

Based on these acute inhalation LD_{50} values, captan requires classification and labelling according to Regulation (EC) 1272/2008 as Acute Tox. Category 4 ($1.0 < LC_5 \leq 5.0$ mg/L), H332 “Harmful if inhaled”.
However, the harmonised classification of captan regarding its acute inhalation toxicity is Acute Tox. 3, H331 “Toxic if inhaled” according to Annex VI of Regulation (EC) No. 1272/2008.

3.3.1.2 Study 2 (1991)

Reference:	Captan Technical: Acute inhalation toxicity study. Four-hour exposure (nose only) in the rat
Author(s), year:	Anonymous (1991)
Report/Doc. number:	Safepharm Laboratories Limited., report No. 306/102 (Company file: R-6292)
Guideline(s):	OECD 403 (1981) and 84/449/EEC (1984)
GLP:	Yes
Deviations from OECD 403 (2009):	No; the essential criteria of OECD 403 are met
Acceptability:	Yes

Material and Methods

Captan technical was administered as a single four-hour exposure (nose only) to Sprague-Dawley rats. Three groups of five males and five females were each exposed to nominal concentrations of 0.4, 1.8 and 12.6 mg/L captan, corresponding to mean achieved concentrations of 0.23, 0.94 and 4.81 mg/L. Test atmospheres were generated by the suspension of captan particulate in air using a Wright Dust Feed generator. The air flow rate was 22 L/min, providing 44 air changes/minute. Animals were observed for mortality and clinical signs at hourly intervals during exposure, one hour after termination of exposure and then once daily for 14 days. Body weight was recorded on the day of exposure and seven and 14 days after exposure or at death. Rats were necropsied and observed for gross pathological changes at the end of the observation period.

Findings

A summary of the nominal and measured concentrations of captan and particle size distribution is presented in Table . Acute inhalation toxicity is summarised in Table 3.3.1.1-1.

Table 3.3.1.2-1 Acute inhalation toxicity of captan in rats: particle size distribution

Concentration (mg/L)		MMAD[2] (µm)	Respirable fraction (%) (< 4µm)
Nominal	Achieved[1]		
0.4	0.23 ± 0.04	1.6	88.1
1.8	0.94 ± 0.05	1.8	87.4
12.6	4.81 ± 0.36	1.8	88.2

[1] Mean ± SD.
[2] Mass mean aerodynamic diameter.

Table 3.3.1.2-2 Acute inhalation toxicity of captan in rats: summary of toxicity

Concentration		Toxicological results[2]	Duration of signs[3]	Time of death[3]	LC_{50} mg/L (14 days)[6]
Nominal	Achieved[1]				
male					
0.4	0.23 ± 0.04	0/5/5	during exposure - 4 d[4]	-	0.90 (0.43 - 1.90)
1.8	0.94 ± 0.05	3/5/5	during exposure - 6 d[4]	during exposure[5]-1 h[4]	
12.6	4.81 ± 0.36	5/5/5	during exposure[5]	during exposure[5]	
female					
0.4	0.23 ± 0.04	0/5/5	during exposure - 3 d[4]	-	0.67 (0.36 - 1.22)
1.8	0.94 ± 0.05	4/5/5	during exposure - 6 d[4]	1 h - 1 d[4]	
12.6	4.81 ± 0.36	5/5/5	during exposure[5]	during exposure[5]	

[1] Mean ± sd.
[2] Number of animals which died/number of animals with clinical signs/number of animals in dose group.
[3] Hours or days after exposure.
[4] Post-exposure.
[5] Deaths occurred during exposure or immediately following removal from test chamber at 4 hours.
[6] Acute inhalation median lethal concentration (95% confidence limits)

Mortality: All animals exposed to 4.81 mg/L died or were killed in extremis between 10 and 220 minutes after the start of exposure. There were seven deaths in the group exposed to 0.94 mg/L. There were no deaths at 0.23 mg/L.

Clinical signs: Common abnormalities noted during exposure were wet fur and decreased respiratory rate. Three of the males killed in extremis upon removal from the chamber showed gasping respiration and were comatose. Upon removal from the test chamber common signs of toxicity were hunched posture, lethargy, pilo-erection, ataxia and ptosis. Incidences of gasping, laboured and noisy respiration and brown/red stains around the snout were apparent. Isolated incidents of pallor of the extremities, dehydration and frequent sneezing were observed.

Bodyweight: During the first week after exposure, one animal from the 0.94 mg/L group showed normal body weight gain and two showed body weight loss. Normal weight gain was resumed during week two. Body weight gain in all animals from the 0.23 mg/L group was normal.

Necropsy: Animals that died or were killed in extremis showed common abnormalities including lungs that appeared haemorrhagic, swollen and fluid filled. An incident of pale lungs and patchy pallor of the liver and reddening or congestion of the small intestine was also noted in animals exposed to 4.81 mg/L. Additional observations in two animals in the 0.94 mg/L group that died were signs of fluid in the nasal and/or oral tract, fluid in the lung cavity, dark liver and haemorrhage, reddening or congestion of the small intestine.
Surviving animals showed no abnormalities at necropsy.

Conclusion

The acute (4-hour) inhalation LC_{50} for rats exposed to captan was 0.90 mg/L and 0.67 mg/L in males and females, respectively. The combined sex LC_{50} for captan was 0.78 mg/L (95% confident limits 0.49 - 1.23 mg/L).

Based on the acute inhalation LD_{50} value of 0.67 mg/L, captan requires classification and labelling according to Regulation (EC) 1272/2008 as Acute Tox. Category 3 ($0.5 < LC_{50} \leq 1.0$ mg/L), H331 "Toxic if inhaled". This is in line with the current harmonised classification of captan according to Annex VI of Regulation (EC) No. 1272/2008.

3.3.1.3 Study 3 (1985)

Reference:	Acute Inhalation Study with Captan technical
Author(s), year:	Anonymous (1985)
Report/Doc. number:	Stauffer Environmental Health Center Inhalation Facility T- 11933
Guideline(s):	US EPA (1982)
GLP:	No. However, in the study report information on quality assurance inspections is available.
Deviations from OECD 403 (2009):	- 10 animals per sex and concentration were tested instead of 5 - Particle size of low and high concentration was measured once instead of twice during exposure - MMAD > 4 µm for mid and high concentration - geometric standard deviation > 3 for mid concentration
Acceptability:	Yes

Material and Methods

Captan technical (Lot. # DCC-0303, unknown purity)was administered as a single four-hour exposure (whole body) to Sprague-Dawley rats. Three groups of ten males and ten females were each exposed to nominal concentrations of 0, 2.69, 3.92 and 6.81 mg/L captan, corresponding to mean achieved concentrations of 0, 0.56, 0.71 and 1.39 mg/L. Test atmospheres were generated by the suspension of captan particulate in air using an extensively modified NBS dust generator. The air flow in the chamber ranged from 22 to 27 air/changes/hour. Within 10-12 minutes >99% of the final test concentration was attained, this test material atmosphere was presented to the test animals for 4 hours. Animals were observed frequently for mortality and clinical signs during exposure, and then twice daily for 14 days.

Body weight was recorded immediately prior to exposure, and on Days 2, 7 and 13 after exposure or at death. Rats were necropsied and observed for gross pathological changes at the end of the observation period.

Findings

A summary of the nominal and measured concentrations of captan and particle size distribution is presented in Table . Acute inhalation toxicity is summarised in Table 3.3.1.1-1.

Table 3.3.1.3-1 Acute inhalation toxicity of captan in rats: particle size distribution

Concentration (mg/L)		MMAD[1] (µm)	geometric standard deviation
Nominal	Achieved		
0	0	-	-
2.69	0.56	5.7[2]	2.98[2]
3.92	0.71	5-5.8	3.19-3.6
6.81	1.39	2.95[2]	2.14[2]

[1] Mass mean aerodynamic diameter.
[2] Measured only once due to insufficient sample collected on impactor to determine particle size distribution

Table 3.3.1.3-2 Acute inhalation toxicity of captan in rats: summary of toxicity

Concentration		Toxicological Results[1]	Duration of signs	Time of death	LC_{50} mg/L (14 days)[3]
Nominal	Achieved				
male					
0	0	0/0/10	-	-	0.72 (0.54-0.95)
2.69	0.56	3/10/10	during exposure - 13 d[2]	during exposure - 2 d[2]	
3.92	0.71	5/10/10	during exposure - 14 d[2]	during exposure - 2 d[2]	
6.81	1.39	10/10/10	during exposure - 2 d[2]	during exposure - 2 d[2]	
female					
0	0	0/0/10	-		0.87 (0.67-1.13)
2.69	0.56	1/10/10	during exposure - 14 d[2]	1 d[2]	
3.92	0.71	5/10/10	during exposure - 14 d[2]	during exposure - 3 d[2]	
6.81	1.39	8/10/10	during exposure - 5 d[2]	during exposure - 3 d[2]	

[1] Number of animals which died/number of animals with clinical signs/number of animals in dose group.
[2] Post-exposure.
[3] Acute inhalation median lethal concentration (95% confidence limits)

Mortality: Mortality was observed at all concentrations and it was dose-related. Males indicated a slightly higher sensitivity than female rats.

Clinical signs: During the captan exposures, the animals were lethargic, appeared depressed, were salivating and had severe difficulty in breathing. After the exposures, nearly all rats exposed to captan Technical exhibited stains about the face and many showed laboured breathing for varying lengths of time.
Among surviving rats most of the clinical signs were temporary. Additional clinical signs included reduced activity, chromodacryorrhea, chromorhinorrhea and stains about the facial area.

Bodyweight: Body weights for males on day 2, 7 and 13 and for females on days 7 and 13 at 1.39 mg/l could not be compared to controls because of insufficient data due to mortality. The males at 0.56 and 0.71 mg/l showed significant depression on Days 2, 7 and 13. The female rats showed significant depression only on study Day 2 at all three exposure levels. The female rats at 0.56 and 0.71 mg/l dose levels had recovered to control level by study Day 7. The body weights for male rats, although still significantly depressed, exhibited partial recovery by study Day 7.

Necropsy: Animals that died showed common abnormalities including lungs that appeared reddened, mottled, failed to collapse properly. These observations appeared dose-related. The eyes were cloudy-white in several of the rats that died at the 0.71 and 1.39 mg/l exposure levels during observation period.

Few males and females rats, surviving to the end of the study hat abnormal findings, which were considered incidental by the study author.

Conclusion

The acute (4-hour) inhalation LC_{50} for rats exposed to captan was 0.72 mg/L (95th confidence interval : 0.54-0.95) and 0.87 mg/L (95th confidence interval : 0.67-1.13) in males and females, respectively.

Based on the acute inhalation LD_{50} value of 0.72 mg/L, captan requires classification and labelling according to Regulation (EC) 1272/2008 as Acute Tox. Category 3 ($0.5 < LC_{50} \leq 1.0$ mg/L), H331 "Toxic if inhaled". This is in line with the current harmonised classification of captan according to Annex VI of Regulation (EC) No. 1272/2008.

3.3.1.4 Study 4 (2000)

Reference:	Captan Technical- Acute Inhalation Toxicity Study in Rats
Author(s), year:	Anonymous (2000)
Report/Doc. number:	Huntington Life Sciences, New Jersey, 00-5439
Guideline(s):	US EPA OPPTS 870.1300 (1998)
GLP:	Yes
Deviations from OECD 403 (2009):	- Housing temperature was outside the desired range (16-27°C) - Housing relative humidity was outside the desired range (up to 87%) - MMAD of 7.399 for non-milled test substance
Acceptability:	Yes (for the milled test substance), supplementary information only (for the non-milled test substance due to the limited amount of respirable fraction)

Material and Methods

Captan technical (Lot. PJE0805LS, purity : 91.66%) was administered as a single four-hour exposure (nose-only) to Sprague-Dawley rats. The study was designed to include a comparison of testing the milled test substance (three exposure levels), with smaller particle size and the non-milled (typical manufactured product) test substance (one exposure level) with larger particles. Three groups of five males and five females were each exposed to nominal concentrations of 0.21, 1.4 and 4.5 mg/L milled captan, corresponding to mean achieved concentrations of 0.072, 0.648 and 2.28 mg/L. One group of five males and five females were exposed to a nominal concentrations of 5.1 mg/L non-milled captan, corresponding to a mean achieved concentration of 0.668 mg/L. The milled test substance was packed into Wright dust feeder cups. The non-milled test substance was place into the AccuRate Dry Material Feeder and fed into the NBS dust feeder. The air flow in the chamber ranged from 25-27.4 litres/ minute. This flow rate provided one complete air change approximately every 1.5-1.6 minutes. Animals were observed at 15 minutes intervals during the first hour of exposure and hourly for the remainder of exposure for mortality and clinical signs. Detailed physical observations for signs of toxicity were performed on all animals just prior to exposure, immediately upon removal form the chamber, hourly for two hours post exposure and once daily for a 14-day post-exposure observation period. Body weight was recorded prior to exposure, and weekly thereafter. Rats were necropsied and observed for gross pathological changes at the end of the observation period.

Findings

A summary of the nominal and measured concentrations of captan and particle size distribution is presented in Table . Acute inhalation toxicity is summarised in Table 3.3.1.1-1.

Table 3.3.1.4-1 Acute inhalation toxicity of captan in rats: particle size distribution

Concentration (mg/L)		MMAD[1] µm	Respirable fraction (%) (< 4µm)
Nominal	Achieved		
Milled test-substance			
0.072	0.21	2.545	68.36
0.648	1.4	2.611	68.99
2.28	4.5	2.979	63.26
Non-milled test-substance			
5.1	0.668	7.399	25.06

[1] Mass mean aerodynamic diameter

Table 3.3.1.4-2 Acute inhalation toxicity of captan in rats: summary of toxicity

Concentration		Toxicological Results[1]	Duration of signs	Time of death	LC_{50} mg/L (14 days)[3]
Nominal	Achieved				
male					
Milled test- substance					
0.072	0.21	1/5/5	during exposure - 14 d[2]	1 d[2]	0.22 (0.084-0.579)
0.648	1.4	4/5/5	during exposure - 14 d[2]	during exposure - 2 d[2]	
2.28	4.5	5/5/5	during exposure - 1 d[2]	during exposure - 1 d[2]	
Non-milled test- substance					
5.1	0.668	0/5/5	during exposure - 14 d[2]	-	> 0.668
Female					
Milled test- substance					
0.072	0.21	0/5/5	during exposure - 8 d[2]	-	0.322 (0.156-0.664)
0.648	1.4	4/5/5	during exposure - 14 d[2]	during exposure - 3 d[2]	
2.28	4.5	5/5/5	during exposure	during exposure	
Non-milled test- substance					
5.1	0.668	0/5/5	during exposure - 14 d[2]	-	> 0.668

[1] Number of animals which died/number of animals with clinical signs/number of animals in dose group.
[2] Post-exposure.
[3] Acute inhalation median lethal concentration (95% confidence limits)

Mortality: The 2.28, 0.648 and 0.072 mg/L exposures to the milled substance resulted in respective mortalities of 100, 80 and 10% within 3 days after exposure. No mortality occurred in the group exposed to the non-milled test substance.

Clinical signs: The only sign of toxicity during the exposure period to the milled test substance was laboured breathing (0.648 and 2.28 mg/L) and poor condition (2.28 mg/L). There were no signs of toxicity noted during the 0.0720 mg/L exposure to the milled test substance.
The only sign of toxicity during the exposure period to the non-milled test substance was laboured breathing.
Among surviving animals from all exposure groups signs of toxicity noted immediately following the exposure included clear or red nasal discharge, chromodacryorrhea, excessive salivation, laboured breathing and moist rales. Among surviving animals from all exposure groups, responses similar to those seen immediately after the exposure were seen during the first week after exposure with recovery, in general, during the second week after exposure.

Bodyweight: Most surviving animals exposed to the milled substance lost weight during the first week after exposure. However, all these animals gained weight during the second week after exposure. All animals exposed to the non-milled substance gained weight during both weeks after exposure.

Necropsy: Macroscopic test substance-related findings were red mucinous material in the nasal cavity and fluid in the trachea and lung. Animals found dead had red lungs. The relationship, if any, of this finding to the test substance is not clear because red lungs are a common finding in animals which are not exsanguinated prior to post-mortem examination. Scattered red foci in the lungs of rats terminally sacrificed are usually due to agonal haemorrhages.

Conclusion

The acute (4-hour) inhalation LC_{50} for rats exposed to milled captan was 0.22 mg/L (95th confidence interval : 0.084-0.579) and 0.322 mg/L (95th confidence interval : 0.156-0.664) in males and females, respectively. The combined sex LC_{50} for milled captan was 0.272 mg/L (95% confident limits 0.159-0.466 mg/L). LC_{50} of non-milled captan was greater than 0.668 mg/L for both sexes combined.

Based on the acute inhalation LD_{50} value of 0.22 mg/L, captan requires classification and labelling according to Regulation (EC) 1272/2008 as Acute Tox. Category 2 ($0.05 < LC_{50} \leq 0.5$ mg/L), H330 "Fatal if inhaled". This is not in line with the current harmonised classification of captan according to Annex VI of Regulation (EC) No. 1272/2008.

3.3.2 Human data

No relevant studies.

3.3.3 Other data

No relevant studies.

3.4 Skin corrosion/irritation

3.4.1 Animal data

3.4.1.1 Study 1 (1991)

Reference:	Captan technical: Acute dermal irritation test in the rabbit
Author(s), year:	Anonymous (1991c)
Report/Doc. number:	Safepharm Lab. Ltd., report No. 306/103 (Company file: R-6293)
Guideline(s):	OECD 404 (1981) and 84/449/EEC method B4 (1984)
GLP:	Yes
Deviations from OECD 404 (2015):	No; the essential criteria of OECD 404 are met
Acceptability:	Yes

Material and Methods

Captan was applied as a single 0.5 g dermal dose to three New Zealand White rabbits. The test substance, moistened with 0.5 mL distilled water, was introduced under a 2.5 x 2.5 cm gauze patch onto shorn skin under semi-occluded conditions. After four hours the patch was removed and any residual test material removed. The area was examined at 1, 24, 48 and 72 hours after the removal of the patch and scored according to Draize (1959).

Findings

Very slight erythema was noted at all treated skin sites 1 and 24 hours after patch removal and in one animal at the 48.hour observation. All treated skin sites appeared normal 72 hours after treatment. Scores are given in Table 3.4.1.1-1.

Table 3.4.1.1-1 Dermal irritation responses in rabbits: individual dermal responses

Animal No.			52	54	55	Mean scores
Draize grade after	1 h	O	0	0	0	
		E	1	1	1	
	24 h	O	0	0	0	
		E	1	1	1	
	48 h	O	0	0	0	
		E	0	1	0	
	72 h	O	0	0	0	
		E	0	0	0	
	mean[1]	O	0	0	0	
		E	0.33	0.66	0.33	
Overall average means[1]					**O** **E**	**0** **0.44**

O = oedema.
E = erythema and eschar formation.
[1] Mean for individual animals over three time points 24, 48 and 72 hours for each lesion, calculated as mean score at (24 hours + 48 hours + 72 hours)/3.

Conclusion

Based on the skin reaction observed in this study (mean skin irritation scores 24 to 72 hours after removal of test substance) captan does not require classification and labelling as irritating to the skin according to Regulation (EC) 1272/2008.

3.4.2 Human data

No relevant studies.

3.4.3 Other data

No relevant studies.

3.5 Serious eye damage/eye irritation

3.5.1 Animal data

3.5.1.1 Study 1 (1991)

Reference:	Captan technical: Acute eye irritation test in the rabbit
Author(s), year:	Anonymous (1991d)
Report/Doc. number:	Safepharm Lab. Ltd., report No. 306/104 (Company file: R-6294)
Guideline(s):	OECD 405 (1987) and 84/449/EEC method B5 (1984)
GLP:	Yes
Deviations from OECD 405 (2012):	No; the essential criteria of OECD 405 are met
Acceptability:	Yes

Material and Methods

A single dose of 82 mg (equivalent to 0.1 mL) captan was administered into the elevated lower lid of the right eye of one female New Zealand white rabbit. The left eye remained untreated and served as a control. Immediately after administration of the test material, an assessment of the initial pain reaction was made. Assessment of ocular damage/irritation was made 1 and 5 hours following administration according to Draize (1959).

Findings

A dulling of the normal lustre of the corneal surface was noted in the treated eye one hour after treatment with areas of diffuse corneal opacity at the 5 hour observation. Iridial inflammation as well as severe conjunctival irritation was noted in the treated eye. After 5 hours, haemorrhage of the nictitating and conjunctival membranes was also noted. Scores for ocular irritation are given in Table 3.5.1.1 -1.

The animal was killed immediately after the 5-hour assessment for humane reasons in accordance with UK Home Office Guidelines. No further animals were treated.
There was no evidence of recovery during the observation period following administration of the test substance since the study was terminated after 5 hours.

Table 3.5.1.1 -1 Ocular irritation in the rabbit

Tissue	Signs	Draize grades	
		1 h	5 h
Cornea	Opacity	D	1
	Area	4	4
Iris	Iritis	1	1
Conjunctiva	Chemosis	3	4
	Redness	2	2[H]
	Discharge	3	3

D Dulling of the normal lustre of the corneal surface.
H Haemorrhage of the nictitating and conjunctival membrane.

Conclusion

According to the criteria laid down in Regulation (EC) 1272/2008 and based on the severity and irreversibility of the effects in one animal, the test substance captan is classified for Serious eye damage Category 1, H318, “Causes serious eye damage”. This is in line with the current harmonised classification of captan according to Annex VI of Regulation (EC) No. 1272/2008.

3.5.1.2 Study 2 (1982)

Reference:	The eye irritation potential of Chevron Captan technical
Author(s), year:	Bullock, C.H. (1982b)
Report/Doc. number:	Chevron Chemical Company, report No. S-1918 (Company file: R-6334 / TMN-0726)
Guideline(s):	In house method
GLP:	Yes
Deviations from OECD 405 (2012):	No measures for animal welfare were taken, no sequential testing; however, the essential criteria of OECD 405 are met
Acceptability:	Yes

Material and Methods

A single dose of 100 mg technical captan powder was administered into the conjunctival sac of one eye of each of nine female New Zealand white rabbits. The other eye served as the control. Thirty seconds after treatment, eyes of three of the rabbits were rinsed with 250 mL distilled water over a period of one minute. All eyes were assessed for ocular reaction at 1 hour and 1, 2, 3, 4, 7, 10, 14, 17 and 21 days after administration according to the method of Draize et al (1944).

Findings

Summaries of the ocular responses following administration of captan are given in Table 3.5.1.2-1for unrinsed eyes and in Table 3.5.1.2-2 for rinsed eyes.

Table 3.5.1.2-1 Ocular irritation in rabbits: individual ocular responses (unrinsed eyes)

Time	Rabbit No.	Cornea			Conjunctivae		
		Opacity	Area	Iris	Redness	Chemosis	Discharge
1 hour	20	0	0	0	2	3	2
	22	0	0	1	2	3	2
	53	0	0	0	2	2	1
	54	0	0	0	1	2	1
	55	0[a]	0	0	2	3	1
	56	0	0	0	2	3	1
24 hours	20	1	1	1	3[b]	4	3
	22	1	2	1	3[b]	4	3
	53	0	0	1	3[b]	3	2
	54	1	2	1	3[b]	3	3
	55	0	0	0	3[b]	3	3
	56	1	3	1	3[b]	4	3
	Mean score	**0.67**	**-**	**0.83**	**3.00**	**3.50**	**-**
48 hours	20	3	4	1	3[b]	2	3
	22	3	4	1	3[b]	3	3
	53	1	1	1	3[b]	2	2
	54	2[a]	4	1	3[b]	2	3
	55	1	4	1	3[b]	2	3
	56	3	4	1	3[b]	3	3
	Mean score	**2.17**	**-**	**1.00**	**3.00**	**2.33**	**-**
72 hours	20	3	4	1	3[b]	2	3
	22	3	4	1	3[b]	3	3
	53	0	0	1	3[b]	2	2
	54	4	2	1	3[b]	2	3
	55	1	2	0	3	1	2
	56	4	2	1	3[b]	2	3
	Mean score	**2.50**	**-**	**0.83**	**3.00**	**2.00**	**-**
96 hours	20	3	4	1	3[b]	2	3
	22	3	4	1	3[b]	3	3
	53	0	0	0	3[b]	2	1
	54	4	3	1	3[b]	2	3
	55	1	1	0	3	0	1
	56	4	2	1	3[b]	2	3
7 days	20	4[a,c]	4	1	2	1	3
	22	4[a,c]	4	1	3[b]	2	3
	53	0	0	0	2	1	2
	54	2[c]	2	1	2	0	1
	55	0[d]	0	0	3[e]	0	1
	56	4[a,c]	3	1	2	1	2
10 days	20	4[a,c]	4	1	2	1	3
	22	4[a,c]	4	1	3[b]	2	3
	53	0	0	0	1	1	1
	54	2[c]	2	0	2	0	1
	55	0[d]	0	0	3[e]	0	1
	56	4[a,c]	3	1	2	1	2

Time	Rabbit No.	Cornea			Conjunctivae		
		Opacity	Area	Iris	Redness	Chemosis	Discharge
14 days	20	4[a,c]	4	0[f]	2	0	2
	22	4[a,c]	4	0	3[b]	2	3
	53	1[a,c]	1	0	0	0	0
	54	4[a,c]	2	0	1	0	0
	55	0[d]	0	0	1	0	0
	56	1[c]	4	0	1	0	1
17 days	20	4[a,c,g]	4	0[f]	2	0	2
	22	4[a,c]	4	0	3[b]	2	2
	53	1[a,c]	1	0	0	0	0
	54	3[a,c]	2	0	1	0	0
	55	0[d]	0	0	0	0	0
	56	1[c]	2	0	0	0	0
21 days	20	2[a,c]	4	0[f]	1	0	2
	22	4[a,c]	4	0	3[b]	1	3
	53	0	0	0	0	0	0
	54	3[a]	1	0	0	0	1
	55	0	0	0	0	0	0
	56	1[c]	2	0	0	0	0
MEAN OVERALL SCORE[1]		**1.78**	**-**	**0.89**	**3.00**	**2.61**	-

[1] For each lesion calculated as a mean score at (24 hours + 48 hours + 72 hours)/3.
a = roughened cornea; b = conjunctival bleeding; c = pannus; d = lackluster cornea; e = conjunctival necrosis; f = iris sluggish in light response; g = cornea blistered.

Table 3.5.1.2-2 Ocular irritation in rabbits: individual ocular responses (rinsed eyes)

Time	Rabbit No.	Cornea			Conjunctivae		
		Opacity	Area	Iris	Redness	Chemosis	Discharge
1 hour	57	0	0	0	1	1	1
	58	0	0	0	1	0	1
	59	0	0	0	1	2	1
24 hours	57	0	0	0	1	0	1
	58	0	0	0	1	0	0
	59	0	0	1	3	2	3
	Mean score	**0.00**	**-**	**0.33**	**1.67**	**0.67**	**-**
48 hours	57	0	0	0	0	0	1
	58	0	0	0	0	0	0
	59	0	0	0	3	1	1
	Mean score	**0.00**	**-**	**0.00**	**1.00**	**0.33**	**-**
72 hours	57	0	0	0	0	0	0
	58	0	0	0	0	0	0
	59	0	0	0	2	0	1
	Mean score	**0.00**	**-**	**0.00**	**0.67**	**0.00**	**-**
96 hours	57	0	0	0	0	0	0
	58	0	0	0	0	0	0
	59	0	0	0	1	0	1
7 days	57	0	0	0	0	0	0
	58	0	0	0	0	0	0
	59	0	0	0	0	0	0
OVERALL MEAN SCORE[1]		**0.00**	**-**	**0.11**	**1.11**	**0.33**	**-**

[1] For each lesion calculated as a mean score at (24 hours + 48 hours + 72 hours)/3.

In unrinsed treated eyes, slight to complete corneal opacity along with severe conjunctival irritation were observed in most rabbits by 24 hours after treatment and persisted in some animals throughout the 21-day observation period. Iritis and pannus formation were also observed during the study.

Administration of captan immediately followed by rinsing with distilled water caused only slight reversible irritation. In rinsed eyes no corneal opacity was observed. Iritis was observed at 24 hours in one animal. Slight to severe conjunctival irritation was observed through to 96 hours. All rinsed treated eyes appeared normal by day 7.

Conclusion

Administration of captan without rinsing resulted in significant irritation to eyes which persisted throughout the 21-day observation period. According to the criteria laid down in Regulation (EC) 1272/2008 and based on the irreversibility of irritation to eyes, the test substance captan is classified for Serious eye damage Category 1, H318 “Causes serious eye damage”. This is in line with the current harmonised classification of captan according to Annex VI of Regulation (EC) No. 1272/2008.

3.5.2 Human data

No relevant studies.

3.5.3 Other data

No relevant studies.

3.6 Respiratory sensitisation

3.6.1 Animal data

No relevant studies.

3.6.2 Human data

No relevant studies.

3.6.3 Other data

No relevant studies.

3.7 Skin sensitisation

3.7.1 Animal data

3.7.1.1 Study 1 (1991)

Reference:	Captan technical: Magnusson and Kligman maximisation study in the guinea pig
Author(s), year:	Anonymous (1991e)
Report/Doc. number:	Safepharm Laboratories. Ltd., report No. 306/105 (Company file: R-6295)
Guideline(s):	OECD 406 (1981) and 84/449/EEC method B6 (1984)
GLP:	Yes
Deviations from OECD 406 (1992):	No
Acceptability:	Yes

Material and Methods

The dermal sensitisation of captan was evaluated by means of the Magnusson and Kligman maximisation test in 30 female Dunkin-Hartley guinea pigs (20 test and 10 control animals). The sensitivity of the strain of guinea pigs used was confirmed using the known positive sensitiser 2,4-dinitrochlorobenzene (DNCB). Concentrations used were determined from a series of "sighting tests" to determine the highest concentrations for intradermal induction, topical induction and topical challenge. For the intradermal induction phase, a row of three injections (0.1 mL each) was made on each side of the midline of a shaved area of skin on the shoulder region:

i) Freunds complete adjuvant (FCA) plus distilled water in a ratio of 1:1;

ii) 0.1% captan (w/v) suspension in arachis oil BP;

iii) 0.1% captan (w/v) suspension in 1:1 preparation of FCA in distilled water.

After 7 days, a topical induction of 50% captan (w/w) in arachis oil was applied to the same area. The area was covered with an occlusive dressing which was held in place for 48 hours and the area was examined after one and 24 hours.

A topical challenge of 0.1 to 0.2 mL 10% captan (w/w) in arachis oil was applied to the right shorn flank on day 21. The area was covered with an occlusive dressing for 24 hours. The vehicle alone was similarly

applied to the left shorn flank. The area was examined 24 and 48 hours after the removal of the challenge dressing.

Control animals were treated in the same way as the test animals, except that the vehicle alone (arachis oil BP) was used instead of captan during the induction phases.

Findings

Following challenge with the test material, positive skin responses were noted in (at least) 16 and 14 animals after 24 and 48 hours, respectively. Adverse reactions prevented the accurate evaluation of erythema at test material sites in 4 of the 20 animals at 24 hours. At 48 hours, these reactions persisted in one animal and increased in severity in the other three animals.
The individual skin reactions of test animals are summarised in the following table.

Table 3.7.1.1-1 Magnusson Kligman maximisation study in the guinea pig: skin reactions of individual test animals following topical challenge with 10% w/w captan in arachis oil

Animal number	Skin reactions (hours after removal of dressing)			
	24 hours		48 hours	
	Test	Vehicle	Test	Vehicle
1	2	0	?eD	0
2	2 R	0	2 RD	0
3	2 R	0	1 R	0
4	?eWeSp	0	?eSt	0
5	?eWe	0	2 ROeSp	0
6	2 Oe	0	2 ROe	0
7	2 R	0	?eDR	0
8	2	0	1	0
9	1	0	0	0
10	1	0	0	0
11	2	0	1	0
12	?eDWeR	0	2 RD	0
13	2	0	1 RD	0
14	2 R	0	?eCrD	0
15	2 R	0	2 R	0
16	2	0	1 R	0
17	1	0	1 D	0
18	2	0	1	0
19	?eDWe	0	2 SsD	0
20	1	0	1 D	0

0 = no reaction; 1 = scattered mild redness; 2 = moderate and diffuse redness.
R = extended reaction; ?e = adverse reactions prevent accurate evaluation of erythema; We = well defined erythema surrounding test site; Sp = hardened light brown coloured scab; Oe = oedema extends ventrally below treatment site; D = desquamation; St = hardened dark brown/black coloured scab; Cr = superficial cracking of the epidermis; Ss = small superficial scattered scabs.

No adverse reactions were noted at the vehicle control sites in test animals or at any sites of control animals at the 24- and 48-hour observations.

Conclusion

Under the conditions of the Magnusson Kligman test, captan produced a 100% sensitisation rate in the guinea pigs and was considered an extreme sensitiser.

According to the criteria laid down in Regulation (EC) 1272/2008 and based on the skin reaction of 100% of animals tested following challenge, and considering an intradermal dosing of 0.1% (w/v), the test substance captan is classified as Skin sensitisation Category 1A, H317 “May cause an allergic skin reaction”.

3.7.2 Human data

No relevant studies.

3.7.3 Other data

No relevant studies.

3.8 Germ cell mutagenicity

3.8.1 In vitro data

3.8.1.1 Study 8 (2017)

Reference:	Reverse mutation assay using Bacteria (Salmonella typhimurium and Escherichia coli) with Captan technical
Author(s), year:	Anonymous (2017)
Report/Doc. number:	Document No: 171623, R-38477
Guideline(s):	OECD 471 (1997), EC B.13/14, OPPTS 870.5100
GLP:	Yes
Deviations:	None
Acceptability:	Yes

Executive Summary

The mutagenic potential of Captan Technical was investigated in a reverse gene mutation assay in bacteria according to the testing guideline OECD 471. Strains TA98, TA100, TA1535 and TA1537 of *Salmonella typhimurium* and tester strain *E. coli* WP2 uvrA were exposed to Captan Technical in DMSO. The test article was tested with and without metabolic activation at 9 dose levels (0.050 – 500 µg/plate) in experiment I. Experiment II was not applicable.
Under the experimental conditions, Captan Technical caused gene mutations by base pair changes and frameshifts in the genome of the tester strains used, tested up to the top concentration of 500 µg/plate in the 5 test strains with and without metabolic activation.
Captan Technical was mutagenic when tested on *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 and tester strain *E. coli* WP2 uvrA with or without S9-mix activation in the present bacterial reverse mutation assay. Captan's potency to induce gene mutations was decreased under conditions of metabolic activation.

Material and Methods

A. MATERIALS

1. Test Material:	Captan Technical
Description:	Off-white solid
Batch #:	941381319
Purity:	96.5%
Storage conditions:	room temperature
Expiry date:	01.01.2020
Solvent used:	Dimethyl sulfoxide (DMSO, AppliChem, Lot No.: 0000978834)

2. Positive control Materials:
Without metabolic activation:
Sodium azide (NaN_3) Sigma batch No. BCBN6481V, in H_2O: 10 µg/plate – TA100, TA1535
4-nitro-o-phenylene-diamine (4-NOPD) Fluka batch No. MKBM5257V, in DMSO: 10 µg/plate – TA98, 40 µg/plate – TA1537

Methylmethanesulfonate (MMS) Sigma batch No. MKBX5165V, in H_2O: 1 µL/plate – *E.coli* WP2 uvrA

With metabolic activation:

2-aminoanthracene (2-AA) Aldrich batch No. STBD3302V, in DMSO:
2.5 µg/plate – TA98, TA100, TA1353, TA1357
10 µg/plate – E.coli WP2 uvrA

3. Test system:

Bacteria:	*Salmonella typhimurium*: TA 98, TA 100, TA 1535, TA 1537 *E.coli* WP2 uvrA
Source:	TA98, TA1535, *E.coli* WP2 uvrA from MOLTOX, INC. NC28607, USA TA100, TA1537 from Xenomertix AG, Switzerland
Exposure duration:	48 hours

4. Metabolic activation:

S9 derived from rats (phenobarbital and β-naphthoflavone induced rat liver)
Protein content: 32.4 mg/mL S9 (Eurofins Munich)
33.7 mg/mL S9 (Trinova Biochem)
Component/Content: 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP in 100 mM Na-Phosphate-buffer (pH 7.4)

5. Test concentrations:

Pre-experiment: 3.16, 10.0, 31.6, 100, 316, 1000, 2500, 5000 µg/plate
Experiment I: 0.050, 0.158, 0.500, 1.58, 5.00, 15.8, 50.0, 158 and 500 µg/plate
Experiment II: not applicable

B. TEST PERFORMANCE

1. In-life dates

19. April – 12. May 2017 at Eurofins BioPharma Product Testing Munich GmbH, Planegg, Germany.

2. Preliminary experiment for cytotoxicity

The toxicity of Captan Technical was determined by clearing or rather diminution of the background lawn or a reduction in the number of revertants down to a mutation factor of approximately ≤ 0.5 in relation to the solvent control with the tester strains TA 98 and TA 100 in a pre-experiment. Eight concentrations were tested for toxicity and induction of mutations with three plates each. The experimental conditions were the same as described below for the main experiment (plate incorporation test).

The following concentrations were employed: 3.16, 10.0, 31.6, 100, 316, 1000, 2500, 5000 µg/plate

3. Plate incorporation mutation assay

The test item concentrations were chosen according to the results of the pre-experiment, 500 µg/plate was selected as the maximum concentration. All tester strains were incubated with or without activation by S9 mix as well as the corresponding controls. For each strain and dose level, including controls, three plates were used. After solidification, the plates were inverted and incubated at 37°C for at least 48 h in the dark. Strain specific positive controls were included, in order to demonstrate an effective performance of the test.

Test item concentrations experiment: 0.050, 0.158, 0.500, 1.58, 5.00, 15.8, 50.0, 158 and 500 µg/plate

4. Evaluation

The Mutation Factor is calculated by dividing the mean value of the revertant counts by the mean values of the solvent control (the exact and not the rounded values are used for calculation).

The test item is considered as mutagenic if:

- a clear and dose-related increase in the number of revertants occurs and/or

- a biologically relevant positive response for at least one of the dose groups occurs in at least one tester strain with or without metabolic activation.

A biologically relevant increase is described as follows:
- if in tester strains TA98, TA100 and E. coli WP2 uvrA the number of reversions is at least twice as high
- if in tester strains TA1535 and TA1537 the number of reversions is at least three times higher as compared to the reversion rate of the solvent control.

Findings

A. PRE-EXPERIMENT
Pronounced cytotoxicity was noted from concentrations of 31.6 µg/plate onwards in presence of S9 and 100 µ/plate in absence of S9 for both test strains. No precipitation of the test item was observed in both strains, T98 and T100.

B. PLATE-INCORPORATION TEST
Eight concentrations ranging from 0.05 to 500 µg Captan/plate were employed in the plate-incorporation test, with and without metabolic activation.
No precipitation of the test item was observed in any tester strain used in the experiment (with and without metabolic activation). Toxic effects of the test item were noted in all tester strains evaluated in the experiment. Toxic effects of the test item were observed in tester strain TA100, TA1535 and E coli WP2 uvrA at concentrations of 50.0 µg/plate and higher (without metabolic activation) and at 158 µg/plate and higher (with metabolic activation). In tester strains TA98, toxic effects were noted at concentrations of 158 µg/plate and higher (with and without metabolic activation). In tester strains TA1537 toxic effects were observed at concentrations of 50.0 µg/plate and higher (with and without metabolic activation).
Biologically relevant increases of revertant colony numbers were observed in tester strains TA98 at concentrations of 1.58 - 50.0 µg/plate (without metabolic activation) and at concentrations of 15.8 - 50.0 µg/plate (with metabolic activation). The threshold value of 2.0 was exceeded and a maximum mutation factor of 15.3 was reached at a concentration of 50.0 µg/plate (with metabolic activation). Biologically relevant increases of revertant colony numbers were observed in tester strain TA100 and TA1535 at concentrations of 0.5 – 15.8 µg/plate (without metabolic activation) and at concentrations of 15.8 – 50.0 µg/plate (with metabolic activation). The threshold value of 2.0 was exceeded and a maximum mutation factor of 6.9 was reached at a concentration of 15.8 µg/plate (without metabolic activation) in tester strains TA100. The threshold value of 2.0 was exceeded and a maximum mutation factor of 14.2 was reached at a concentration of 50.0 µg/plate (with metabolic activation) in tester strain TA1535. Biologically relevant increases of revertant colony numbers were observed in tester strain TA1537 at concentrations of 1.58 – 15.8 µg/plate (without metabolic activation) and at concentrations of 15.8 µg/plate (with metabolic activation). The threshold value of 3.0 was exceeded and a maximum mutation factor of 17.2 was reached at a concentration of 15.8 µg/plate (without metabolic activation) in tester strain 1537.
Biologically relevant increases of revertant colony numbers were observed in tester strain E. coli WP2 uvrA at concentrations of 1.58 – 50.0 µg/plate (without metabolic activation) and at concentrations of 15.8 - 158 µg/plate (with metabolic activation). The threshold value of 2.0 was exceeded and a maximum mutation factor of 18.4 was reached at a concentration of 50.0 µg/plate (with metabolic activation).
Moreover, a dose-response relationship was found in all tester strains (with and without metabolic activation).

Table 3.8.1.1-1 Results from plate incorporation mutagenicity assay on Captan Technical

Dose [µg/plate]	Mutation factor[a]									
	TA98		TA100		TA1535		TA1537		*E.coli* WP2 uvrA	
	- (S9)	+ (S9)	- (S9)	+ (S9)	- (S9)	+ (S9)	- (S9)	+ (S9)	- (S9)	+ (S9)
Experiment I										
H_2O	0.9	1.2	1.1	1.1	1.0	0.7	1.3	0.9	1.1	1.0
DMSO	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
0.050	0.9	1.0	1.2	1.1	0.9	1.4	1.9	1.5	0.8	0.8
0.158	0.8	1.1	1.3	1.1	1.2	1.0	2.0	1.4	1.1	1.0
0.500	1.0	1.3	2.0	1.1	2.3	0.9	1.7	0.5	1.4	0.8
1.58	2.0	1.1	4.2	1.1	5.1	0.9	5.4	0.7	2.5	1.0
5.00	4.4	1.1	5.0	1.0	3.3	0.8	5.6	0.9	3.8	0.9
15.8	12.6	7.9	6.9	3.4	4.0	12.1	17.2	3.2	11.3	3.0
50.0	2.5	15.3	0.4	3.9	0.1	14.2	0.0	1.3	3.9	18.4
158	0.0	0.2	0.0	0.0	0.0	0.0	0.0	3.8	1.7	4.1
500	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.8
4-NODP[b]	14.1	-	-	-	-	-	19.5	-	-	-
NaN_3[c]	-	-	10.2	-	58.5	-	-	-	-	-
2-AA[d]	-	65.9	-	12.0	-	9.4	-	24.6	-	2.6
MMS[e]	-	-	-	-	-	-	-	-	9.9	-

[a] Mutation factor = mean revertants (test item) / mean revertants (vehicle control)
[b] 4-nitro-o-phenylene-diamine: 10 µg/plate – TA98, 40 µg/plate – TA1537
[c] Sodium azide: 10 µg/plate - TA100, TA1535
[d] 2-aminoanthracene: 2.5 µg/plate – TA98, TA100, TA1353, TA1357; 10 µg/plate – E.coli WP2 uvrA
[e] Methylmethanesulfonate: 1 µL/plate – E.coli WP2 uvrA

Conclusions

Captan Technical caused gene mutations by base pair changes or frameshifts in the genome of all tester strains used. Captan Technical is considered to be mutagenic in this bacterial reverse mutation assay. Captan's potency to induce gene mutations was decreased under conditions of metabolic activation, with the exception of strain TA1535.

3.8.1.2 Study 9 (1985)

Reference:	Microbial/mammalian microsome mutagenicity plate incorporation assay: comparison of Captan Technical (SX-1086), Chevron Folpet Technical (SX-1388), and Chevron Captafol Technical (SX-945)
Author(s), year:	Anonymous (1985)
Report/Doc. number:	Chevron Chemical Company, report No. 2042 (Company file: R-9246/TMN-0808)
Guideline(s):	In-house method according to the procedures described by Ames et al (1975)1
GLP:	Yes, but with exceptions (homogeneity, concentration and stability of the test materials under the test conditions were not determined for this study)
Deviations from OECD 471 (1997):	Numerous deviations, e.g.: only one strain (TA100) tested instead of the recommended four S. typhimurium strains and one E. coli strain; not tested in presence of an appropriate metabolic activation system (S9); only two instead of at least five different analysable concentrations tested.
Acceptability:	Very limited

Material and Methods

The mutagenicity of captan (purity 92.4%, batch no. not specified in report) in *Salmonella typhimurium* strain TA100 was investigated in the presence and absence of cysteine and glutathione and the non-thiol analogs (alanine, glycine, methionine, serine and threonine) to test their ability to inhibit the mutagenic response induced by captan. The test substance was dissolved in dimethylsulphoxide (DMSO). Plates were run in triplicate at a nominal concentration of 7.5 and 10 µg/plate without S-9 mix. Cysteine and glutathione were tested at different concentrations, ranging from molar ratios of inhibitor:captan of 0 (no inhibitor) to 30. Non-thiol inhibitors were tested at molar ratios of inhibitor:captan of 3 to 12. Solvent controls were run concurrently.

Findings

Captan showed a mutagenic response by increasing the number of revertants approximately 5-fold and 6 to 9-fold at concentrations of 7.5 and 10 µg captan/plate, respectively, compared to the solvent (DMSO) control in the strain TA100.

The non-thiol amino acids had no effect on the observed mutagenic response when tested at any ratio. In contrast both glutathione and cysteine inhibited the mutagenic response induced by captan in a dose-dependent manner. The molar ratios required to inhibit captan induced mutagenicity were the following: Glutathione inhibited the mutagenicity of captan (to the solvent control level) at, starting at molar ratios of 6 and 9, respectively. Cysteine was inhibitory, starting at ratios of 5 and 6, respectively. A summary of the findings is given in Table 3.8.1.2-1.

[1] Ames, B N, McCann, J, Yamasaki, E (1975) Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research, 31, pp 347 - 364.

Table 3.8.1.2-1 Effect of glutathione and cysteine on the mutagenic response induced by captan in the Salmonella typhimurium strain TA100: number of revertants as percent of solvent control[1]

	7.5 µg/plate (0.025)[3]		10.0 µg/plate (0.033)[3]		
Molar ratio[2]	**Glutathione**	**Cysteine**	**Glutathione**	**Glutathione[4]**	**Cysteine**
0.0	499 ± 11	499 ± 22	601 ± 30	896 ± 37	601 ± 26
1.0	473 ± 42	-	474 ± 24	-	-
2.0	195 ± 13	-	-	356 ± 47	-
2.5	-	265 ± 20	-	-	309 ± 25
3.0	262 ± 28	-	153 ± 24	-	-
4.0	191 ± 37	-	-	177 ± 22	-
5.0	158 ± 22	111 ± 9	-	-	142 ± 9
6.0	-	-	159 ± 26	123 ± 26	-
7.5	-	-	-	-	-
9.0	-	-	128 ± 13	-	-
10.0	-	101 ± 23	-	-	108 ± 14
12.0	-	-	119 ± 15	-	-
15.0	-	134 ± 27	-	-	-
18.0	-	-	-	-	-
20.0	-	121 ± 19	-	-	98 ± 13
21.0	-	-	-	-	-
24.0	-	-	-	-	-
25.0	-	-	-	-	-
30.0	-	-	-	-	92 ± 9

[1] Number of revertants per plate as a percentage of the solvent control (DMSO); actual revertants per plate for controls were 116 ± 21 (mean ± S.D.).
[2] Ratio of inhibitor:captan.
[3] Concentration of test material µmoles/plate.
[4] Experiment repeated at later date.

Conclusions

Captan showed a mutagenic response by increasing the number of revertants of *Salmonella typhimurium* strain TA100 at concentrations of 7.5 and 10.0 µg/plate. Glutathione and cysteine inhibited the *in vitro* mutagenic potential of captan in a dose-dependent manner.

3.8.1.3 Carere et al. (1978)

Reference:	Microbiological mutagenicity studies of pesticides in vitro
Author(s), year:	Carere, A. et al. (1978)
Report/Doc. number:	Instituto Superiore di Sanita, Italy; published report in 'Mutation Research', 57, pp 277 - 286 (Company file: R-2615/TMN-806)
Guideline(s):	In-house method according to the procedures described by Ames et al (1975)[2]
GLP:	No
Deviations from OECD 471 (1997):	Numerous deviations, e.g.: testing in the S. typhimurium strains TA1536 and TA1538 is not recommended anymore; the test substance was not tested in the recommended S. typhimurium strains TA98 and TA100 and in one E. coli strain; only one instead of at least five different analysable concentrations was tested.
Acceptability:	Very limited

Material and Methods

[2] Ames, B N, McCann, J, Yamasaki, E (1975) Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research, 31, pp 347 - 364.

The mutagenicity of captan (purity 93%, batch no. not specified in report) in *Salmonella typhimurium* strains TA1535, TA1536, TA1537 and TA1538 was investigated in the presence and absence of metabolic activation (sodium phenobarbital induced rat liver S9). The test substance was dissolved in dimethylsulphoxide (DMSO). Plates were run in triplicate at a nominal concentration of 0 and 20 µg/plate in at least three independent experiments.

Findings

Captan was positive for mutagenicity in presence and absence of metabolic activation in strain TA1535 at a concentration of 20 µg/plate. However, no increase in the number of revertants was observed in the other tested strains TA1536, TA1537 and TA1538. The number of *His*$^+$ revertants per plate in the presence and absence of metabolic activation is summarised in Table 3.8.1.3-1.

Table 3.8.1.3-1 Summary of revertants in the presence of captan with and without metabolic activation

Captan (µg/plate)	S-9	*His*$^+$ revertants per plate[1]			
		TA1535	TA1536	TA1537	TA1538
20	+	200	0	10	20
0	+	30	0	13	18
20	-	500	1	27	19
0	-	37	0	16	21

[1] Mean of three replicates in one of at least 3 independent experiments with similar results.

Conclusions

Captan was positive in the *Salmonella typhimurium* strain TA1535 with and without metabolic activation. Captan showed no mutagenic response in the *Salmonella typhimurium* strains TA1536, TA1537 and TA1538.

3.8.1.4 Study 10 (2018)

Reference:	In vitro Mammalian chromosome aberration test in human lymphocytes with Captan technical
Author(s), year:	Anonymous (2018)
Report/Doc. number:	Document No: 171625, R-38479
Guideline(s):	OECD 473 (2016), EC B.10, OPPTS 870.5375
GLP:	Yes
Deviations:	None
Acceptability:	Yes

Executive Summary

A chromosome aberration assay in conformance with OECD guideline 473 was carried out in order to investigate a possible potential of Captan Technical to induce structural chromosome aberrations in human lymphocytes. Two independent trials in both the absence and the presence of S9-mix activation system were conducted at the concentration range from 0.01 – 0.06 mM.
In the experiment without and with metabolic activation, toxic effects (decrease below 70% relative mitotic index) were seen at a concentration of 0.04 mM and 0.02 mM, respectively. A biologically relevant decrease of the proliferation index was observed with or without metabolic activation. In the experiment without metabolic activation, a biologically relevant increase of the aberration rates was noted after treatment with the test item. With metabolic activation, no biologically relevant increase was noted after treatment with the test item.
A statistically significant increase ($p < 0.05$, Fisher's Exact Test) of cells with chromosomal aberrations was observed in two test item concentrations evaluated in the experiment without metabolic activation.
The $\chi 2$ Test for trend found a statistically significant increase in the experiment without metabolic activation. Moreover, in the experiment with and without metabolic activation no biologically relevant

increase in the frequencies of polyploid cells was found after treatment with the test item as compared to the controls.
In conclusion, it can be stated that during the described in vitro chromosomal aberration test and under the experimental conditions reported, the test item Captan Technical did induce structural chromosomal aberrations in human lymphocyte cells. Captan's potency to induce structural chromosome aberrations was eliminated under conditions of metabolic activation.
Therefore, Captan Technical is considered to be clastogenic in this chromosome aberration test.

Material and Methods

A. MATERIALS
1. Test Material: Captan Technical
Description: White solid
Batch #: 941381319
Purity: 96.4%
Storage conditions: Room temperature
Expiry date: 01.01.2020
Solvent used: Dimethyl sulfoxide (DMSO) 1% (v/v)

2. Control Materials:

Negative: Treatment medium RPMI 1640
Solvent: DMSO (AppliChem Lot No. 0000978834 and 0000950733)
Positive: non activated (-S9): EMS, ethylmethanesulfonate, Sigma Lot No. BCBS6100V at 900 µg/mL activated (+S9): CPA, cyclophosphamide, Sigma Lot No. MKBX1827 at 5 µg/mL

3. Metabolic activation:
S9 derived from rat liver (phenobarbital and β-naphthoflavone induced male rats).
Protein content: 32.4 and 34.1 mg/mL S9
Component/Content: 8 mM $MgCl_2$, 33 mM KCl, 5 mM gluc-6-phosphate, 5 mM NADP in 100 mM Na-Phosphate-buffer (pH 7.4)

4. Test cells:
Human peripheral blood lymphocytes were obtained by venipuncture from a single healthy unmedicated donor and collected in heparinized tubes.

5. Culture medium:
Complete medium: 15% (v/v) fetal bovine serum (FBS)
100 U/100 µg/mL penicillin/streptomycin solution
0.24 g/mL phytohemagglutinin-L
Treatment medium: Complete culture medium without FBS

6. Test compound concentrations:
0.02, 0.04, 0.05 and 0.06 mM (-S9)
0.01, 0.02, 0.04 and 0.06 mM (+S9)

B. TEST PERFORMANCE
1. In-life dates
22 June 2017 –30 January 2018 at Eurofins BioPharma Product Testing Munich GmbH, Planegg, Germany.

2. Pre-Experiment for Toxicity
According to the relevant guidelines the highest recommended concentration to be tested is 10 mM, if no cytotoxicity or precipitation is observed. Due to precipitation found at higher concentrations the following concentrations were tested with and without S9 mix: 0.0005, 0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2 and 0.5 mM.

3. Cytogenetic assay

Cell treatment

After 48 h the culture medium was replaced with serum-free medium containing the test item (without metabolic activation) and serum-free medium containing the test item with 50 µL/mL S9 mix (with metabolic activation). After 4 h the cells were spun down by gentle centrifugation for 10 min. The treatment medium was discarded and the cells were washed in PBS. After washing, the cells were resuspended in complete cell culture medium.

Spindle inhibition

0.2 µg/mL colcemid was administered 2 h before cell harvest.

Cell harvest

Cells were harvested two hours after addition of colcemid for both the non-activated and S9-activated studies. For the chromosome aberration assay dividing cells were harvested at approximately 24 hours from initiation of the treatment.

Slide preparation

The cells were centrifuged and the pellet resuspended in approximately 5 mL hypotonic solution (0.4% KCl). After removal of the hypotonic solution by centrifugation the cells were fixed with 3+1 methanol + glacial acetic acid. Slides were prepared by dropping the harvested cultures on clean slides. All slides were stained with Fluorescent plus Giemsa technique and air dried. The slides were coverslipped using 2 - 3 drops of Eukitt.

Metaphase analysis

For each concentration, 300 metaphases were scored for structural chromosomal aberrations, except for the solvent control (282 cells), concentrations 0.02 mM (267 cells) and 0.04 mM (284 cells) in the experiment without metabolic activation due to few cells present on the slides. In the experiment with metabolic activation, less than 300 metaphases were scored for the concentration 0.06 mM (227 cells) due to strong cytotoxic effects. Only 125 (EMS) and 150 (CPA) metaphases were scored for the positive controls due to a clearly positive result. The mitotic index was determined (percentage of cells in mitosis per 1000 cells). Additionally the number of polyploid cells was scored. The cell cycle of the actual lymphocyte cultures is monitored using a BrdU-labeling technique. In case of mitotic delays observed in the experiments an additional treatment and/or preparation interval of 48 h can be used.

Evaluation criteria

Providing that all acceptability criteria are fulfilled, a test chemical is considered to be clearly positive if, in any of the experimental conditions examined, at least one of the test concentrations exhibits a statistically significant dose-related increase compared with the concurrent negative control and an appropriate trend test and any of the results are outside the 95% control limits of the historical negative control data.

Findings

A. PRELIMINARY CYTOTOXICITY ASSAY

Due to strong precipitation of the test item in cell culture medium, the concentration in the pre-experiment was reduced to 0.5 mM. The relative mitotic index was used as parameter for evaluating toxicity. No precipitation of the test item was noted.

B. CYTOGENETIC ASSAYS

The test item Captan Technical was investigated for a possible potential to induce structural chromosomal aberrations in human lymphocytes in vitro in the absence and presence of metabolic activation by S9 homogenate. The selection of the concentrations used in the main experiment was based on data from the solubility test and the pre-experiment which were performed according to the guidelines.

No precipitation of the test item was noted without and with metabolic activation at the concentrations evaluated. Due to the positive result in the short-term exposure experiment it was not necessary to perform a long-term exposure experiment.

In experiment without metabolic activation, a biologically relevant decrease of the relative mitotic index (decrease below 70% rel. mitotic index) was noted at 0.04 mM and 0.06 mM. In the experiment with

metabolic activation a biologically relevant decrease of the relative mitotic index was noted at 0.02 mM and higher.
In the experiment without metabolic activation, the aberration rates of the negative control (1.0%), the solvent control (1.4%) and the concentrations 0.02 mM (2.6%) and 0.05 mM (1.0%) were within the historical control data of the testing facility (-0.09% – 3.47%). Two concentrations were highly increased above the historical control range with 5.3% (0.04 mM) and 7.7% (0.06 mM) mean aberrant cells. These values were also statistically significantly increased and a concentration-dependency was observed determined with the χ^2 Test for trend. Therefore, the number of aberrant cells found in the concentrations treated with the test item did show a biologically relevant increase compared to the corresponding solvent control.
With metabolic activation, the aberration rates of the negative control (1.0%), the solvent control (3.0%) and most of the concentrations treated with the test item (0.01 mM: 3.0%; 0.04 mM: 2.2% and 0.06 mM: 1.7%) were within the historical control data of the testing facility (-0.41% – 3.42%). The test item concentration 0.02 mM showed a slightly increased amount of aberrant cells to 3.7%. However, no statistical significance or a dose-response relationship was noted. Therefore, the number of aberrant cells found in the concentrations treated with the test item did not show a biologically relevant increase compared to the corresponding solvent control.
No biologically relevant increase in the frequencies of polyploid cells was found after treatment with the test item.
EMS (900 µg/mL) and CPA (5 µg/mL) were used as positive controls and induced distinct and biologically relevant increases in cells with structural chromosomal aberrations, thus proving the ability of the test system to indicate potential clastogenic effects.

Table 3.8.1.4-1 Summary of the results with or without metabolic activation

	Dose Group	Relative Mitotic Index [%]	Proliferation Index	Mean % Aberrant Cells		Historical Laboratory Negative Control Range	Statistical Significance [c]
				incl. Gaps	excl. Gaps		
Without S9	Culture medium	144	1.51	**2.3**	**1.0**	-0.09% - 3.47% aberrant cells	-
	DMSO	100	1.52	**2.5**	**1.4**		/
	0.02 mM	70	/	**7.1**	**2.6**		-
	0.04 mM	49	/	**7.0**	**5.3**		+
	0.05 mM	86	1.07	**1.3**	**1.0**		-
	0.06 mM	54	1.05	**9.0**	**7.7**		+
	EMS [a]	59	/	**24.0**	**24.0**		+
With S9	Culture medium	61	1.44	**1.3**	**1.0**	-0.41% - 3.42% aberrant cells	-
	DMSO	100	1.52	**4.0**	**3.0**		/
	0.01 µM	80	/	**3.7**	**3.0**		-
	0.02 µM	33	/	**4.3**	**3.7**		-
	0.04 µM	59	1.00	**2.2**	**2.2**		-
	0.06 µM	23	1.05	**2.7**	**1.7**		-
	CPA [b]	32	/	**26.4**	**21.6**		+

[a] Ethylmethanesulfonate at 900 µg/mL
[b] Cyclophosphamide at 5 µg/mL
[c] statistical significant increase compared to solvent controls (Fisher's exact test, $p < 0.05$), +: significant; -not significant

The mitotic index was determined in 1000 cells per culture of each test group. The relative values of the mitotic index are related to the solvent controls.

Conclusions

In conclusion, it can be stated that during the described *in vitro* chromosomal aberration test and under the experimental conditions reported, the test item Captan Technical induced structural chromosomal aberrations in human lymphocyte cells. Captan's potency to induce structural chromosome aberrations was eliminated under conditions of metabolic activation.

Therefore, Captan Technical is considered to be clastogenic in this chromosome aberration test.

3.8.1.5 Tezuka et al. (1980)

Reference:	Sister-chromatid exchanges and chromosomal aberrations in cultured Chinese hamster cells treated with pesticides positive in microbial reversion assays
Author(s), year:	Tezuka, H. et al. (1980)
Report/Doc. number:	Institute of Environmental Toxicology, Tokyo, Japan, report published in 'Mutation Research' (1980) 78, pp 177 – 191 (Company file: R-2715/TMN-0816)
Guideline(s):	In-house method
GLP:	No
Deviations from OECD 473 (2014):	Numerous deviations, e.g.: no testing in presence of metabolic activation; cell proliferation was apparently measured by BrdU incorporation; only exposure to captan for 26.5 hours was tested; only cells with chromosomal aberrations excluding gaps were scored; at least 300 metaphases should be scored per concentration and exposure time
Acceptability:	Limited

Material and Methods

Genotoxicity of captan (purity 99.9%, Nishio Ind. Co. Ltd., Japan; batch number not specified in report) was investigated *in vitro* in the Chinese hamster V79 cell line. Cells were incubated with captan (dissolved and diluted in dimethylsulfoxide (DMSO) - final concentration 1%, in a serum-free medium without activation) at concentrations from 6 x 10^{-6} M to 6 x 10^{-5} M for 26.5 hours. 5-Bromodeoxyuridine (BrdUrd) was added to all cultures at the beginning of the incubation at a final concentration of 2 µM. Control cultures containing DMSO and BrdUrd were also prepared. Colcemid (final concentration 0.1 µM) was added to all cultures 2 hours before cells were harvested. Chromosome preparations were made and the number of sister chromatid exchanges (SCEs) per cell and chromosome aberrations were scored. Cells with gaps alone were not evaluated as cells with chromosomal aberrations. Generally 50 metaphases were scored for detecting SCEs and 100 metaphases for aberrations at each concentration.

Findings

A significant increase in the frequency of SCEs was observed at all captan concentrations. Chromosomal aberrations were significantly increased at captan concentrations of 4.5 x 10^{-5}M and above. Summaries of the frequency of sister chromatid exchange and chromosome aberrations of cell cultures treated with captan are given in Table 3.8.1.5-1 and Table 3.8.1.5-2 below.

Table 3.8.1.5-1 *In vitro* mammalian genotoxicity test: frequency of sister-chromatid exchange in cell cultures treated with captan

Concentration (M)	Number of cultures	Number of cells observed for SCEs	SCEs/cell (mean ± SEM)	Induced SCEs/cell/µM
0	4	200	4.7 ± 0.2	-
6 x 10^{-6}	2	50	6.6 ± 0.5***	-
1.5 x 10^{-5}	2	50	11.2 ± 0.8***	0.43[1]
3 x 10^{-5}	2	50	15.0 ± 0.7***	0.34
4.5 x 10^{-5}	2	50	21.1 ± 1.1***	0.36
6 x 10^{-5}	2	50	24.9 ± 1.1***	0.34

[1] Maximal value of induced SCEs/cell/µM.
*** Significantly different to the control ($p < 0.001$).

Table 3.8.1.5-2 *In vitro* mammalian genotoxicity test: frequency of chromosomal aberrations and polyploid cells in cell cultures treated with captan

Concentration of captan (M)	Total number of cells observed	Cells with aberrations		Cells with 10 or more aberrations	Aberrations and chromatid type						Induced frequency of cells with aberrations (%)/µM	Polyploid cells/total cells observed	
		Number	%		cdg	icdg	cdb	icdb	cdx	chromosome type		Number	%
0	400	14	3.5	0	6	1	5	6	1	4	-	16/1426	1.1
6×10^{-6}	100	1	1.0	0	5	0	2	0	0	0	-	8/461	1.7
1.5×10^{-5}	100	4	4.0	0	4	1	0	3	0	1	-	5/489	1.0
3×10^{-5}	100	7	7.0	2	4	1	1	5	4	0	-	7/441	1.6
4.5×10^{-5}	100	23***	23.0	16	2	0	4	3	6	1	0.43[1]	10/402	2.5
6×10^{-5}	100	28***	28.0	13	14	3	11	10	18	1	0.41	8/409	2.0

[1] Maximum value of induced frequency of cells with chromosomal aberrations (%)/µM.
*** Significantly different from the control ($p < 0.001$).
Abbreviations: cdg chromatid gaps, icdg isochromatid gaps, cdb chromatid breaks, icdb isochromatid breaks, cdx chromatid exchanges

Conclusions

Incubation of Chinese hamster C79 cells in serum-free medium with captan without metabolic activation resulted in a significant dose-dependent increase in the frequency of sister chromatid exchange and chromosomal aberrations. Captan can therefore be considered positive in this assay.

3.8.1.6 Tezuka et al. (1978)

Reference:	Cytogenetic and dominant lethal studies on captan
Author(s), year:	Tezuka, H. et al. (1978)
Report/Doc. number:	Institute of Environmental Toxicology, Tokyo, Japan, report published in 'Mutation Research' (1978) 57, pp 201 – 207 (Company file: R-1666/TMN-0824)
Guideline(s):	In-house method
GLP:	No
Deviations from OECD 473 (2014):	Numerous deviations, e.g.: no demonstration of exponential cell growth/no stimulation of cell division; no testing in presence of metabolic activation; only cells with chromosomal aberrations excluding gaps were scored; at least 300 metaphases should be scored per concentration and exposure time
Acceptability:	No

Material and Methods

The genotoxic activity of captan (purity > 98%, batch number not specified) was investigated by an *in vitro* cytogenetic study in human diploid fibroblasts. Cells were incubated in the presence of captan at concentrations of 0, 0.5, 1.5, 3.0 and 4.0 µg/mL for 4 or 24 hours. The test substance was dissolved and diluted in DMSO (final concentration 0.5%). A positive control culture was run with mitomycin C at a concentration of 0.05 µg/mL for 24 hours. Colchicine (0.2 µg/mL) was added to cultures three hours before cells were harvested. Chromosome preparations were made and scored for gaps and breaks. For the determination of the frequency of accumulated metaphases with colchicine pretreatment, at least 3000 cells were counted.

Findings

A summary of the results from the study is given in Table 3.8.1.6-1. Highly significant mitotic inhibition was noted after exposure to captan for 4 and 24 hours at 3.0 and 4.0 µg/mL. Significant mitotic inhibition was also noted after 4 hours exposure at 0.5 µg/mL and after 24 hours at 1.5 µg/mL. Effects on chromosomes were manifested as a dose dependant increase in the number of cells showing stickiness, whereby it is not clear what is meant by "stickiness". However, no chromosomal aberrations were induced in any of the captan treated cultures.

Table 3.8.1.6-1 *In vitro* mammalian cytogenicity: summary of effects.

Dose (µg/mL)	Treatment time (h)	Accumulated metaphases (%)	Total cells observed	Number cells showing stickiness	Number cells with chromosomal aberrations[1]
0	4	1.9	200	0	4
0.5	4	1.1*	200	0	1
1.5	4	1.4	200	1	2
3.0	4	0.6***	77	21***	2
4.0	4	0.8***	60	59***	0
0	24	1.7	200	0	5
0.5	24	1.3	200	0	4
1.5	24	0.8**	200	3	1
3.0	24	0.3***	84	14***	3
4.0	24	-[2]	-	-	-
Mitomycin C (0.05)	24	0.3***	97	0	18***

[1] Cells only with gaps were excluded; [2] complete mitotic inhibition;
*, **, *** Significantly different to the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Conclusions

In cells exposed to captan no chromosomal aberrations were observed. However, there was mitotic inhibition at all tested concentrations which was more pronounced at higher concentrations and after longer duration of exposure.

3.8.1.7 Study 11 (2017)

Reference:	In vitro Mammalian cell gene mutation test (HPRT-Locus) in Chinese Hamster V79 cells with Captan technical
Author(s), year:	Anonymous (2017)
Report/Doc. number:	Document No: 171624, R-38478
Guideline(s):	OECD 476 (2016), EC B.17, OPPTS 870.5300
GLP:	Yes
Deviations:	Due to a typing error, the Sponsor address was corrected. Mammalian microsomal fraction S9 Mix was obtained from Trinova Biochem GmbH, Giessen, Germany from male Sprague Dawley rats, due to change of supplier. Statistical analysis deviation was a request from Sponsor (see B.4). 6.0 x 10^6 cells were exposed to the designated concentrations of the test item as a result of high cytotoxicity. Certificate of analysis in terms of purity and colour characterisation was changed due to additional information provided by the sponsor. Adaptation to OECD test guideline: 95% confidence limits were used to determine historical control data range in negative and/or solvent controls. These deviations did not influence the quality or integrity of the present study.
Acceptability:	Yes

Executive Summary

The test substance Captan Technical was examined for its potential to induce gene mutations at the HPRT-locus using V79 cells of the Chinese Hamster, according to OECD guideline 476. The selection of the concentrations to be tested was based on data from the pre-experiments. Two independent trials in both the absence and the presence of S9-mix activation system were conducted as a 4 h short-term exposure assay. For the cytotoxicity and gene mutation, test substance doses in DMSO ranged from 0.0002 – 0.04 mM.
No precipitation of the test item was noted in the experiments reported. Biologically relevant growth inhibition (relative survival < 70%) was observed in the main experiment with and without metabolic activation. In the main experiment without metabolic activation the relative survival was 18% for the highest concentration (0.004 mM) evaluated. The highest biologically relevant concentration evaluated with metabolic activation was 0.04 mM with a relative survival of 15%.
Biologically relevant increase of mutants was found without metabolic activation after treatment with the test item. Half of the elevated mutant frequencies in the main experiment without metabolic activation were statistical significantly increased over those of the solvent controls and a dose-response relationship was observed. In regards to the experiment with metabolic activation, the mutant frequencies of the concentrations 0.005 mM and 0.03 mM were increased. However, as no concentration-related increase was observed, these inductions were not regarded as biologically relevant although a statistical significant trend was determined.
Under the experimental conditions of this assay, Captan Technical is considered to be mutagenic at the HPRT locus using V79 cells of the Chinese Hamster. Captan's potency to induce gene mutations was eliminated under conditions of metabolic activation.

Material and Methods

A. MATERIALS
1. Test Material: Captan Technical

Description:	White solid
Batch #:	941381319
Purity:	96.5%
Storage conditions:	Room temperature
Expiry date:	01.01.2020
Solvent used:	Dimethyl sulfoxide (DMSO) 1% (v/v)

2. Control Materials:

Negative control: Treatment medium MEM, duplicates
Solvent control: DMSO, Applichem Lot No. 0000978834, duplicates
Positive control without S9: Ethylmethanesulfonate (EMS), Sigma Aldrich batch No. BCBS6100V at 300 µg/mL
Positive control with S9: 7,12-dimethylbenzo[a]anthracene (DMBA), Sigma Aldrich batch No. SLBS6093 at 1 µg/mL

3. Metabolic activation:

S9 derived from rats (phenobarbital and β-naphthoflavone induced rat liver).
Protein content: 33.7 mg/mL S9
Component/Content: 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, 5 mM NADP in 100 mM Na-Phosphate-buffer (pH 7.4)

4. Test cells: V79 cells (ATCC, CCL-93) from Eurofins Munich stock cultures

5. Locus examined: HPRT, the selection agent used was 6-thioguanine

B. TEST PERFORMANCE

1. In life dates:

24. May – 21. July 2017 at Eurofins BioPharma Product Testing Munich GmbH, Planegg, Germany.

2. Pre-experiment for Toxicity

The toxicity of the test item was measured in pre-experiments up to concentration of 5 mM due to precipitation in the higher concentrations as determined in the solubility test. As strong cytotoxic effects were observed during the testing, the pre-experiment was repeated with lower concentrations. Eight concentrations (0.0002, 0.0005, 0.001, 0.002, 0.005, 0.01, 0.02, 0.05 mM) were tested with and without metabolic activation for the 4 h short-term exposure assay. Toxicity of the test item was evaluated using the relative survival (RS).

3. Main experiment

The study was conducted using V79 Chinese hamster cells, screening for forward mutations at the HPRT locus with thioguanine as the selective agent, both in the presence and absence of a liver S9 preparation derived from phenobarbital and β-naphthoflavone treated rats. For testing, Captan Technical was diluted in DMSO 1% (v/v) in treatment medium, which was also used as the vehicle for positive control substances and as negative (solvent) control. As positive controls the directly acting mutagen ethylmethanesulfonate (EMS) which requires metabolic activation and 7,12-dimethylbenzo[a]anthracene (DMBA) without metabolic activation were used.
Based on preliminary dose-ranging studies, 0.004 mM (without metabolic activation) and 0.04 mM (with metabolic activation) were selected as the highest concentrations.

Test item concentrations:
0.0002, 0.0005, 0.0007, 0.001, 0.002, 0.004 mM (-S9 metabolic activation)
0.002, 0.005, 0.01, 0.02, 0.03, 0.04 mM (+S9 metabolic activation)

The treatment was 4 h (short-term exposure assay) without and with S9 mix.
Approx. 24 h after seeding the cells were exposed to designated concentrations of the test item either in the presence or absence of metabolic activation in the mutation experiment. After 4 h (short term exposure) the treatment medium (MEM without serum) containing the test item was removed and the cells were washed twice with PBS, trypsinised and counted with a cell counter. At the end of the expression period (after 7 to 9 days) to select the mutants, about 4×10^5 cells for each treatment group were seeded in cell culture petri dishes with selection medium containing 11 µg/mL thioguanine for further incubation. After incubation for an appropriate time (7-12 days) colonies were fixed with methanol, stained with Giemsa and counted.

4. Statistics

Normality was tested according to Kolmogorov-Smirnov-test. To test equality of variances in one-way ANOVA, the Browne and Forsythe test was performed. For the determination of statistical significances, the mean values of each test item concentration were evaluated with one-way ANOVA (Dunnett's test with multiple comparisons) at the 5% level ($p < 0.05$). Statistical differences between the mean values of the positive control and the negative control were evaluated with the two-tailed unpaired t-test.

Indication for a potentially dose-related trend is given by statistical significance of a linear trend on the mean values over all groups, including the solvent control as part of the follow-up testing after one-way ANOVA at the 5% level ($p<0.05$).

5. Evaluation criteria

a) Assessment of cytotoxicity:

A cytotoxic effect is assumed when the relative survival (RS) decreases below 70%. The RS was calculated based on the cloning efficiency of the approx. 200 cells plated immediately after treatment adjusted by any loss of cells during treatment.

b) Assessment of mutagenicity:

The mutagenicity of the test substance was determined by the mutant frequency (MF). The mutant frequency was calculated based on the number of mutant colonies in selective medium divided by the cloning efficiency in non-selective medium at the time of mutant selection. After incubation for an appropriate time (7 - 12 days) colonies were fixed with methanol, stained with Giemsa and counted.

Assuming acceptability criteria are met, a test chemical is considered to be clearly positive if in any of the experimental conditions examined at least one of the test concentrations exhibits a statistically significant concentration related increase, compared with the concurrent negative control and outside the distribution of the historical negative control data. If there is by chance a low spontaneous mutation rate in the corresponding negative and solvent controls a concentration related increase of the mutations within their range has to be discussed.
A test chemical is considered to be clearly negative if, in all experimental conditions examined, none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control, there is no concentration-related increase when evaluated with an appropriate trend-test, and all results are inside the distribution of the 95th control limits of the historical negative control data

Findings

A. ANALYTICAL DETERMINATIONS
The following quality control determinations were performed in S9 mix by Trinova Biochem GmbH: Alkoxyresorufin-0-dealkylase activities, test for the presence of adventitious agents and promutagen activation. Additionally Eurofins Munich performed biological activity assays.

B. PRECIPITATION AND TOXICITY
No precipitation of the test item was noted in the experiments reported. However, precipitation was observed in the solubility test in higher concentrations (2.5 mM, 5 mM and 10 mM).
A biologically relevant growth inhibition (reduction of relative survival below 70%) was observed after the treatment with the test item in the experiment with and without metabolic activation.
Without metabolic activation the relative survival was 18% for the highest concentration (0.004 mM) evaluated. The highest biologically relevant concentration evaluated with metabolic activation was 0.04 mM with a relative survival of 15%.

C. MUTATION ASSAY
In the main experiment without and with metabolic activation all validity criteria were met. The mutant values of the negative controls fall within the historical data range of the test facility and the cloning efficiencies of the negative and solvent controls are > 50%.
The positive controls, DMBA (1.0 µg/mL) and EMS (300 µg/mL) showed statistically significant increases in mutant frequency, thereby demonstrating both the sensitivity and validity of the test systems.

Main Experiment
Without metabolic activation: The mutant values of the negative controls and the solvent controls were within the historical control data of the test facility Eurofins Munich (about 7.8 - 39.7 mutants per 10^6 cells) with 31.5 and 26.2 mutants per 10^6 cells for the negative controls and 25.9 and 31.7 mutants per 10^6 cells for the solvent controls.
The positive control EMS induced a distinct increase in mutant frequency with 315.1 mutants/10^6 cells. The mutant values of the test item are exceeding the historical control range at concentrations of 0.0005 mM and higher (42.6. to 59.6 mutants per 10^6 cells). A dose-dependent increase was noted. The highest mutant frequency was observed at a concentration of 0.002 mM (59.6 mutants per 10^6 cells) with a relative survival of 66%. The decrease in mutation frequency at the concentration of 0.004 mM, 53.2 mutants per 10^6 cells can be explained by strong cytotoxicity in this concentration.
A statistical analysis displayed that the mutant frequencies were significantly increased over those of the solvent controls for the test item concentrations of 0.001 mM and higher. Due to the exceeded mutant frequencies, the dose effect

relationship and the statistical significance trend obtained in this experiment, this effect is regarded as biologically relevant.

With metabolic activation: The mutant values of the negative controls, the solvent controls and four of six mutant values of the test item concentrations found were within the historical control data of the test facility Eurofins Munich (about 9.2 - 38.8 mutants per 10^6 cells). The mutant frequencies of the negative controls were 26.3 and 30.5 mutants per 10^6 cells and 32.0 and 26.5 mutants per 10^6 cells for the solvent controls. The positive control DMBA induced a distinct increase in mutant frequency with 117.1 mutants/10^6 cells. The mutant frequencies of the test item were in the range of 31.8 to 46.6 mutants per 10^6 cells. The mutant values of the test item exceeded the historical control range at concentrations of 0.005 and 0.03 mM, which were also statistically significant compared to the solvent control. The highest mutant frequency was observed at a concentration of 0.03 mM (46.6 mutants per 10^6 cells) with a relative survival of 93%. However these increases were regarded as biologically not relevant as no concentration-related increase could be determined.
Due to positive results in the first experiment no second experiment was performed.

Table 3.8.1.7-1 Main experiment without metabolic activation

Dose Group	Relative survival (RS) [%]	Mutant frequency per 10^6 cells	Statistical significance [a]
Treatment medium 1	96	31.5	-
Treatment medium 2	103	26.2	
DMSO 1 (1% v/v)	100	25.9	/
DMSO 2 (1% v/v)		31.7	
0.0002 mM	108	28.9	-
0.0005 mM	79	42.6	-
0.0007 mM	97	45.8	-
0.001 mM	73	55.6	+
0.002 mM	66	59.6	+
0.004 mM	18	53.2	+
EMS[b]	122	315.1	+

[a] statistical significant increase compared to solvent controls (one-way ANOVA, $p < 0.05$), +: significant; -: not significant
[b] Ethylmethanesulfonate (EMS) at 300 µg/mL

Table 3.8.1.7-2 Main experiment with metabolic activation

Dose Group	Relative survival (RS) [%]	Mutant frequency per 10^6 cells	Statistical significance [a]
Treatment medium 1	109	26.3	-
Treatment medium 2	98	30.5	
DMSO 1 (1% v/v)	100	32.0	/
DMSO 2 (1% v/v)		26.5	
0.002 mM	102	35.7	-
0.005 mM	112	42.0	+
0.01 mM	112	31.8	-
0.02 mM	115	35.4	-
0.03 mM	93	46.6	+
0.04 mM	15	38.1	-
DMBA[b]	141	117.1	+

[a] statistical significant increase compared to solvent controls (one-way ANOVA, $p < 0.05$), +: significant; -: not significant
[b] 7,12-dimethylbenzo[a]nthracene (DMBA) at 1 µg/mL

Indication for a potentially dose-related trend is given by statistical significance of a linear trend on the mean values over all groups, including the solvent control as part of the follow-up testing after one-way ANOVA at the 5% level ($p < 0.05$).

Table 3.8.1.7-3 Trend-test

Experiment I	p-value	Statistical Significance
without metabolic activation	<0.0001	+
with metabolic activation	0.0299	+

+: significant ($p < 0.05$)
-: not significant

Conclusions

In conclusion, in the described in vitro cell gene mutagenicity test under the experimental conditions reported, the test item Captan Technical is considered to be mutagenic in the HPRT locus using V79 cells of the Chinese Hamster. Captan's potency to induce gene mutations was eliminated under conditions of metabolic activation.

3.8.1.8 Study 12 (1986)

Reference:	An assessment of the mutagenic potential of Merpan technical using an in vitro mammalian cell test system
Author(s), year:	Anonymous (1986)
Report/Doc. number:	Huntingdon Research Centre Ltd., UK, unpublished report No. MBS 9A/851485 (Company file: R-4319)
Guideline(s):	In-house method
GLP:	No
Deviations from OECD 476 (2015):	The current OECD testing guideline 476 does not support the measurement of mutation at the thymidine kinase (TK) locus anymore, only at the hypoxanthine-guanine phosphoribosyl transferase (HPRT) and at the xanthine-guanine phosphoribosyl transferase (XPRT) locus.
Acceptability:	Yes

Material and Methods

Captan (purity not specified, batch no. 610541) was tested for its mutagenic potential in an *in vitro* mammalian cell mutation assay using the mouse lymphoma L5178Y cell mutation test (mouse lymphoma thymidine kinase locus assay). Two independent tests in the absence and three in the presence of exogenous metabolic activation (Arochlor induced rat liver S-9) were carried out. Captan, dissolved in DMSO, was tested without metabolic activation at concentrations of 0.025 to 0.4 µg/mL and with metabolic activation at concentrations of 3.125 to 100 µg/L. DMSO alone was used as solvent control. Positive controls were carried out in the absence of metabolic activation with ethyl methane sulphonate (EMS) dissolved in DMSO at a final concentration of 500 µg/mL and in the presence of metabolic activation with 20-methylcholanthrene at a final concentration of 7 µg/mL. Cells were incubated with test substance for 3 hours, washed and then incubated for an additional 48 hours. Growth in suspension was monitored at 24 and 48 hours after treatment. Cells were then cloned in soft agar and after 12 days incubation colonies with a diameter of more than 400 µm were counted. Four of the most appropriate concentrations were chosen for cloning based on toxicity observed at 24 and 48 hours.

Findings

Treatment with captan induced dose dependant cytotoxicity in both the presence and absence of metabolic activation as evidenced by decreases in mean cell survival compared to the solvent control (Table 3.8.1.8-1).

Table 3.8.1.8-1 *In vitro* gene mutation: summary of growth in suspension of L5178Y cells

Concentration (µg/mL)	% Control[1]				
	Without activation		With activation		
	test 1	test 2	test 1	test 2	test 3
0	100	100	100	100	100
0.025	117[2]	104[2]	-	-	-
0.05	100	97	-	-	-
0.075	-	74	-	-	-
0.1	83	48	-	-	-
0.2	37	8	-	-	-
0.3	11	4[2]	-	-	-
0.4	5[2]	4[2]	-	-	-
3.125	-	-	138[2]	-	-
6.25	-	-	114[2]	87[3]	-
12.5	-	-	111	67[3]	-
15	-	-	-	-	58
20	-	-	-	60	30
25	-	-	53	54	33
30	-	-	-	-	26
35	-	-	-	-	14
40	-	-	-	-	13[2]
45	-	-	-	-	16[2]
37.5	-	-	-	33	-
50	-	-	10	7	-
100	-	-	10	-	-
EMS (500 µg/mL)	60	37	-	-	-
20-methyl-cholanthrene (7 µg/mL)	-	-	100	81	45

[1] Mean of eight replicates in controls and two replicates in treatments.
[2] Cultures disregarded prior to cloning in agar in favour of cultures of more acceptable levels of toxicity.
[3] Cultures discarded proir to cloning in agar in favour of cultures treated with higher concentrations of captan

A summary of the viability of cells in soft agar is given in Table 3.8.1.8-2. In the absence of metabolic activation, captan caused highly significant dose-related increases in mean mutant frequency at concentrations ≥ 0.075 µg/L (Table). In the presence of metabolic activation, captan failed to give rise to statistically significant increases in mean mutation frequency in two of three tests. Significant differences in mean mutation frequency were observed in a third test at concentrations of 25 and 30 µg/mL. At 30 µg/mL a doubling in mutant frequency occurred in one culture. However, no two-fold increases in mean mutant frequency were achieved at any treatment concentration. The positive controls produced highly significant increases in mutant frequency.

Table 3.8.1.8-2 *In vitro* gene mutation: summary of viability of L5178Y cells in soft agar

Concentration (µg/mL)	% Mean survival[1]				
	Without activation		With activation		
	test 1	test 2	test 1	test 2	test 3
0	100	100	100	100	100[2]
0.05	98	81	-	-	-
0.075	-	63	-	-	-
0.1	83	45	-	-	-
0.2	32	5	-	-	-
0.3	6	-	-	-	-
12.5	-	-	107	-	-
15	-	-	-	-	46
20	-	-	-	70	16
25	-	-	48	58	19
30	-	-	-	-	14
35	-	-	-	-	3[3]
37.5	-	-	-	37	-
50	-	-	<1	3	-
100	-	-	<1	-	-
EMS (500 µg/mL)	45	23	-	-	-
20-methyl-cholanthrene (7 µg/mL)	-	-	101	88	22

[1] Mean of eight replicates in controls and two replicates in treatments, except where specified.
[2] Four replicates.
[3] Only one replicate.

Table 3.8.1.8-3 *In vitro* gene mutation: summary of mutation of L5178Y cells in soft agar

Concentration (µg/mL)	Mean mutant frequency/10^6 viable cells[1]				
	Without activation		With activation		
	test 1	test 2	test 1	test 2	test 3
0	57	48	57	45	60[2]
0.05	60	57	-	-	-
0.075	-	79***	-	-	-
0.1	100**	80***	-	-	-
0.2	119***	239***	-	-	-
0.3	244***	-	-	-	-
12.5	-	-	47	-	-
15	-	-	-	-	78
20	-	-	-	60	84
25	-	-	43	46	108**
30	-	-	-	-	116**
35	-	-	-	-	56[3]
37.5	-	-	-	42	-
50	-	-	490[4]	45	-
100	-	-	0	-	-
EMS (500 µg/mL)	418***	388***	-	-	-
20-methyl-cholanthrene (7 µg/mL)	-		195***	220***	260***

[1] Mean of eight replicates in controls and two replicates in treatments, except where specified.
[2] Four replicates.
[3] Only one replicate, hence disregarded from statistical analysis.
[4] Percentage survival less than 1%, therefore values omitted from statistical analysis.
significant difference compared to the control: ** p = 0.01; *** p = 0.001.

Conclusions

In the absence of exogenous metabolic activation, captan demonstrated mutagenic activity *in vitro* under the conditions of the test system. In the presence of exogenous metabolic activation, no reproducible evidence of mutagenic activity was observed.

3.8.1.9 O'Neill et al. (1981)

Reference:	Cytoxicity and mutagenicity of the fungicides captan and folpet in cultured mammalian cells (CHO/HGPRT system)
Author(s), year:	O'Neill, J.P. et al. (1981)
Report/Doc. number:	Oak Ridge National Laboratory, Tennessee, USA, report published in 'Environmental Mutagenesis', 3, pp 233 - 237 (Company file: R-3487/TMN-0810)
Guideline(s):	In-house method
GLP:	No
Deviations from OECD 476 (2015):	No testing in presence of metabolic activation; no historical control data is available
Acceptability:	Yes

Material and Methods

The cytoxicity and mutagenicity of captan (purity not specified) was investigated *in vitro* in the Chinese Hamster Ovary (CHO) cell strain (CHO-K_1-BH_4). Cells in the exponential growth stage were exposed to captan at concentrations of 0, 0.1, 0.25, 0.5, 1.0, 2.0 and 4.0 µg/mL. Captan was dissolved and diluted in DMSO. The solvent control culture contained 0.1% DMSO only. Ethyl methane sulphonate (EMS) was used as a positive control. Cells were incubated for five hours with test substance, washed and incubated for an additional 19 hours, and then sub-cultured for the assessment of cytotoxicity and 6-thioguanine-resistant mutants.

Findings

Mutant frequency increased with increasing concentrations of captan. The mutant frequencies at concentrations of 0.25 µg/mL and above were significantly higher than the controls (p = 0.01). The results are summarised in Table 3.8.1.9-1.

Table 3.8.1.9-1 In vitro mammalian cell gene mutation test: cytoxicity and mutagenicity of captan

Dose (µg/ mL)	Relative cloning efficiency (%)				Mutants/10^6 cells			
	exp 1	exp 2	exp3	mean	exp 1	exp 2	exp 3	mean
0	100	100	100	100	1.3	1.2	4.6	2.4
0.1	87.5	102.2	90.6	93.4	7.3	1.4	4.0	4.2
0.25	93.3	100.4	94.6	96.1	9.8	19.2	34.2	21.1**
0.5	85.2	90.4	80.7	85.4	27.9	30.3	34.4	30.9**
1.0	65.6	64.7	55.9	62.1	44.1	33.8	40.2	39.4**
2.0	12.1	4.2	7.3	7.9	50.7	85.0	61.9	65.9**
4.0	11.1	6.2	3.6	6.9	69.9	-[2]	145.1	107.5**
EMS[1]	90.2	86.5	83.2	86.6	201.2	176.5	209.4	195.7**

[1] Ethyl methane sulphonate at a concentration of 200 µg/mL.
[2] No data due to contamination.
** Significantly different from the control (p = 0.01).

Conclusions

Captan was found to be mutagenic in the CHO/HGPRT assay, in the absence of any exogenous metabolic activation. In this study, the mutagenic potential of captan in mammalian cells was not tested in presence of metabolic activation.

3.8.2 Animal data

3.8.2.1 Study 1 (1985)

Reference:	Merpan: Mouse micronucleus test
Author(s), year:	Anonymous (1985)
Report/Doc. number:	Life Science Research Israel Ltd., report No. MAK/072/MER (Company file: R-3666)
Guideline(s):	OECD 474 (year not specified)
GLP:	No
Deviations from OECD 474 (2014):	None
Acceptability:	Yes

Material and Methods

Captan (purity 94%, batch number 610541) was evaluated for its ability to induce micronucleated polychromatic erythrocytes (MNPCEs) in the bone marrow of CD-1 strain mice. Captan was formulated as a suspension in 0.5% carboxymethyl cellulose (CMC) and 0.5% acetic acid. Fasted mice received doses of 0, 40, 200 and 1000 mg/kg bw by oral gavage (15 animals/sex in the control and high dose group and 5 animals/sex in the intermediate and low dose group). Doses were determined based on a preliminary toxicity test. A positive control group (5 animals/sex) received chlorambucil (in 10% ethanol) at a dose rate of 30 mg/kg bw. In-life observations included body weight measurements and clinical signs. Five male and five female mice in each group were sacrificed 24 hours after treatment. Further lots of five males and five females from the control and the 1000 mg/kg bw groups were sacrificed at 48 and 72 hours. Bone marrow smears were prepared for each timepoint. The incidence of micronucleated cells per 1000 polychromatic cells was scored for each animal.

Findings

General observations: Treatment with captan did not result in any significant reduction in body weight. There were no adverse treatment-related effects or mortalities. Two of the 200 mg/kg bw males were found dead. Necropsy revealed

congested lungs, which indicated misdosing. One male dosed at 1000 mg/kg bw had decreased motor activity and was killed *in extremis*. Necropsy revealed a tympanic stomach.

Bone marrow counts: Captan did not result in any significant alteration in the frequency of MNPCEs. Mice treated with the positive control chlorambucil showed a significant increase in the number of MNPCEs compared to the untreated control at the same timepoint. The ratio of polychromatic to normochromatic cells was similar in all groups.
A summary of the number of micronuclei per 1000 polychromatic erythrocytes is given **Fehler! Verweisquelle konnte nicht gefunden werden.**.

Table 3.8.2.1 -1 Mouse micronucleus test: number of micronuclei per 1000 polychromatic erythrocytes

Dose (mg/kg bw)	Time (hours post-dose)	Sex	Number of animals	MNPCE[1]
0	24	M	5	0.6
		F	5	1.4
40	24	M	5	1.2
		F	5	1.4
200	24	M	3	1.0
		F	4	1.3
1000	24	M	5	1.0
		F	5	1.0
0	48	M	5	0.8
		F	5	1.0
1000	48	M	5	1.2
		F	5	1.6
0	72	M	5	1.4
		F	5	1.2
1000	72	M	5	1.2
		F	5	2.2
Chlorambucil	24	M	5	61.9***
		F	5	58.2***

[1] Number of micronuclei per 1,000 polychromatic erythrocytes.
*** Statistically significant to the control at 24 hours ($p < 0.001$).

Conclusions

Captan did not induce an increase in micronuclei in the bone marrow cells of mice and was, therefore, considered negative under the conditions of this mouse micronucleus test.

As the result of the mouse micronucleus test was negative and as the test substance did not induce toxic effects in the bone marrow, it has to be demonstrated that exposure of the target tissue occurred. Therefore, all available evidence of bone marrow exposure was assessed in accordance with the published EFSA Scientific Opinion from 2017 (Clarification of some aspects related to genotoxicity assessment ; 16 Nov 2017).

- Toxicity to bone marrow observed in mammalian erythrocytes (i.e. decrease in the PCE/(NCE+PCE) ratio): no evidence
- Toxicity to the bone marrow observed in repeated dose toxicity studies : no evidence
- Test substance detected in the bone marrow in toxicokinetic studies :
  In the same species (mouse), there is evidence that six hours after administration of a single oral dose of 900 mg/kg bw of [^{35}S]-captan to CD-1 strain male mice, radioactivity was detected in the bone marrow (Study 5, 1991, section 3.9.4.1).
  In rats, there is evidence from ADME studies with captan that radioactivity reaches blood/plasma and bone after oral administration. In studies with oral administration of [^{14}C]-ring-labelled-captan, the animals were sacrificed 7 days following dosing. Due to the rapid excretion of captan, the levels of radioactivity detected were very low or even below the limit of detection (Study 1-3, section 2.1-3). However, the metabolite THPI, which is formed in the first degradation step of captan through cleavage of the trichloromethylthio moiety (thiophosgene) from captan, was thoroughly tested and was clearly negative in a series of genotoxicity tests. Rationale for considering data from different species, i.e. rat data : In a comparative metabolism study, biotransformation and excretion

was similar in rats and mice, however, absorption and elimination rate in mouse was faster than in rat (Study 6, 1985, section 2.6). Furthermore, two in vivo chromosomal aberration assays in rat bone marrow is available (Study 3, 1979, section 2.8.2.3 and Tezuka, 1978) showing both negative results.

- Systemic toxicity observed in the bone marrow micronucleus test : no evidence
- Systemic toxicity observed in toxicity studies : In an acute oral toxicity study in mice, clinical signs were observed in all tested dose groups (i.e. dosages of 1500 mg/kg bw and higher), essentially abdominal ache syndrome, reduced activity, reduced frequency of respiration, titubation, tremors. Surviving animals exhibited signs of sedation and reduced readiness for reflexing.
- Test substance detected systemically in toxicokinetic studies : Evidence from comparative metabolism study (Study 6, 1985, section 2.6) that mice excrete radioactivity via urine and expired air after single oral administration of [^{14}C]-trichloromethyl labelled captan.

The *in vitro* mutagenicity of captan is ascribed to thiophosgene which *in vivo* is rapidly neutralized by thiol-containing molecules (Arce et al., 2010). Therefore, the ADME studies with oral administration of [^{14}C]-trichloromethyl-labelled captan (Study 5, 1988, see 2.5) can be considered more relevant with regard to bone marrow exposure. In this study in rats, radioactivity was detected in blood and bone following a single oral low dose, repeated oral low dose and a single oral high dose of radiolabelled captan (tables 2.5-4-6; sacrifice after 96, 96 and 120 hours, respectively).
In general, the systemic bioavailability of captan and its metabolites is demonstrated in the ADME studies by the wide distribution of radioactivity following initial absorption and by the large amount of urinary excretion (implying exposure in the blood).
Overall, there is sufficient evidence available to conclude that the bone marrow was exposed.

3.8.2.2 Study 2 (1981)

Reference:	Mutagenicity evaluation of captan in the somatic cell mutation assay
Author(s), year:	Anonymous (1981)
Report/Doc. number:	Litton Bionetics, Inc. unpublished report No. 20951 (Company file: TMN-0834)
Guideline(s):	In-house method
GLP:	No
Deviations from OECD 484 (1986):	Not relevant ; Test guideline OECD 484 was deleted in April 2014
Acceptability:	Yes

Material and Methods

Captan (purity 92.2%, batch no. SX-944) was evaluated for its ability to induce specific-locus somatic mutations in mice. Pregnant C57B1/6J mice (mated with T-strain males) were fed captan in the diet from day 8 through to day 12 of pregnancy at dose rates of 100, 1000 and 5000 ppm, corresponding to 10.8, 104.6 and 419 mg/kg bw/day captan, respectively. Positive control animals were administered a single dose of ethylnitrosourea in 0.9% saline at a dose rate of 50 mg/kg bw on gestation day 10. Negative control animals received laboratory chow only. The body weight of females was measured before and after the captan feeding period (days 8 and 12 of pregnancy). All exposed embryos were allowed to develop to birth. Litter size was determined and external morphologies examined for any gross abnormalities. New-borns were scored for spots on day 12 and again at the time of weaning.

Frequencies of coat color spots in the treated groups were compared against the negative control according to Fisher`s exact test to determine any significant increase.

Findings

A summary of body weight changes and food consumption is given in the following table**Fehler! Verweisquelle konnte nicht gefunden werden.**. Mice fed captan in the diet at a concentration of 5000 ppm over gestation days 8 to 12 consumed less food and lost body weight. Weight gain and food consumption was similar among other treated groups and the control.

Table 3.8.2.2-1 Mouse spot test: summary of body weight changes and food consumption (mean ± sd)

Dose (ppm)	Mean body weight (g)		Body weight change (g)	Mean feed consumption[1] (g)
	Gestation day 8	Gestation day 12		
0	21.3 ± 1.6	22.1 ± 2.4	+ 0.8	11.2 ± 1.4
100	21.5 ± 1.5	22.3 ± 2.5	+ 0.8	12.1 ± 1.8
1000	21.4 ± 1.5	21.9 ± 2.2	+ 0.5	11.7 ± 2.2
5000	21.3 ± 1.6	21.0 ± 2.0	- 0.3	8.8 ± 1.1

[1] Feed consumption over the period of dosing.

Pregnancy rates, average litter size and survival rates of pups at 12 days old were similar among all groups. The incidence of gross morphological abnormalities was low and there was no evidence of a treatment-related effect. The frequency of white midventral spots (WMVS), which are considered to represent melanocyte toxicity and not mutation induction, was similar among captan treated groups and did not differ significantly from the negative control ($p > 0.05$). Although the frequency of recessive spots (RS) in the 100 ppm group (4.4%) was slightly higher than the negative control (2.9%), frequencies in the 1000 and 5000 ppm groups were both lower than the negative control. There was no significant difference between the frequency of RS in any of the captan treated groups compared to the negative control. The frequencies of WMVS and RS in the ethyl-nitrosourea positive control group were significantly increased compared to the negative control. A summary of the parameters measured is given in **Fehler! Verweisquelle konnte nicht gefunden werden.** below.

Table 3.8.2.2-2 Mouse spot test: summary of findings

Treatment	Dose	Pregnant/plugged females (%)	Number of animals			Observed Coat Colour Spots[3]	
			Born (ALS)[1]	Surviving to 12 days (%)	Abnormal morphology	White midventral spots (%)	RS[2] (%)
Control	0 ppm	51/105 (49%)	297 (5.8)	275 (93%)	2-umbilical masses 1-crooked tail	3 (1.1%)	8 (2.9%)
Captan	100 ppm	50/106 (47%)	302 (6.0)	294 (97%)	2-umbilical masses	7 (2.4%)	13 (4.4%)
Captan	1000 ppm	52/106 (49%)	292 (5.6)	278 (95%)	none	9 (3.2%)	6 (2.2%)
Captan	5000 ppm	51/106 (48%)	321 (6.3)	308 (96%)	1-no bottom lip	9 (2.9%)	6 (1.9%)
Ethyl-nitrosourea	50 mg/kg bw	26/53 (49%)	143 (5.5)	134 (94%)	none	15 (11.2%)*	26 (19.4%)**

[1] ALS = average litter size.
[2] RS = individual recessive somatic mutation spots.
[3] Frequency of spots calculated as number of RS divided by the number of pups surviving to day 12 for each corresponding group.
* Significant at $p = 0.0002$ (Fischer's Exact Test).
** Significant at $p < 0.0001$ (Fischer's Exact Test).

Conclusions

The administration of captan did not result in an increase in the frequency of recessive somatic mutant spots and therefore captan was considered to be negative in the mouse spot test.

3.8.2.3 Study 3 (1979)

Reference:	Captan-Spritzpulver AA Captan: Investigation of effects on bone marrow chromosomes of the rat after sub-acute oral administration
Author(s), year:	Anonymous (1979)
Report/Doc. number:	Life Science Research, Essex, UK, report No. 79/MAK008/292 (Company file: R-1735)
Guideline(s):	In-house method
GLP:	No
Deviations from OECD 475 (2014):	Cytotoxicity (mitotix index) was not determined; only 100 metaphases per animal instead of 200 were examined; no historical control data is available
Acceptability:	Yes

Material and Methods

The effect of captan (purity not specified, batch number 702/38) on chromosomal structure was investigated in the rat. Captan prepared as a suspension in 0.5% gum tragacanth, was administered by oral gavage on five occasions, at 24-hour intervals, to male CD rats at dose rates of 200, 400 and 800 mg/kg bw (6 animals/dose). The dose volume was 10 mL/kg bw. Six negative control animals received vehicle only. A positive control group of six animals received two doses of chlorambucil 24 hours apart at a dose rate of 15 mg/kg, in a vehicle of 10% ethanol. Bone marrow cell division was arrested by an intraperitoneal administration of colchicine, at a dose rate of 4 mg/kg bw (dose volume 5 mL), three hours after the last treatment. Animals were killed by cervical dislocation two hours after colchicine administration. Bone marrow cells were collected, slides prepared and 100 metaphases examined for each animal. The following characters were recorded: chromosome number, number of normal and aberrant chromosomes, specific types and numbers of aberrations.

Findings

A summary of the observed aberrations is given in Table 3.8.2.3-1. Statistical analysis of the number of scored metaphases per animals with one or more aberration was carried out. There was no significant difference between any of the captan treated groups and the negative control ($p > 0.05$). Animals treated with chlorambucil showed a significantly higher number of metaphases with one or more aberration compared to the negative control ($p < 0.001$). No statistical comparison was made between treated animals and the controls for the frequency of cells with aberrations other than gaps. However, little difference between the vehicle control and captan treated groups was found for this parameter. Conversely, there was a marked difference between the vehicle control and chlorambucil treated group.

Table 3.8.2.3-1 In vivo bone marrow cytogenetic test: summary of observed aberrations

Dose (mg/kg bw/d)	Total no. cells scored	Totals and percentages[1]		Total number of specific aberrations seen							
		cells with aberrations	cells with aberrations other than gaps	chromatid gaps	chromosome gaps	chromatid breaks	chromosome breaks	exchanges	Frag-ments	Multi-ple aberrations	other aberrations
Control	600	18 3.00 ± 1.26	6 1.0 ± 0.63	12	0	4	0	0	2	0	0
200	600	17 2.83 ± 3.19	7 1.17 ± 1.17	9	2	2	0	0	6	0	1[2]
400	600	12 2.00 ± 2.00	4 0.67 ± 1.21	7	1	0	0	1	2	0	1[2]
800	581	9 1.50 ± 1.05	8 1.34 ± 0.82	1	0	2	0	2	5	0	0
Chlorambucil (15)	464	181*** 41.00 ± 32.69	165 38.33 ± 33.24	32	5	116	5	68	74	63	8[3,4]

[1] Totals (upper figures) include all animals; percentages (lower figures) are means of percentages in individual animals, with standard errors
[2] Abnormal chromosomes.
[3] Two dicentric chromosomes.
[4] Six pulverised metaphases.
*** Significantly different from the control ($p < 0.001$).

Conclusions

Under the conditions of this test, captan produced no evidence of damage to chromosomal structure in bone marrow cells of rats after oral administration of five daily doses of captan at up to 800 mg/kg bw/day. Captan was therefore negative in the *in vivo* mammalian bone marrow genotoxicity test.

3.8.2.4 Feng, J. and Lin, B. (1987)

Reference:	Cytogenetic effects of an agricultural antibiotic, captan, on mouse bone marrow and testicular cells
Author(s), year:	Feng, J. and Lin, B. (1987)
Report/Doc. number:	Jiangsu Provincial Sanitary and Anti-Epidemic Station, Nanjiing, China, published in 'Environmental Research' 43, pp 359 - 363 (Company file: TMN-0826)
Guideline(s):	In-house method
GLP:	No
Deviations from OECD 474 (2014):	Interval between collection of bone marrow samples and final treatment should be between 18 and 24 hours instead of 6 hours; proportion of immature among total erythrocytes was not determined; poor reporting of methods and results.
Deviations from OECD 475 (2014):	Cytotoxicity was not determined; very poor reporting of methods and results
Acceptability:	Limited

Material and Methods

The *in vivo* cytogenetic effects of captan were investigated in mouse (i) in a micronucleus test (MNT) of PCE in bone marrow, (ii) in a chromosome aberration test on bone marrow cells and (iii) in a chromosome aberration test in

spermatogonia and spermatocytes. Captan (purity 96.5%, batch number not specified), emulsified with Tween 40, was administered by oral gavage at various dosages.

For the MNT, five mice per group were administered captan at dose rates of 10, 50, 100, 400 and 800 mg/kg bw for two days. A negative control group received the vehicle only and a positive control group received Bis-A-TPA (*N,N´*-methylenebis(2-amino-1,3,4-thiadiazole)). All mice were sacrificed 30 hours after the first gastric tubing. Bone marrow cells were treated according to Schmid (1975).

For the chromosome aberration test on bone marrow cells, mice were administered captan by oral gavage at dose rates of 100, 400, 600, 800 and 1,000 mg/kg bw for five consecutive days. A group of negative control animals received vehicle only and a positive control group 1.5 mg/kg benzene. Bone marrow cell division was arrested by an intraperitoneal administration of colchicine, at a dose rate of 4 mg/kg bw, four hours after the last treatment and animals were sacrificed two hours later.

Findings

In the MNT, there was a dose-dependent increase in the frequency of MN in mice treated with captan, which showed statistical significance at the doses of 100 mg/kg bw and above.

In the chromosome aberration test in bone marrow of captan treated mice, the lowest dosage that induced chromosome aberration was 400 mg/kg bw. The principle structural abnormality was monosomic fragmentation in the two lower tested groups, with centromere rings appearing over 600 mg/kg bw. There was also a linear relationship between dose and genotoxic effects.

Table 3.8.2.4-1 In vivo micronucleus test: frequency of polychromatic erythrocytes (PCE) with micronuclei (MN)

Dose (mg/kg)	No. animals scored	Cells scored	No. Of PCE with MN	Percentage	P value
Control	5	5000	21	4.3	-
10	5	5000	19	3.9	> 0.05
50	5	5000	31	6.2	> 0.05
100	5	5000	58	11.6	< 0.01
400	5	5000	67	13.4	< 0.01
800	5	5000	112	22.4	< 0.01
Bis-A-TPA (200)	5	5000	67	13.4	< 0.01

Table 3.8.2.4-2 In vivo chromosome aberration test: effects of captan on bone marrow cells

Dose (mg/kg)	No. animals scored	Cells scored	Gap	Chromosome aberration					P value
				Chromatid break	Fragment	Ring	Total no.	%	
Control	6	600	1	1	-	-	1	0.16	-
100	5	500	1	2	-	-	2	0.4	> 0.05
400	5	500	4	5	-	-	5	1.0	< 0.05
600	5	500	2	8	1	1	10	2.0	< 0.01
800	7	700	10	6	5	2	13	2.6	< 0.01
1000	5	500	12	8	4	1	13	2.6	< 0.01
Benzene (1.5)	3	300	5	6	2	2	10	3.34	< 0.01

Conclusions

Under the conditions of the test, captan was genotoxic to cells of mouse bone marrow. A significant increase in the frequency of micronuclei in polychromatic erythrocytes in the bone marrow was observed at 100 mg/kg bw and above, and chromosome aberration in mouse bone marrow cells was significant at 400 mg/kg bw/day and above.

3.8.2.5 Fry, S.M. and Ficsor, G. (1978)

Reference:	Cytogenetic test of captan in mouse bone marrow
Author(s), year:	Fry, S.M. and Ficsor, G. (1978)
Report/Doc. number:	Department of Biomedical Sciences, Western Michigan University, Michigan, USA ; report published in 'Mutation Research', 58, pp 111 - 114 (Company file: R-2576/TMN-0825)
Guideline(s):	In-house method
GLP:	No
Deviations from OECD 475 (2014):	Numerous deviations, e.g.: Maximum tolerated dose was exceeded; only one dose level was tested; irrelevant route of exposure (intraperitoneally); at least 200 metaphases per animal should be analysed; a product (Captan 50 WP) containing 50% technical captan was tested instead of the technical active substance, therefore this study can be used as additional information only.
Acceptability:	No

Material and Methods

Captan 50 WP (50% technical captan, batch number 01007-10) was tested in an *in vivo* mammalian cytogenicity test. Swiss albino mice were administered captan in 0.9% saline by intraperitoneal injection at a dose rate of 250 mg/kg bw (dose volume 1 mL/kg bw). Control animals received 1 mL/kg vehicle only. Positive control animals were administered triethylenemelamine (TEM) in 0.9% saline at dose rates of 0.5 and 1.0 mg/kg bw by intraperitoneal injection (dose volume 1 mL/kg bw). Treated and control animals were sacrificed at 6, 12, 30 and 54 hours after treatment. Five hours prior to sacrifice, animals were administered 1 mg/kg bw colchicine in 0.9% saline by intraperitoneal injection (dose volume 1 mL/kg bw). Bone marrow cells were extracted, slides prepared and 100 metaphases per animal, if possible, were examined.

Findings

General observations: Signs of toxicity noted within 12 hours of captan administration were oedema, decreased body temperature, sluggishness and bluish skin colour. Mortality was 0, 25, 20 and 68% in the captan treated animals scheduled for sacrifice at 6, 12, 30 and 54 hours after treatment, respectively.

Cytogenicity: A summary of the findings is given in the following table. The incidence of chromosome aberrations in bone marrow cells from animals treated with captan did not differ significantly from the control at any time point. However, three metacentric chromosomes (one in each of the 6, 30 and 54 hour time point groups) in 1001 metaphases from the captan treated groups were observed, whereas none appeared in the 1495 control spreads. Since mouse chromosomes are telocentric, the metacentric chromosomes may have originated from Robertsonian fusion and, therefore, because of this unique rearrangement it cannot be stated with certainty that chromosomal breakage does not occur *in vivo* following the intraperitoneal administration of captan. Captan appears to be mutagenic in test systems which do not metabolise the test article. Studies in which captan is dosed orally, demonstrate that it loses mutagenic ability when metabolised.

Table 3.8.2.5-1 In vivo bone marrow cytogenetic test: summary of observed aberrations

Treat-ment	Time (hours)	No. of animals	No. of cells	Number of cells						Abnor-mal cells (%)
				Gaps	Chromatid breaks	Chromo-some breaks	Rearrangements	Rings	Multiple breaks	
Control	6	4	391	2	4	1	0	0	0	1.79
Captan	6	3	300	0	0	0	1	0	0	0.33
TEM (0.5)	6	3	307	9	39**	13**	7*	1	1	17.92**
Control	12	3	300	0	2	3	0	0	0	1.67
Captan	12	3	300	2	5	0	0	0	0	2.33
TEM (0.5)	12	3	259	6	40**	9	8*	0	0	19.31**
TEM (1.0)	12	2	195	30**	60**	13**	39**	24**	42**	58.97**
Control	30	4	400	2	1	2	0	0	0	1.25
Captan	30	3	300	3	1	1	1	0	0	1.67
TEM (0.5)	30	3	303	21**	44**	6	34**	4	50**	35.6**
TEM (1.0)	30	1	100	1	5*	0	9**	5**	72**	77.0**
Control	54	4	404	3	3	1	0	0	0	1.73
Captan	54	2	101	0	1	0	1	0	0	1.98
TEM (0.5)	54	3	300	1	0	6	0	1	0	2.67
TEM (1.0)	54	1	100	0	1	0	0	1	0	2.00

[1] TEM = triethylenemelamine
Significant difference between the control and treated group; * ($p < 0.05$), ** ($p < 0.01$).

Conclusions

The results of the study suggest that captan did not result in chromosomal damage *in vivo* after intraperitoneal injection at a dose rate of 250 mg/kg bw. However, it cannot be stated with certainty that captan does not result in chromosomal breakage due to the presence of metacentric chromosomes in some captan treated animals.

3.8.2.6 Study 4 (2016)

Reference:	Captan: Transgenic Mice (Muta™ Mouse) Mutation Assay
Author(s), year:	Anonymous (2016)
Report/Doc. number:	Biosafety Research Center (BSCR), unpublished report No. G514 (564-030)
Guideline(s):	OECD 488 (July 2013)
GLP:	Yes
Deviations from OECD 488 (2013):	None
Acceptability:	Yes

Executive Summary

A gene mutation assay with transgenic male mice (Muta™ Mouse) was conducted to assess the potential of Captan to induce gene mutation (reporter gene: *lacZ*) in the liver and duodenum.

Based on the following data: 1) dietary administration of Captan technical induced incidences of duodenal tumors in male and female mice at ≥ 6000 ppm in a mouse carcinogenicity feeding study (Study 3, 1981, section 3.9.1.3); 2) duodenal crypt hyperplasia was observed in mice treated with 6000 ppm Captan in diet for 28 days in an investigative study of mouse duodenal hyperplasia (Allen, 1994); and 3) body weight reduction was observed at the 6000 ppm exposure level

(Study 3, 1981, section 3.9.1.3, 1981), the maximum tolerated dose was considered to be 6000 ppm. Therefore, the following dose levels were selected for the gene mutation assay: 600, 2000, and 6000 ppm. The test substance was administered in diet to transgenic mice for 28 consecutive days, and then after an additional 3 days for mutant manifestation following the last administration, the liver and duodenum were removed and mutant frequencies were determined.

As a result, there were no significant differences in the mutant frequencies in the liver and duodenum in any of the groups treated with Captan as compared to the negative control group.

The mutant frequencies in the liver and duodenum in the positive control group, which was treated with N-ethyl-N-nitrosourea (ENU, dosage level of 100 mg/kg, i.p.), increased and these increases were statistically significant compared with those of the negative control group.

It was, therefore, concluded that Captan did not induce gene mutations in the liver or duodenum of transgenic mice (was negative) under the conditions in this study.

Material and Methods

1. Test Material: Captan
   - Description: White crystalline powder
   - Lot/Batch #: 94138718
   - Purity: 94.96%
   - CAS #: 133-06-2

   Stability of test compound:
   After storage for 2 days in a well-closed container in the cold storage room followed by 8 days in an open-container in animal room, samples of diet formulation were analysed. Captan was shown to be stable in diets.

2. Control materials:
   - Negative: Basic diet
   - Positive: N-ethyl-N-nitrosourea (ENU) in Phosphate Buffer-Saline (PBS) to make a 10 mg/mL solution (i.p.)

3. Test animals:
   - Species: Mouse (transgenic mouse)
   - Strain: CD_2-LacZ80/HazfBR (Muta^{TM}Mouse) [SPF]
   - Age: 9 weeks of age
   - Weight at dosing: 24.7 to 26.5 g
   - Source: Japan Laboratory Animals, Inc.
   - Number of animals per dose: 6 Treated, 5 Evaluated
   - Acclimation Period: 7 days
   - Diet: CRF-1; lot No. 151112, Oriental Yeast, *ad libitum*
   - Water: Tap water, *ad libitum*
   - Housing: All animals were individually housed in an econ cage (W 18.2 × D 26.0 × H 12.8 em) with bedding (ALPHA-dri1M, lot No. 05115, Shepherd Specialty Papers).

   Environmental conditions:
   - Temperature: 20 to 26°C (actual values: 22.8 to 23.2°C)
   - Humidity: 35 to 70% RH (actual values: 43 to 64% RH)
   - Ventilation: 12 times or more/h
   - Photoperiod: Alternating 12-hour light and dark cycles

4. Test compound concentrations:
   0, 600, 2000, and 6000 ppm captan in diet (equivalent to mean test substance intakes of 0, 85.3, 273, and 776 mg/kg bw/day, respectively);
   100 mg/kg positive control ENU (intraperitoneally once daily for 2 consecutive days)

TEST PERFORMANCE
Biosafety Research Center (BSRC), Japan, conducted the study during the period of January 2016 to June 2016.

Treatment and sampling times:
The test substance in the diet was administered for 28 days, available continuously for *ad libitum* consumption. The organs were removed 3 days after last dosing (Day 31).
Positive control was administered on test day 2 and 3. Tissues were removed 10 days after last dosing (Day 13).

Tissues:
The liver (major site of xenobiotic metabolism) and duodenum (site of tumor formation in mouse oncogenic feeding study; Study 3, 1981, section 3.9.1.3, 1981) were removed and analyzed for mutant frequency.

Details of tissue analysis:
Following sacrifice, the liver and duodenum were removed and frozen in an ultra-low temperature freezer. The following steps were then followed during tissue processing: (1) Genomic DNA extraction, (2) Bacterial test strains preparation, (3) *In vitro* packaging of genomic DNA, (4) Plating of packaging DNA, (5) Calculation of total number of plaques, (6) Calculation of total number of mutant plaques, (7) Calculation of mutant frequency for each animal.

Evaluation Criteria:
To determine whether a statistically significant response in mutant frequency was treatment related the following criteria were applied:
The results were evaluated as positive when the mutant frequency in the test substance-treated group was significantly different from that in the concurrent negative control group.

Final judgment was made in consideration of biological relevance under the test conditions.

Statistical methods:
The data on the mutant frequency from the negative control group and the positive control group were tested by F test for homogeneity of variance (two-sided, significance level of 0.05) first. If homogeneity of variance was determined (not significant on F test), Student's t test (two-sided, significance level of 0.05) was performed to assess the statistical significance of differences between the negative control group and the positive control group. If there was no homogeneity (significant on F test), Aspin-Welch's t test (two-sided, significance level of 0.05) was performed to analyze the differences.

Findings

General observations:
No deaths occurred. There was no change in the general condition in any of the captan treated groups.
In the 6000 ppm (776 mg/kg bw/day) dose group body weights were significantly lower than in the negative control group from day 15 to 31, and body weight gain in this group during the administration period was significantly lower than that of the negative control group. There were no apparent decreases in body weights of the 600 and 2000 ppm dose groups.
In the 6000 ppm (776 mg.kg bw/day) dose group food consumption was significantly lower than that in the negative control group at weeks 1 and 3. There were no apparent decreases in food consumption of the 600 and 2000 ppm dose groups.

<u>TGR Mutant Frequency</u>:

<u>Liver</u>
In the negative control group, the mean ± SD of mutant frequency among the individuals was 45.7 ± 12.5 ($\times 10^{-6}$).
The mean ± SD of mutant frequencies in the Captan treated groups, 600, 2000 and 6000 ppm, were 39.7 ± 9.2 ($\times 10^{-6}$), 45.6±12.8 ($\times 10^{-6}$) and 58.2 ± 31.3 ($\times 10^{-6}$), respectively, and no statistically significant increase was observed compared with the negative control group. The mutant frequency of 1/5 animals (animal ID No. 1305) in the 6000 ppm group was high. However, the increase was judged to be incidental, because it was observed only in one mouse in one group and there was no dose-relationship.
In the positive control group, the mean ± SD of mutant frequency among the individuals was 112.0 ± 14.0 ($\times 10^{-6}$) and a statistically significant increase was observed compared with the negative control group.

<u>Duodenum</u>

In the negative control group, the mean ± SD of mutant frequency among the individuals was 83.9 ± 13.1 ($\times 10^{-6}$). The mean ± SD of mutant frequencies in the Captan treated groups, 600, 2000 and 6000 ppm, were 87.5 ± 10.8 ($\times 10^{-6}$), 95.5 ± 9.3 ($\times 10^{-6}$) and 104.3 ± 25.2 ($\times 10^{-6}$), respectively, and no statistically significant increase was observed compared with the concurrent negative control group.
In the positive control group, the mean ± SD of mutant frequency among the individuals was 812.0 ± 101.7 ($\times 10^{-6}$) and a statistically significant increase was observed compared with the negative control group.

Table 3.8.2.6-1 Summary of Transgenic Rodent Mutant Frequency Results

Substance	Dose (ppm)	Liver Group mean ± S.D ($\times 10^{-6}$)	Duodenum Group mean ± S.D ($\times 10^{-6}$)
CRF-1	0	45.7 ± 12.5	83.9 ± 13.1
Captan	600	39.7 ± 9.2	87.5 ± 10.8
	2000	45.6 ± 12.8	95.5 ± 9.3
	6000	58.2 ± 31.3	104.3 ± 15.2
ENU	100 mg/kg	112.0 ± 14.0* (S)	812.0 ± 101.7* (AW)

CRF-1: Negative Control
ENU: Positive control (N-ethyl-N-nitrosourea)
*: Significant difference from negative control ($p \leq 0.05$)
(AW): Aspin-Welch's t test; (S): Student's t test

Conclusions

In this transgenic rodent assay, the captan was dietary administered at doses of 0, 600, 2000, and 6000 ppm (equivalent to mean test substance intakes of 0, 85.3, 273, and 776 mg/kg bw/day, respectively) to transgenic mice for 28 consecutive days, and then after an additional 3 days for mutant manifestation following the last administration, the liver and duodenum were removed and mutant frequencies were determined.
There were no significant differences in the mutant frequencies in the liver and duodenum in any of the groups treated with captan as compared to the negative control group.

The mutant frequencies in the liver and duodenum in the positive control group, which was treated with N-ethyl-N-nitrosourea (ENU, dosage level of 100 mg/kg bw/d, i.p. for 2 days), were significantly increased compared with those of the negative control group.

It was, therefore, concluded that captan did not induce gene mutations in the liver or duodenum of transgenic mice under the conditions in this study.

3.8.2.7 Chidiac, P. and Goldberg, M.T. (1986)

Reference:	Lack of inductions of nuclear aberrations by captan in mouse duodenum
Author(s), year:	Chidiac, P. and Goldberg, M.T. (1986)
Report/Doc. number:	Zeneca Ag Products, USA, report published in 'Environmental mutagenesis' (1987), 9, pp: 297-306 (Company file: R-6346/TMN-0833)
Guideline(s):	In-house method
GLP:	No
Deviations:	Not applicable
Acceptability:	Limited

Material and Methods

The genotoxicity of captan was studied *in vivo* in the proximal small intestine of the mouse, the site of its oncogenicity, using the small intestine nuclear aberration assay. Captan was administered in a variety of ways:

1. Technical captan (purity 94%) was administered to male C57B1/6J mice in the diet at levels of 0, 8000 and 16000 ppm of the diet for seven days. Animals wer.e sacrificed on the eighth day of the experiment.

2. Technical grade captan (purity 94%) suspended in a medium containing 1% gum tragacanth and 0.05% Tween 40, was administered by oral gavage to fasted male CD1 mice on five consecutive days. Cumulative doses were up to 5000 mg/kg bw. Animals were sacrificed 4 hours after the final treatment.
3. Technical grade captan (purity 92.4%), analytical grade captan (purity 99%), and a formulation containing 50% captan, suspended in a medium containing 1% gum tragacanth and 0.05% Tween 40, were administered by oral gavage to fasted CD1 male mice at doses equivalent to 0, 200 and 2000 mg/kg bw. Animals were sacrificed 24 hours after treatment.
4. Captan, prepared in suspension as described above was administered by oral gavage followed immediately by the intraperitoneal administration of 1,2-dimethyl-hydrazine (DMH) dissolved in 1 mM EDTA (pH adjusted to 6.5 with 1N NaOH). Animals were sacrificed 24 hours after treatment.
5. Technical grade captan (94%) at doses of up to 4000 mg/kg bw, was administered by oral gavage to mice four hours after pre-treatment with 1300 mg/kg bw L-buthionine-S,R-sulphoximine (BSO) or vehicle (distilled water). Five animals from each of the pretreatment groups were sacrificed at the time of captan treatment for the determination of duodenal glutathione concentration. Remaining animals were sacrificed 24 hours after treatment with captan.

Further groups of fasted mice were administered bis-(trichloromethyl)disulphide dissolved in medium chain triglyceride oil by oral gavage at doses up to 100 mg/kg bw and sacrificed 24 hours after treatment. Tetrahydrophthalimide (THPI) suspended in 1% gum tragacanth and 0.05% Tween 20 was administered by oral gavage to fasted CD1 mice at doses up to 1500 mg/kg bw.

Following sacrifice of the animals, slides were prepared from the duodenum and sections were examined for the presence of aberrant nuclei (apoptic bodies and micronuclei) in crypts. Ten crypts were scored per section, starting as near as possible to the pylorus and moving distally.

Findings

Neither feeding dietary concentrations of captan at concentrations up to 16000 ppm captan for 7 days (Table 3.8.2.7-1), the administration of captan for five consecutive days by oral gavage at doses of up to 1000 mg/kg bw/day (**Fehler! Verweisquelle konnte nicht gefunden werden.**), nor the administration of the three grades of captan (technical, analytical and formulation) at doses equivalent to 200 and 2,000 mg/kg bw (**Fehler! Verweisquelle konnte nicht gefunden werden.**) resulted in an increase in the incidence of nuclear aberrations in crypts of the small intestine compared to the controls.

Table 3.8.2.7-1 The incidence of nuclear aberrations: one week dietary captan (94%)

Dose (ppm)	Number of animals	NA/crypt[1]
0	5	0.04 ± 0.05
8000	6	0
16000	5	0.04 ± 0.09

[1] NA = nuclear aberration (mean ± sd).

Table 3.8.2.7-2 The incidence of nuclear aberrations: consecutive oral administration of captan (94%) over five days

Dose (mg/kg bw/day)	Mortality[1]	N	NA/crypt[2]
0	0/10	10	0.05 ± 0.08
20	2/10	8	0.10 ± 0.11
200	3/10	7	0.06 ± 0.08
1000	6/10	4	0.05 ± 0.06

[1] Number dead/number treated.
[2] NA = nuclear aberration (mean ± sd).

Table 3.8.2.7-3 The incidence of nuclear aberrations: single oral administration of different grades of captan

Dose (mg/kg bw)	Number of animals	NA/crypt[1]
Control	5	0.04 ± 0.05
Analytical grade (99%)		
200	6	0.07 ± 0.08
2000	6	0.10 ± 0.09
Technical grade (92.4%)		
200	6	0.08 ± 0.08
2000	6	0.07 ± 0.08
Formulation (50%)		
200	5	0.12 ± 0.08
2000	6	0.02 ± 0.04

[1] NA = nuclear aberration (mean ± sd).

The administration of DMH alone and with captan resulted in dose-related increases in nuclear aberrations (Table 3.8.2.7-4). However, the presence of captan did not have a significant effect on the incidence of nuclear aberrations in animals treated with DMH.

Glutathione levels in the duodenal epithelium, measured four hours after the administration of BSO were reduced by 55 ± 11% ($p < 0.01$). However, no differences in the incidence of nuclear aberrations were detected between animals pre-treated with BSO and the vehicle control at any dose level of captan (Table 3.8.2.7-5).

Table 3.8.2.7-4 The incidence of nuclear aberrations: administration of DMH with and without captan (94%).

Dose (mg/kg bw)	Number of animals	NA/crypt[1]
without captan		
0	5	0.12 ± 0.11
10	5	1.12 ± 0.50*
20	5	2.18 ± 0.50*
with captan (400 mg/kg bw)		
0	4	0.03 ± 0.05
10	4	1.30 ± 0.18*
20	5	1.86 ± 0.39*

[1] NA = nuclear aberration (mean ± sd).
* Significantly different to the control ($p < 0.01$).

Table 3.8.2.7-5 The incidence of nuclear aberrations: administration of captan with and without glutathione depletion

Dose (mg/kg bw)	N	NA/crypt[1]
pre-treatment with vehicle only		
0	5	0.06 ± 0.09
50	5	0.02 ± 0.04
100	5	0.02 ± 0.04
200	5	0.16 ± 0.15
400	3	0
2000	5	0.16 ± 0.18
4000	4	0.08 ± 0.10
pre-treatment with BSO (1300 mg/kg bw)		
0	5	0.06 ± 0.09
50	4	0.13 ± 0.10
100	5	0.06 ± 0.09
200	4	0.03 ± 0.05
400	5	0
2000	6	0.23 ± 0.43
4000	4	0.05 ± 0.10

[1] NA = nuclear aberration (mean ± sd).

THPI did not result in an increase in the incidence of nuclear aberrations in duodenal crypts (Table 3.9.3.7-6).

Table 3.8.2.7-6 The incidence of nuclear aberrations: single oral administration of THPI

Dose (mg/kg bw)	N	NA/crypt[1]
0	5	0.04 ± 0.09
250	5	0.14 ± 0.11
500	5	0.14 ± 0.11
750	5	0.08 ± 0.08
1000	5	0.10 ± 0.17
1500	4	0.15 ± 0.19

[1] NA = nuclear aberration (mean ± sd).

The administration of bis-(trichloromethyl)disulphide at 25 mg/kg bw resulted in a significant difference in the incidence of nuclear aberrations in crypts compared to the control ($p < 0.01$) (**Fehler! Verweisquelle konnte nicht gefunden werden.**). However, there was no dose-response relationship observed with known small intestine carcinogens, indicating that bis-(trichloromethyl)disulphide was negative in this assay. Higher doses of THPI than those reported in the current study have been found to result in mortalities (no figures reported in study report).

Table 3.8.2.7-7 The incidence of nuclear aberrations: single oral administration of bis-(trichloromethyl)disulphide

Dose (mg/kg bw)	N	NA/crypt[1]
0	6	0.03 ± 0.03
25	6	0.13 ± 0.08*
50	6	0.05 ± 0.04
100	6	0.06 ± 0.04

[1] NA = nuclear aberration (mean ± sd).
* Significantly different to the control ($p < 0.01$).

Conclusions

Captan administered in the diet or by oral gavage did not produce nuclear aberrations in duodenal crypts under the conditions of the small intestine nuclear aberration assay, with or without glutathione depletion. The captan impurities bis-(trichloromethyl)disulphide and 1,2,3,6-tetrahydrophthalimide were also negative in this assay. The potent intestinal

carcinogen 1,2-dimethyl-hydrazine significantly increased the incidence of nuclear aberrations. Captan did not affect the genotoxicity of 1,2-dimethyl-hydrazine.

3.8.2.8 Study 5 (1990)

Reference:	Captan: Assessment for the induction of unscheduled DNA synthesis in rat hepatocytes in vivo
Author(s), year:	Anonymous (1990)
Report/Doc. number:	ICI Central Toxicology Laboratories, unpublished report No. CTL/P/2995, (Company file: TMN-0835)
Guideline(s):	In-house method
GLP:	Yes
Deviations from OECD 486 (1997):	The study met the essential criteria of the test guideline.
Acceptability:	Yes

Material and Methods

An assay was carried out to evaluate the ability of captan to induce DNA repair *in vivo*. Male Alderley Park (Alpk:APfSD) rats were administered a single oral dose of captan (purity 91.2%, batch number WRC 11240-37-1) in corn oil by oral gavage at dose levels of 500, 1000 and 2000 mg/kg bw (total 5 animals/dose/time point in two independent experiments). In the absence of toxicity, a limit dose of 2000 mg/kg bw was used. Animals were sacrificed 4 and 12 hours after treatment. Negative control animals received vehicle only (sacrificed at 4 and 12 hours) and positive control animals were treated with 6-p-dimethylaminophenylazo-benzthiazole (6BT) in corn oil or N-nitrosodimethylamine (NDMA) in deionised water and were sacrificed at 12 hours (6BT) and 4 hours (NDMA) after treatment (two animals/dose/time point in two independent experiments). The dosing volume was 10 mL/kg bw. Following sacrifice, hepatocytes were extracted and cultures of hepatocytes incubated with [^{3}H]-thymidine for 4 hours at 37°C after which the cells were washed three times and incubated overnight with medium containing an excess of unlabelled thymidine. Slides were prepared (three per animal), processed and examined microscopically. Only the two highest dose levels not showing undue cytotoxicity were selected to be examined for UDS. The number of nuclear grains, the number of cytoplasmic grains and the net nuclear grain count (= nuclear grain count minus cytoplasmic grain count) was determined for at least 25 (normally 50) cells per slide. Two slides (or a total of 100 cells) per animal were scored.

Findings

Cytotoxicity: No apparent signs of excessive cytotoxicity were observed. Therefore the two highest concentrations tested were selected for assessment of unscheduled DNA synthesis (UDS).

Unscheduled DNA synthesis: A summary of the findings is given in Table 3.8.2.8-1. Captan did not cause a significant increase in mean net nuclear grain count or the percentage of cells in repair compared to the control. Mean net nuclear grain counts of hepatocytes from captan treated animals were less than zero. The sensitivity of the system was confirmed by the appropriate responses of 6BT and NMDA showing induction of UDS.

Table 3.8.2.8-1 Unscheduled DNA synthesis in rat hepatocytes: summary of findings

Dose (mg/kg bw)	Number of animals	Nuclear grain count[1]	Cytoplasmic grain count[1]	Net nuclear grain count[1]	Mean %cells in repair[2]
4 hours post-treatment					
0	2	8.6 ± 0.5	12.8 ± 0.4	-4.2 ± 0.9	3
1000	5	9.3 ± 2.0	14.8 ± 3.8	-5.5 ± 2.0	1
2000	5	8.7 ± 1.4	14.0 ± 2.2	-5.2 ± 1.2	2
NMDA (10)	2	37.3 ± 0.7	9.7 ± 1.2	+27.6 ± 0.5	93
12 hours post-treatment					
0	2	5.2 ± 1.5	7.9 ± 0.4	-2.7 ± 1.1	2
1000	5	5.1 ± 0.6	7.8 ± 0.5	-2.8 ± 0.6	1
2000	5	5.3 ± 1.2	8.7 ± 1.6	-3.4 ± 0.8	0
6BT (40)	2	25.0 ± 6.0	9.1 ± 0.4	+15.9 ± 5.6	91

[1] Mean ± SD
[2] A cell in repair = net nuclear grain count ≥ 5.

Conclusions

Captan did not induce unscheduled DNA synthesis in rats following a single oral dose of up to 2000 mg/kg bw in either of two independent experiments and it can therefore be considered to be negative in this assay.

3.8.2.9 Study 6 (1977)

Reference:	Dominant lethal study with captan technical in albino mice exposed for 8 weeks to the chemical in the diet
Author(s), year:	Anonymous (1977)
Report/Doc. number:	Industrial Bio-test Laboratories, Inc., unpublished report No. IBT 623-05998 (Company file: TMN-0850)
Guideline(s):	In-house method
GLP:	No
Deviations from OECD 478 (2016):	Unclear whether the negative control and the positive control would meet the current acceptability criteria; according to OECD 478, the Dominant Lethal factor is estimated as: (post-implantation deaths/total implantations per female) x 100; in contrast, only early resorptions were considered for mutagenicity in this study, whereas the frequency of late deaths was considered "non-genetic" by the study author; unclear whether statistical significance was analysed for mutagenicity data in this study
Acceptability:	Not reliable

Material and Methods

A dominant lethal mutagenicity study was carried out with captan (purity 89.8%, batch no.: SX-648) in Charles River strain albino mice. Male mice were administered the test substance in the diet for a period of eight weeks at concentrations of 0, 500, 3000 and 7000 ppm. A group of positive control animals was administered methyl methanesulphonate in diet at a concentration of 1000 ppm for 8 weeks. A total of 15 males per group were treated. The majority of animals in each group were proven sires (11 in the control group, 13 in each of the captan treated groups and 12 in the positive control group). Parameters measured during treatment were clinical signs and mortality (daily) and body weight (weekly). At the end of the treatment period each male was housed with three untreated virgin females for one week, after which females were removed and replaced by three new virgin females. The procedure was continued for eight consecutive weeks. Females were sacrificed 14 days after the initiation of cohabitation and autopsied. Fetal swellings, implantation sites and resorption sites (early and late differentiated) were recorded. Upon completion of the mating trials all males were sacrificed. Gross pathological examination of the testes was carried out.

The mutation rate was calculated as "(number of early resorption sites)/(number of implantation sites) x 100".

Findings

General observations: Males fed 7000 ppm captan exhibited weight loss during the third week of pre-mating period and the total weight gain in 7000 ppm males was less than controls. Body weights and body weight gains were obtained for the 500 and 3000 ppm males were comparable to controls. Food consumption was not altered by the ingestion of captan in any treatment group. No test substance-related mortalities were observed.

Reproductive and mutagenicity data: No alterations were noted in the ability of males to sire litters. No consistent reductions in male fertility were noted which could be correlated with captan exposure. The number of implantation sites was not altered by captan.

The number of resorption sites (early and late) and viable embryos were not statistically significantly altered by captan. However, an increase in the number of early resorptions was noted in the high dose group during week 8 (not considered test substance-related by study author). Positive control animals exhibited an increase in the number of early resorptions during the first two weeks of the mating period, which was statistically significant in week 2.

At the time of study conduction, only early resporptions were considered for the calculation of the mutation rate. According to the latest version of the relevant guideline OECD 478 (2016) the sum of late and early resorptions is considered and the Dominant Lethal factor (DLF) is estimated as "(post-implantation deaths/total implantations per female) x 100". Therefore, the DLF was calculated in accordance to the current guideline and included in the following table (last column).

Gross pathology: No treatment-related gross effects on testes were observed.

Table 3.8.2.9-1 Dominant lethal test in albino mice: summary of reproductive and mutagenicity data

Group	Week no.	Pregnangt females examined	Total implantation sites	Early reporptions	Late reporptions	Mutation rate (early resorpions) %	DLF (early + late)[2] %
Negative control	1	32	396	14	6	3.5	5.1
	2	30	336	19	4	5.7	6.8
	3	31	385	17	7	4.4	6.2
	4	29	357	19	5	5.3	6.7
	5	30	351	18	0	5.1	5.1
	6	22	233	12	2	5.2	6.0
	7	20	198	11	0	5.6	5.6
	8	29	401	22	5	5.5	6.7
Captan 500 ppm	1	26	316	13	0	4.1	4.1
	2	29	364	15	6	4.1	5.8
	3	30	400	22	6	5.5	7.0
	4	32	400	23	3	5.8	6.5
	5	28	329	14	3	4.3	5.2
	6	20	221	19	1	8.6	9.0
	7	14	151	7	3	4.6	6.6
	8	29	344	20	4	5.8	7.0
Captan 3000 ppm	1	35	423	17	7	4.0	5.7
	2	36	450	34	2	7.6	8.0
	3	37	474	23	9	4.9	6.8
	4	37	444	14	1	3.2	3.4
	5	26	307	12	1	3.9	4.2
	6	25	276	19	1	6.9	7.2
	7	22	245	11	1	4.5	4.9
	8	28	360	29	4	8.1	9.2
Captan 7000ppm	1	34	414	14	2	3.4	3.9
	2	38	459	27	5	5.9	7.0
	3	33	420	32	8	7.6	9.5
	4	36	443	20	4	4.5	5.4
	5	28	333	8	2	2.4	3.0
	6	21	260	29[1]	1	11.2	11.5
	7	13	133	3	1	2.3	3.0
	8	30	399	40	2	10.0	10.5
Positive control (methyl methane-sulfonate 1000 ppm)	1	33	383	46	1	12.0	12.3
	2	31	341	56**	1	16.4	16.7
	3	34	402	32	5	8.0	9.2
	4	35	438	34	2	7.8	8.2
	5	27	314	13	2	4.1	4.8
	6	12	133	6	0	4.5	4.5
	7	22	237	10	1	4.2	4.6
	8	32	392	32	1	8.2	8.4

** Statistically significant difference noted at the 99% confidence level utilizing a Chi-Square test of independence.
[1] One female accounted for 16 resorption sites.
[2] According to the current test guideline OECD 478 (2016).

Conclusions

According to the study author, there were no differences in the percentage of early deaths between treated and control groups, and there were no differences between the number of embryos per female in treated group and the number of embryos per female in the control that could be correlated to treatment with captan. Under the conditions of the test, captan was considered to be negative in the dominant lethal assay in the mouse.

At the time of study conduction, only early resporptions were considered for the calculation of the mutation rate, whereas the frequency of late deaths was considered "non-genetic" by the study author. According to the latest version of the relevant guideline OECD 478 (2016) the complete post-implantation loss has to be considered (early and late) and the Dominant Lethal factor (DLF) is estimated as "(post-implantation deaths/total implantations per female) x 100". Therefore, the DLF was calculated in accordance to the current guideline. As shown in the table above (last column), this does, however, not substantially change the conclusion.

Furthermore, it is unclear whether the negative control and the positive control would meet the current acceptability criteria, and whether statistical significance was analysed for mutagenicity data in this study. Overall, the study is considered inconclusive.

Note from original DAR: the results of the above study provided by the applicant are not taken into account in the evaluation of captan genotoxicity, as the work was performed at the Industrial BIO-TEST Laboratories Inc., later inquired for unfaithful reporting of experimental data.

3.8.2.10 Tezuka et al. (1978)

Reference:	Cytogenetic and dominant lethal studies on captan
Author(s), year:	Tezuka, H. et al. (1978)
Report/Doc. number:	Institute of Environmental Toxicology, Tokyo, Japan, report published in 'Mutation Research' (1978) 57, pp 201 – 207 (Company file: R-1666/TMN-0824)
Guideline(s):	In-house method
GLP:	No
Deviations from OECD 478 (2016):	Poor reporting, information missing (e.g. whether administration via gavage or feed, body weights, food consumption and clinical sings not measured or not reported, no rationale for selected doses, etc.); at least three treated dose groups should be analysed (instead of two) in order to provide sufficient data for dose response analysis; insufficient number of pregnant females (should provide at least 400 implants); for a single treatment up to five daily doses, the mating period should be 8 weeks for mice (instead of 6 weeks); females should remain with the males for at least the duration of one oestrus cycle/1 week (instead of 2-4 days); statistical method not stated
Acceptability:	Limited

Material and Methods

The genotoxic activity of captan (two suppliers: Wako Pure Chemical Industries, Japan (purity: >98%), and Nishio Industry Co., Japan (purity: 99.9%); batch number not specified) was investigated *in vivo* in the mouse dominant lethal assay. Groups of 15 male C3H mice were treated orally with captan at a daily dose of 0, 200 or 600 mg/kg bw for five successive days. Untreated mice received the vehicle alone (5% water solution of Arabic gum). The positive control group was treated with a single i.p. dose of 300 mg/kg bw ethylmethanesulfonate (EMS). Immediately after the treatment, each male was mated with one untreated SLC-ICR (random bread) virgin female. Replacement of females was made at intervals of 2 to 4 days and continued for 6 succesive weeks. Females were checked for vaginal plugs every morning and mated females were sacrificed 12-13 days after copulation for examination of uterine contents. The numbers of corpora lutea, live embryos, and early or late embryonic deaths were scored and recorded every week.

The frequency of dead implants (%) was calculated.
Furthermore, the induced dominant lethal (DL) mutations were calculated as follows:
Frequency of DL mutations (%) = (1 – live embryos per test female/live embryos per control female) x 100

Findings

In the mouse dominant lethal study, daily doses of 200 or 600 mg/kg bw of captan did not affect fertility of the male mice. The mean number of corpora lutea, implants, and live embryos were within the normal range for both groups treated with captan. No dose-dependent increases in the frequency of induced lethal mutations and of litters with early deaths were observed. The positive control substance EMS elicited a distinct effect on the number of liver embryos per female, on the frequency of dead implants and on the frequency of induced dominant lethal mutations in the first two weeks of the mating period.

Table 3.8.2.10-1 Dominant lethal mutation test with captan in mice

Week	Chemical	Dose (mg/kg/day)	Number of females with implants	Number of corpora lutea per pregnancy[a]	Number of implants per pregnancy[a]	Number of live embryos per pregnancy [a]	Dead implants (%)	Dominant lethal muta-tions (%)
1	Captan	0	24	13.7 ± 1.3	12.8 ±1.4	12.1 ± 1.4	5.5	
		200	24	14.1 ± 1.4	11.9 ± 9.6	10.9 ± 3.7	8.4	3.1
		600	26	13.6 ± 2.2	11.1 ± 3 [b]	10.5 ± 3.5 [b]	5.5	0
	EMS	300	24	14.6 ± 1.5	12.7 ± 2.4	9.1± 4.4[c]	28.0 [d]	23.8
2	Captan	0	26	14.5 ± 2.1	12.2 ± 3.1	11.4 ± 2.9	6.6	
		200	25	14.2 ± 1.5	12.6 ± 3.2	12.0 ± 3.1	4.4	-2.4
		600	28	14.8 t 1.5	13.1 ± 2.6	12.2 ± 2.6	7.1	0.5
	EMS	300	10	13.7 2 2.2	8.5 ± 4.0 [c]	3.5 ± 3.8 [d]	58.8 [d]	55.9
3	Captan	0	21	13.6 ± 1.6	10.3 ± 6.2	9.5 ± 4.8	7.8	
		200	22	14.6 ± 1.9	13.1 ± 3.1 [b]	11.7 ± 3.0	10.4	2.8
		600	24	14.4 ± 1.8	12.7 ± 2.9	11.8 ± 3.1	6.9	-1.0
	EMS	300	22	14.5 ± 2.9	11.8 ± 2.4	10.7 ± 2.6	9.6	2.0
4	Captan	0	23	14.8 ± 1.5	11.4± 3.7	10.5 ± 3.7	8.0	
		200	27	14.8 ± 2.2	12.1 ± 3.3	11.4 ± 3.3	5.8	-2.4
		600	25	14.4 ± 2.4	11.9 ± 9.5	11.4 ± 3.6	4.0	-4.3
	EMS	300	22	14.0 ± 2.4	11.6 ± 3.3	10.9 ± 3.3	6.3	-2.0
5	Captan	0	22	14.9 ± 2.4	12.2 ± 2.9	11.6 ± 2.8	6.3	
		200	25	13.9 ± 1.5	10.2 ± 4.1	9.4 ± 4.1	8.6	2.5
		600	25	13.7 ± 1.6 [b]	10.1 ± 4.3	9.4 ± 4.7	7.5	1.3
	EMS	300	21	14.0 ± 1.4	10.2 ± 3.8	9.5 ± 4.0	7.4	1.2
6	Captan	0	22	14.1 ± 1.9	10.8 ± 3.8	9.9 ± 3.6	8.4	
		200	25	14.2 ± 2.2	11.3 ± 3.0	10.7 ± 2.9	5.3	-3.4
		600	25	13.9 ± 1.7	11.4 ± 2.9	10.8 ± 9.1	4.9	-3.8
	EMS	300	17	14.4 ± 1.4	10.9 ± 3.5	10.5 ± 3.6	3.8	-5.0

a Values given as mean ± S.D.
b,c and d Significantly different from control at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Calculation of the dominant lethal factor (according to the current OECD test guideline 478) determined as
"(post-implantation deaths/total implantations per female) × 100"
is not possible since only processed mean values are reported in the publication.

Conclusions

Under the conditions of this study, the dominant lethal study did not show any mutation induction after treatment of male mice with daily oral doses of 200 or 600 mg/kg bw of captan for five days.

3.8.2.11 Collins, T. F. X. (1972)

Reference:	Dominant lethal assay. I. Captan
Author(s), year:	Collins, T. F. X. (1972)
Report/Doc. number:	Food and Cosmetics Toxicology, Vol. 10 (1972), 353 - 361
Guideline(s):	In-house method (pre-guideline study)
GLP:	No
Deviations from OECD 478 (2016):	Poor reporting, information missing (e.g. body weights, food consumption and clinical sings, no rationale for selected doses, etc.); only early fetal deaths were reported instead of complete post-implantation deaths (early + late)
Acceptability:	Limited

Material and Methods

In a study carried out at the U.S. FDA, technical-grade captan (provided by Chevron Chemical Co., Richmond, Cal.; purity not stated) was suspended in carboxymethyl-cellulose (CMC) and given for five days either i.p. (2.5, 5 and 10 mg/kg bw/day) or by oral intubations (50, 100, and 200 mg/kg bw/day) to groups of 15 male rats and 15 male mice. Triethylenemelamine (TEM) was used as positive control (i.p. at a dose of 0.05 mg/kg bw/day or orally at 0.2 mg/kg bw/day for 5 days). The treated male rats and mice were mated to untreated virgin females for 10 and 12 weeks, respectively. Treated male rats were mated with one female per week, mice with two females per week. Uterine content was examined at day 13 (rats) or 12 (mice) of pregnancy for total implantations, and early and late foetal deaths.

Statistical analysis of results: For the average number of early deaths, the data were transformed by using the Freeman-Tukey arc-sine transformation for binomial proportions. A t test between the control and dose levels was performed on the transformed data. Early deaths were divided into categories of one or more and of two or more early deaths. A hypergeometric distribution was used to test for significant differences between the control and various levels. In addition, a test of linearity of proportions was used to determine whether any significant linear trends, indicative of a dose-relationship, existed.

Findings

No acute toxicity and no treatment-related mortality was observed after captan administration. No captan-related antifertility effects or decrease of total implants per pregnant female were observed.
After oral administration of captan, a statistically significant increase in the mean number of early deaths was observed at the highest dose at week 1, and at the two highest doses at week 2 in mice. In rats, a statistically significant increase in the mean number of early deaths was observed at the highest dose in week 1, 2 and 5, and at the mid dose in week 4. A summary of these results after oral administration of captan to mice and rats is shown in the following tables.
Although not statistically significant, an increase in the mean number of early foetal deaths per pregnancy was seen in rats during the first 7 weeks after i.p. administration of captan at the highest dose level. In mice there was also an increase in the mean number of early foetal deaths per pregnancy was seen in rats during the first 7 weeks after i.p. administration of captan, and the increase was significant at weeks 4 and 5.

Table B.3.8.2.11-1 Dominant lethal mutation test with captan in mice: pregnancy rate, total implantations and early foetal deaths after 5 days treatment with captan by i.p. injection or oral intubation

Parameter	Test compound	Dose level (mg/kg/d)	Values at wk									
			1	2	3	4	5	6	7	8	9	10
			Ip administration									
Incidence of pregnancy (%)	Captan	0	80.0	70.0	70.0	73.3	70.0	66.6	63.3	66.6	60.0	60.0
		2.5	80.0	70.0	73.3	73.3	73.3	64.3	71.4	75.0	75.0	64.3
		5.0	63.3	70.0	73.3	60.0	60.0	60.0	66.6	60.0	70.0	60.0
		10.0	73.3	66.7	73.3	60.0	60.0	66.6	70.0	73.3	66.6	56.7
	TEM	0.05	66.6	56.7	40.0	36.7	53.3	53.3	63.3	56.7	63.3	60.0
Mean total implantations/pregnancy	Captan	0	8.4	8.9	9.2	8.7	9.4	8.6	8.9	8.8	9.2	9.5
		2.5	8.5	9.1	9.0	9.1	9.1	9.4	9.4	9.8	9.0	9.4
		5.0	8.6	9.2	9.6	8.8	8.7	8.5	9.2	8.9	8.8	8.8
		10.0	7.9	9.0	9.1	8.8	7.9	9.2	8.9	8.7	9.1	9.3
	TEM	0.05	8.6	6.2	6.8	5.0	5.7	8.6	8.7	7.9	8.6	8.9
Mean early deaths/pregnancy	Captan	0	0.33	0.38	0.43	0.41	0.33	0.30	0.37	0.25	0.50	0.39
		2.5	0.33	0.19	0.32	0.32	0.36	0.33	0.20	0.33	0.33	0.44
		5.0	0.42	0.38	0.45	0.56	0.50	0.56	0.60	0.33	0.29	0.28
		10.0	0.64	0.75	0.68	**0.89***	**0.78***	0.65	0.67	0.48	0.40	0.41
	TEM	0.05	4.30[1]*	3.24**	4.83**	1.64[1]*	1.57**	1.12*	0.68	0.65	0.47	0.50
			Oral intubation									
Incidence of pregnancy (%)	Captan	0	66.0	63.3	63.3	70.0	66.6	66.6	70.0	66.6	66.6	73.3
		50	73.3	66.7	63.3	70.0	70.0	73.3	70.0	66.6	66.6	70.0
		100	63.3	70.0	73.3	63.3	76.7	66.6	66.6	70.0	63.3	70.0
		200	63.3	63.3	53.3	50.0	70.0	66.6	66.6	73.3	73.3	63.3
	TEM	0.2	50.0	26.7	23.3	43.3	56.7	60.0	70.0	60.0	60.0	70.0
Mean total implantations/pregnancy	Captan	0	9.5	8.4	8.3	9.1	8.8	8.7	9.1	8.5	8.5	8.8
		50	8.7	8.8	8.5	8.3	8.2	8.0	7.8	8.8	9.2	9.0
		100	9.0	8.0	8.5	9.3	8.7	9.1	8.7	8.8	8.8	9.2
		200	8.9	8.0	8.7	9.1	8.3	8.2	8.7	8.7	8.1	8.7
	TEM	0.2	7.1	6.9	5.9	6.7	6.8	6.6	8.3	8.1	7.6	8.7
Mean early deaths/pregnancy	Captan	0	0.40	0.37	0.37	0.33	0.40	0.45	0.38	0.40	0.45	0.45
		50	0.50	0.45	0.37	0.29	0.43	0.45	0.38	0.35	0.45	0.38
		100	0.63	**0.76****	0.55	0.53	0.52	0.30	0.45	0.33	0.53	0.57
		200	**0.95***	**0.84****	0.86	0.87	0.62	0.45	0.55	0.27	0.55	0.53
	TEM	0.2	4.13**	4.75**	3.86**	3.23**	1.76**	1.72**	0.76	0.94**	0.89	1.10

* $p < 0.01$, test?
** $p < 0.001$, test?

Table B. 3.8.2.11-2 Dominant lethal mutation test with captan in rats: pregnancy rate, total implantations and early foetal deaths after 5 days treatment with captan by i.p. injection or oral intubation

Parameter	Test compound	Dose level (mg/kg/d)	Values at wk 1	2	3	4	5	6	7	8	9	10
			Ip administration									
Incidence of pregnancy (%)	Captan	0	93.3	100	93.3	93.3	93.3	93.3	93.3	86.7	80.0	86.7
		2.5	100	100	100	93.3	93.3	86.7	100	86.7	86.7	80.0
		5.0	86.7	100	93.3	93.3	93.3	86.7	86.7	93.3	86.7	80.0
		10.0	93.3	100	86.7	93.3	86.7	86.7	86.7	86.7	73.3	80.0
	TEM	0.05	73.3	86.7	86.7	80.0	86.7	93.3	93.3	80.0	86.7	86.7
Mean total implantations/pregnancy	Captan	0	10.9	11.0	10.5	10.7	10.8	11.4	10.9	11.6	11.3	11.0
		2.5	101	10.8	10.7	10.9	11.1	11.2	10.9	10.7	10.0	10.8
		5.0	10.7	10.4	10.3	10.4	9.6	12.0	11.5	11.1	11.5	11.9
		10.0	10.5	11.5	10.9	11.0	10.4	11.7	11.0	11.5	11.5	11.1
	TEM	0.05	7.8	5.5	4.5	4.5	8.1	10.8	11.3	10.8	10.6	121
Mean early deaths/pregnancy	Captan	0	0.36	0.47	0.50	0.43	0.36	0.36	0.38	0.46	0.58	0.54
		2.5	0.40	0.33	0.33	0.43	0.43	0.54	0.47	0.31	0.31	0.50
		5.0	0.38	0.27	0.50	0.43	0.50	0.38	0.62	0.64	0.38	0.50
		10.0	0.71	0.87	0.69	0.64	0.77	0.85	0.62	0.36	0.45	0.58
	TEM	0.05	6.18**	5.25**	4.38**	4.25**	4.23**	1.79**	1.43*	0.58	1.00	0.77
			Oral intubation									
Incidence of pregnancy (%)	Captan	0	93.8	87.5	100	93.8	100	87.5	100	93.8	87.5	100
		50	93.8	87.5	87.5	81.3	87.5	81.3	93.8	87.5	87.5	100
		100	87.5	93.8	93.8	86.7	93.8	87.5	100	93.8	93.8	81.3
		200	94.1	82.4	88.2	88.2	88.2	88.2	70.6	82.4	88.2	81.3
	TEM	0.2	81.3	81.3	87.5	87.5	93.8	87.5	81.3	75.0	81.3	87.5
Mean total implantations/pregnancy	Captan	0	11.5	12.1	12.9	13.0	12.2	12.7	12.5	11.9	12.4	11.7
		50	12.3	12.3	13.1	12.6	13.1	13.0	12.1	12.7	12.3	12.7
		100	11.6	12.7	13.7	13.1	12.3	12.9	13.0	12.8	12.7	11.6
		200	11.4	11.9	12.9	12.9	12.7	12.9	12.3	13.3	12.4	12.2
	TEM	0.2	9.9	8.7	9.4	8.1	9.8	10.4	12.6	12.2	12.5	13.1
Mean early deaths/pregnancy	Captan	0	0.47	0.43	0.50	0.47	0.44	0.57	0.38	0.40	0.57	0.31
		50	0.80	0.64	0.86	0.85	0.79	0.92	0.67	0.79	0.64	0.80
		100	1.14	1.13	1.07	**1.00***	0.80	0.79	0.69	0.93	0.53	0.62
		200	**1.38***	**1.21***	1.13	1.07	1.00*	0.80	0.75	0.79	0.73	0.71
	TEM	0.2	5.69**	7.31**	8.36**	6.21**	4.87**	2.79**	1.46*	0.92	1.08	0.57

* $p < 0.01$, test?
** $p < 0.001$, test?

Conclusions

Under the conditions of this dominant lethal study, the increase in mean early deaths observed after administration of captan to rats and mice suggests that captan may exhibit mutagenic properties when orally administered in repeated doses for five days.

In a statement submitted by the notifier (Anonymous, 2017; report number 562961-CA-050403-1), the following issues were mentioned with regard to the reliability/acceptability of the study:

- Purity of the test material is not given in the published report.
- Individual data are not accessible for re-analysis only processed data.
- Biological plausibility of the findings is questionable since apparently positive effects were reported upon oral dosing but not upon intraperitoneal administration.
- Experience of the laboratory with the dominant lethal assay appears to be limited; neither before nor following publication of this paper, another dominant lethal assay from this laboratory was published (in parallel a paper on the DLA with folpet and difolatan was published which gave almost identical equivocal results).
- Statistical analysis of results. For the average number of early deaths, the data were transformed using the Freeman-Tukey arc-sine transformation for binomial proportions.
  A t test between the control and dose levels was then performed on the transformed data. Early deaths were divided into categories of one or more and of two or more early deaths.
  A hypergeometric distribution was used to test for significant differences between the control and various levels. In addition, a test of linearity of proportions was used to determine whether any significant linear trends, indicative of a dose-relationship, existed.

- Comment: The Type I-error rate was not controlled for the multiple comparisons against the control. Further, the family wise error rate was not controlled for tests of multiple weeks. Therefore, it is highly likely to have multiple statistically significant results just by chance, due to the amount of statistical comparisons performed. Further it is interesting that stratification for "Litter with two or more early death %", with a lot of "Zero"-observations, resulted in more statistical significant effects than the stratification for "Litters with one or more early deaths %". This suggests that the applied model "t test" does not fit the data appropriately, which is to be expected for a mixedmodel design. Together, the statistical analysis is not appropriate for the experimental set-up and results with apparent statistically significance have to be assessed with scrutiny.

3.8.2.12 Epstein et al. (1972)

Reference:	Detection of chemical mutagens by the dominant lethal assay in the mouse
Author(s), year:	Epstein, S., Arnold, E., Andrea, J., Bass, W., Bishop, Y. (1972)
Report/Doc. number:	Toxicology and Applied Pharmacology. 23. 288 – 325 (1972)
Guideline(s):	In-house method
GLP:	No
Deviations from OECD 478 (2016):	No result data was reported for captan except the mortality data for the tested males; at least three treated dose groups should be analysed in order to provide sufficient data for dose response analysis; vehicle not clearly stated; low number and relatively high mortality of tested males; for two out of three i.p. studies, the mating period was only 3 weeks instead of 8; only early fetal deaths were considered instead of complete post-implantation deaths (early + late)
Acceptability:	No

Material and Methods

Captan was one of 174 test agents tested for mutagenicity in mice using a modified dominant lethal assay.
Captan technical (source: California Chemical Corporation) was tested in the dominat lethal assay in ICR/Ha Swiss mice (from Charles River Breeding Laboratories). The test agent were freshly dissolved in tricaprylin or distilled water (not specifically stated for captan). Captan was administered by a single i.p. injection at 9, 12, 15 and 30 mg/kg bw or by a single oral intubation at 500 and 800 mg/kg bw or by oral intubation for five consecutive days at 25 and 50 mg/kg bw/day. Dosing was adjusted in order to approximate LD_{25} and LD_5 doses, determined in preliminary toxicity test. Groups of 5 to 11 male mice per dose were used. For the oral administration for five consecutive days, 10 and 11 males were used for the 25 and the 50 mg/kg bw/day dose groups, respectively.
After treatment, each male was mated with 3 virgin females per week for a mating period of 3 to 8 weeks. For the oral administration for five consecutive days, the mating period was 8 weeks. Pregnant feamles were sacrificed at gestation day 13 and scored for pregnancy, for the number of total implants, and for early and late fetal deaths. The parameter analysed were total implants and early fetal deaths.

Findings

Captan was classified as "agent tested but not meeting any screening criteria for mutagenic effects" based on the number of total implants and early foetal deaths (raw data not reported in the review). This "category" comprised 74% of the 174 agents tested. However, it is stated that such negative results do not, of course, preclude the possibility of an effect beyond the dose range selected or an effect in another strain, species or test system.

The numbers of total implants and early foetal deaths were not reported in the study. However, mortality of the dosed males was reported which ranged from 1/9 (dead/tested male) at a single intraperitoneal dose of 12 mg/kg bw to 5/5 at a single intraperitoneal dose of 30 mg/kg bw. For the oral administration for five consecutive days, the mortality of males was 3/10 and 4/11 for the 25 and the 50 mg/kg bw/day dose groups, respectively.

Conclusions

Under the conditions of the modified dominant lethal study in mice reviewed in this study, captan was negative at oral doses of up to 50 mg/kg bw/day for five consecutive days. This is in line with the results obtained by Collins (1972) in a dominant lethal study, which however was positive in mice at oral doses of 100 mg/kg bw/day and above under comparable study conditions.

3.8.2.13 Feng, J. and Lin, B. (1987)

Reference:	Cytogenetic effects of an agricultural antibiotic, captan, on mouse bone marrow and testicular cells
Author(s), year:	Feng, J. and Lin, B. (1987)
Report/Doc. number:	Jiangsu Provincial Sanitary and Anti-Epidemic Station, Nanjiing, China, published in 'Environmental Research' 43, pp 359 - 363 (Company file: TMN-0826)
Guideline(s):	In-house method
GLP:	No
Deviations to OECD 483 (2016):	Poor reporting; no positive control tested (only in the assay of sperm morphology); the interval between colchicine injection and sacrifice was 2 hours instead of 3-5 hours; the ratio of spermatogonial mitosis to first and second meiotic metaphases was not reported; statistical method not stated
Acceptability:	Limited

Material and Methods

The *in vivo* cytogenetic effects of captan on germ cells were investigated in mouse (i) in a chromosome aberration test in spermatogonia and spermatocytes, and (ii) in an assay of sperm morphology. Captan (purity 96.5%, batch number not specified), emulsified with Tween 40, was administered by oral gavage at various dosages.

For the chromosome aberration test in mouse testis, six groups with five sexually mature male mice per group were administered captan by oral gavage at dose rates of 10, 50, 100, 400, 800 and 1000 mg/kg bw/day for five consecutive days. A group of negative control animals received vehicle (Tween 40) only. All animals were killed 6 hours after the last gastric tubing. Two hours prior to sacrifice, the mice received an intraperitoneal administration of 6 mg/kg bw colchicine to arrest cell division of testis cells. The testes were treated by the method of Evans et al. (1964) and Meredith (1969). 500 cells were scored per group and cell type.

For the assay of sperm morphology, groups of mice (5 per group) were treated with captan at doses of 50, 200 and 800 mg/kg bw/day for five consecutive days. A group of negative control animals received vehicle (Tween 40) only, and positive control animals received cyclophosphamide at a dose of 40 mg/kg bd/day. All mice were sacrificed 35 days after the first administration and treated according to amodified procedure of Wryobek and Bruce (1978). The frequencies of morphological abnormalities of sperm were scored.

Findings

In the chromosomal aberration test in mouse spermatogonia, there was a dose-dependent increase in the frequency of aberrant cells in mice treated with captan, which showed statistical significance at the highest dose tested of 800 mg/kg bw/day. In primary spermatocytes, the mutagenic dosage was remarkably lower than in spermatogonia, indicating higher sensitivity. In the mouse primary spermatocytes, there was a dose-dependent increase in the frequency of aberrant cells showing statistical significance at the doses of 50 mg/kg bw/day and above.

In assays of sperm morphology, a statistically significant increase in the frequency of sperm-head abnormality was observed at 200 mg/kg bw/day and above.

Table 3.8.2.13-1 *In vivo* chromosomal aberration in mouse spermatogonia induced by captan

Captan dose (mg/kg)	No. of animals scored	Cells scored	Gap	Chromosome aberration				P value
				Monosomic fragmentation	Trans-location	Total no.	%	
0	5	500	2.0	2.0	0	2.0	0.4	-
10	5	500	1.0	1.5	0	1.5	0.3	> 0.05
50	5	500	1.5	2.0	0	2.8	0.4	> 0.05
100	5	500	1.5	2.5	0	2.5	0.5	> 0.05
400	5	500	1.0	6.0	0	6	1.2	> 0.05
800	5	500	10.5	11.0	2.0	13	2.6	< 0.01

Table 3.8.2.13-2 *In vivo* chromosomal aberration in mouse primary spermatocytes induced by captan

Captan dose (mg/kg)	No. of animals scored	Cells scored	Chromosome aberration					P value
			Fragmentation	Ring of quadrivalent	Other type	Total no.	%	
0	5	500	2.5	0	0	2.5	0.5	-
10	5	500	3.5	0	0	3.5	0.7	> 0.05
50	5	500	10.0	1.0	0	11.0	2.2	< 0.05
100	5	500	10.0	1.5	0	11.5	2.3	< 0.05
400	5	500	10.0	3.0	0.5	13.5	2.7	< 0.01
800	5	500	10.0	2.5	1.0	13.5	2.7	< 0.01
1000	5	500	13.5	0.5	0	14	2.8	< 0.01

Table 3.8.2.13-3 Sperm-head abnormalities in the mouse induced by captan

Dose (mg/kg)	No. animals scored	Sperm scored	Sperm-head abnormalities		P value
			Number	%	
Solvent control	5	5000	41	0.82	-
Captan 50	5	5000	65	1.30	> 0.05
Captan 200	5	5000	73	1.46	< 0.05
Captan 800	5	5000	82	1.64	< 0.01
Cyclophosph-amid (40)	5	5000	251	5.02	< 0.01

Conclusions

Under the conditions of the test, captan induced chromosomal aberrations in spermatogonia and primary spermatocytes of mice orally administered captan at doses of 800 and 50 mg/kg bw/day or higher, respectively, for 5 consecutive days. Furthermore, captan induced sperm-head abnormalities were observed in mice 35 days after 200 mg/kg bw/day captan administration and above. Results indicate that primary spermatocytes (cells in which the first meiotic cells division occurs in the process of spermatogenesis) are far more sensitive to captan than sperms and spermatogonia.

In a statement submitted by the notifier (Anonymous, 2017; report number 562961-CA-050403-1), the following was mentioned with regard to the reliability/acceptability of the study:

The overall conclusion is that the study is not reliable based on considerable deviations from the relevant guidelines and deficiencies on the reporting:

The study was not conducted according to GLP and no regulatory guideline was followed. The purity of the test item was rather low and the impurity profile unknown. The reporting is very brief and lacks important information such as strain of mice employed, conditions of animals maintenance, in some assays the number of treated animals is unclear or contradictory, for others the timing of treatment is unclear. Some assays lack a positive control, others give questionable dose levels for the positive control. Historical control data are not given. There is no information on toxicity (clinical signs, mortality) despite the fact that dose levels were rather high and exceeding the LOAEL in other studies with Captan. Result tables contain several calculation mistakes: micronuclei in bone marrow (table 1): percentage of micronuclei is miscalculated by a factor of 10; chromosome aberration in bone marrow (table 3) shows incorrect values for dose level 800 and the solvent control. Moreover, the absolute number of aberrations in primary spermatocytes (table 4) for several dose levels is given as fraction (half a cell, half a fragmentation?). Also there is no mention of whether scoring was done blinded.

3.8.2.14 Study 7 (2017)

Reference:	Captan Genotoxicity – In-vivo studies in germ cells, Annex Point CA 5.4.3
Author(s), year:	Anonymous (2017)
Report/Doc. number:	562961-CA-050403-1
Guideline(s):	Not applicable
GLP:	Not applicable
Deviations:	Not applicable
Acceptability:	Not applicable

The notifier submitted a statement, where the studies on germ cell genotoxicity, both published and unpublished, were summarised and critically evaluated. Four dominant lethal assay in mice, one dominant lethal assay in rats and one report on chromosomal aberration in male germ cells were evaluated. The studies were conducted more than 40 years ago and do not comply with current guidelines, have considerable flaws in reporting and, therefore, lack reliability.
Deviations from the guideline included: only two dose levels tested in one study, poor reporting on methods and/results, incomplete information on animals or purity of test substance. Positive effects observed lacked biological plausibility since the transient findings upon oral administration were not observed following intraperitoneal dosing, also statistical evaluation of data.
In summary, there is overwhelming evidence that the genotoxic potential of captan is not expressed in vivo: It is concluded that captan does not present a risk of genotoxicity in humans, neither in somatic cells nor in germ cells.

3.8.2.15 Study 13 (1999)

Reference:	The Stability of Captan and Folpet in Whole Blood
Author(s), year:	Anonymous (1999)
Report/Doc. number:	Horizon Laboratories, unpublished report 10238 (Company file R-11143/TMN-0392A)
Guideline(s):	None
GLP:	Yes
Deviations:	Not applicable
Acceptability:	Yes

Material and Methods

1,2-^{14}C-captan (0.936 µg/mL, 0.11 µCi) and benzene ring-U-^{14}C-folpet (1.056 µg/mL, 0.074 µCi) were incubated in vitro in heparinised whole human blood at 37°C for time intervals ranging from zero to ca. 31 seconds. All blood came from the same volunteer, considered representative of all human genotypes and phenotypes. Samples were taken following overnight fasting. After incubation with test material, the reaction was rapidly terminated by injection of the samples into phosphoric acid and acetone. Sample clean-up was by silica gel/Florisil column chromatography, and fractionation by HPLC/UV chromatography to separate radiolabelled parent materials from degradates. Fractions were assayed by scintillation to quantifiy captan, folpet and their purported degradates 1,2,3,6-tetrahydrophthalimide (THPI, from captan) and phthalimide (PI, from folpet). These degradates were identified in earlier rat studies. Control samples of captan and folpet were incubated in saline and analysed, to determine if the method of extraction, clean-up and analysis resulted in conversion of either molecule to the purported degradates.

Findings

Degradation of captan in human blood was time-dependent and exceptionally rapid, with a calculated half-life of 0.97 seconds, modelled by a first-order equation (given as µg captan = 0.643(e-0.718t)). Loss of captan was accompanied by a time-dependent appearance of THPI. All captan was converted to THPI by the end of the 31 second incubation period. Incubation of radiolabelled captan in normal saline for 60.4 seconds at 37°C was quantitatively recovered as parent material, demonstrating that whole blood was required for conversion.

For captan, radiolabel in final extracts was quantitatively recovered in eluted fractions, indicating that no sample radiolabel was retained by the HPLC column, and that no significant peaks other than parent and/or the named degradate were noted. These findings indicate that degradation of captan to THPI is direct, i.e. that no intermediate compounds are formed. THPI is not degraded to other compounds in the short time intervals of this study. The degradates in human blood were those also seen in rat metabolism studies.

Conclusions

Captan degrades rapidly in whole human blood at 37°C to THPI with a half-life of 0.97 seconds. No other short term degradates or intermediates were present. There was no significant degradation of captan in saline at 37°C, demonstrating that whole blood was required for conversion. This study demonstrated that any systemically available captan will degrade rapidly in whole human blood, to degradates also seen in rat studies. Dermal risk assessment models require absorption and assume systemic transport to other sites. This study shows that any captan absorbed dermally will degrade rapidly in the blood, such that effective exposure of other organs or tissues to the molecule is zero.

3.8.2.16 Study 14 (2009a)

Reference:	MUTAGENICITY STUDY OF THPI IN THE SALMONELLA TYPHIMURIUM REVERSE MUTATION ASSAY (IN VITRO)
Author(s), year:	Anonymous (2009a)
Report/Doc. number:	LPT Laboratory of Pharmacology and Toxicology GmbH, Hamburg Rep. No 23733 R-24810
Guideline(s):	OECD 471, EC method B.13/14 (2000/32/EC)
GLP:	Yes
Deviations to OECD 471 (1997):	No historical control data provided.
Acceptability:	Yes

Summary

THPI was examined in 5 *Salmonella typhimurium* strains TA 98, TA 100, TA 102, TA 1535 and TA 1537 in two independent experiments, each carried out without and with metabolic activation (a microsomal preparation derived from Aroclor 1254-induced rat liver). The first experiment was carried out as a plate incorporation test and the second as a preincubation test.
THPI was dissolved in dimethyl sulfoxide (DMSO).

Preliminary test
THPI was examined in a preliminary cytotoxicity test without metabolic activation in test strain TA 100 employing a plate incorporation test. Ten concentrations ranging from 0.316 to 5000 µg/plate were tested. No signs of cytotoxicity were noted up to the top concentration of 5000 µg/plate.
Hence, 5000 µg/plate were chosen as the top concentration for the main study.

Main study
Five concentrations ranging from 100 to 5000 µg/plate were employed in independent experiments each carried out without and with metabolic activation.

Cytotoxicity:
No signs of cytotoxicity were noted up to the top concentration of 5000 µg/plate in the plate incorporation test and the preincubation test, each carried out without and with metabolic activation in any test strain.

Mutagenicity:
No mutagenic effect (no increase in revertant colony numbers as compared with control counts) was observed for THPI tested up to a concentration of 5000 µg/plate in any of the 5 test strains in two independent experiments without and with metabolic activation (plate incorporation and preincubation test, respectively). The results for the solvent controls were within the range of historical control data of the laboratory. A 4- to 95-fold increase in revertant colonies was observed in the positive controls compared to the solvent controls.

In conclusion, under the present test conditions THPI tested up to a concentration of 5000 µg/plate caused no mutagenic effect in the *Salmonella typhimurium* strains TA 98, TA 100, TA 102, TA 1535 and TA 1537 neither in the plate incorporation test nor in the preincubation test each carried out without and with metabolic activation.

Material and Methods

1. Test Material: THPI (1.2.3.6-Tetrahydrophthalimide)
   Description: Off white powder
   Lot/Batch #: 279-071-01

Purity: 98.9%
Stability: Expiry date 19.02.2011
Storage conditions: Ambient temperature, cool and dry conditions

2. Control Materials:
Vehicle and negative control: Dimethyl sulfoxide (DMSO)
Positive controls
without metabolic activation: Sodium azide in H_2O, 2-nitro-fluorene in DMSO, 9-amino-acridine in ethanol, cumene hydroperoxide in DMSO
with metabolic activation: 2-amino-anthracene in DMSO, cyclophosphamide in *aqua ad iniectabilia*

3. Test System:
Species: *Salmonella typhimurium* (kept as frozen permanents in nutrient broth containing 8% DMSO in liquid nitrogen)
Strains: TA 98, TA 100, TA 102, TA 1535, TA 1537
Concentrations: 100, 316, 1000, 3160 and 5000 µg/plate
Plates: 3 per concentration and experiment, 2 independent experiments without and with metabolic activation
Metabolic activation system: Post-mitochondrial fraction (S9 fraction) from rats treated with Aroclor 1254, prepared according to MARON and AMES

TEST PERFORMANCE:
15.01. – 13.02.2009 at LPT Laboratory of Pharmacology and Toxicology GmbH & Co. KG Redderweg, 8, 21147 Hamburg, Germany.

1. Main Test Procedure
First independent experiment - Plate Incorporation Method
Sterile top agar containing 0.6% agar and 0.5% NaCl was molten on the day of the test. 10 mL of a sterile solution of 0.5 mM L-histidine HCl/0.5 mM biotin were added to 100 mL of molten agar. 2 mL of this top agar were distributed into culture tubes held at 45°C in a heating block. 0.1 mL of *Salmonella* cell suspension (containing approximately 10^8 viable cells in the late exponential or early stationary phase), 0.1 mL of test item solution (or 0.1 mL solvent or 0.1 mL positive control) and 0.5 mL of S9 mix were added to these culture tubes. In the assay without metabolic activation, the S9 mix was substituted with 0.5 mL phosphate buffer mentioned above.
The test components were mixed by vortexing the soft agar for 3 sec at low speed and then poured onto a coded 27.5 mL minimal glucose agar plate (Vogel-Bonner medium E). To achieve a uniform distribution of the top agar on the surface of the plate, the uncovered plate was quickly tilted and rotated and then placed on a level surface with the cover on and finally allowed to harden.
Immediately, the plates were inverted and placed in a dark 37°C incubator for 48 to 72 hours. The revertant colonies on the test plates and on the control plates were counted with a colony counter, and the presence of the background lawn on all plates was confirmed. A lawn that was thin compared with the lawn on the negative control plate was evidence of bacterial toxicity.
Routine examination of the background lawn of bacterial growth resulting from the trace of histidine added to the top agar can be an aid in determining the presence of toxic effects. If massive cell death has occurred, the background lawn on the test plates will be sparse compared with control plates.
In this case more histidine is available to the individual surviving bacteria and they undergo more cell divisions, consequently appearing as small colonies which can be mistaken for revertants if the absence of a normal background lawn is not noted.

Second independent experiment - Preincubation Method
The test item/test solution was preincubated with the test strain (containing approximately 10^8 viable cells in the late exponential or early stationary phase) and sterile buffer or the metabolic activation system (0.5 mL) for 20 minutes at 37°C prior to mixing with the overlay agar and pouring onto the surface of a minimal agar plate. 0.1 mL of the test item solution, 0.1 mL of bacteria, and 0.5 mL of S9 mix or sterile buffer, were mixed with 2 mL of overlay agar. Tubes were aerated during preincubation by using a shaker. The remaining steps were the same as described for the plate incorporation method.

2. Evaluation
According to the criteria followed in the test facility, a test item is considered to show a positive response if:

- the number of revertants is significantly increased ($p \leq 0.05$, U-test according to MANN and WHITNEY) compared with the solvent control to at least 2-fold of the solvent control for TA 98, TA 100 and TA 102 and 3-fold of the solvent control for TA 1535 and TA 1537 in both independent experiments;
- in addition, a significant ($p \leq 0.05$) concentration (log value)-related effect (Spearman's rank correlation coefficient) is observed;
- positive results have to be reproducible and the histidine independence of the revertants has to be confirmed

Cytotoxicity is defined as a reduction in the number of colonies by more than 50% compared with the solvent control and/or a scarce background lawn.

Findings

Preliminary test

THPI was examined in a preliminary cytotoxicity test without metabolic activation in test strain TA 100 employing a plate incorporation test. Ten concentrations ranging from 0.316 to 5000 µg/plate were tested. No signs of cytotoxicity were noted up to the top concentration of 5000 µg/plate. Hence, 5000 µg/plate were chosen as the top concentration for the main study.

Main study

Five concentrations ranging from 100 to 5000 µg/plate were employed in independent experiments each carried out without and with metabolic activation.

No signs of cytotoxicity were noted up to the top concentration of 5000 µg/plate in the plate incorporation test and the preincubation test, each carried out without and with metabolic activation in any test strain.

No mutagenic effect (no increase in revertant colony numbers as compared with control counts) was observed for THPI tested up to a concentration of 5000 µg/plate in any of the 5 test strains in two independent experiments without and with metabolic activation (plate incorporation and preincubation test, respectively).

Table 3.8.2.16-1 Plate incorporation test without metabolic activation

Test substance (µg/plate)	Number of reverted colonies (mean of three replicates)									
	TA98		TA100		TA102		TA1535		TA1537	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
THPI (5000)	29.0	2.6	143.3	7.6	272.0	9.8	23.7	3.2	8.0	1.0
THPI (3160)	27.3	3.2	159.7	9.1	282.7	7.6	28.0	2.0	7.3	1.5
THPI (1000)	33.7	1.5	147.7	8.3	272.3	9.6	30.3	3.1	7.3	2.1
THPI (316)	31.0	1.0	127.7	7.8	280.7	7.2	24.0	3.0	7.3	1.5
THPI (100)	37.0	9.2	146.7	3.1	284.0	6.1	29.0	2.6	7.7	0.6
DMSO (100 µL/plate)	27.0	5.3	147.7	5.5	278.3	8.1	27.0	2.6	7.0	1.0
2-nitro-fluorene (10)	558.0	27.6	-	-	-	-	-	-	-	-
Sodium azide (10)	-	-	674.7	9.1	-	-	575.7	8.5	-	-
Cumene hydroperoxide (10)	-	-	-	-	1132.3	70.2	-	-	-	-
9-Amino-acridine (100)	-	-	-	-	-	-	-	-	477.3	11.6

Table 3.8.2.16-2 Plate incorporation test with metabolic activation

Test substance (µg/plate)	Number of reverted colonies (mean of three replicates)									
	TA98		TA100		TA102		TA1535		TA1537	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
THPI (5000)	45.0	5.0	106.3	8.1	270.3	6.8	26.0	6.0	4.7	2.1
THPI (3160)	36.0	3.0	121.0	6.1	275.0	8.5	26.7	2.5	5.0	1.0
THPI (1000)	36.3	3.2	127.7	0.6	276.3	17.0	26.0	7.0	4.3	0.6
THPI (316)	31.7	2.3	111.7	4.6	282.0	8.7	27.7	3.5	6.3	2.1
THPI (100)	40.3	3.5	127.0	5.0	283.7	8.3	27.3	2.1	5.0	1.0
DMSO (100 µL/plate)	37.0	3.6	144.0	15.1	281.3	7.1	25.3	3.5	5.0	1.0
2-Amino-anthracene (2)	602.3	16.7	-	-	1128.7	53.4	-	-	475.3	12.3
Cyclophosphamide (1500)	-	-	714.3	19.5	-	-	666.0	39.2	-	-

Table 3.8.2.16-3 Pre-incubation test without metabolic activation

Test substance (µg/plate)	Number of reverted colonies (mean of three replicates)									
	TA98		TA100		TA102		TA1535		TA1537	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
THPI (5000)	48.0	6.2	144.7	29.9	261.0	5.0	20.0	2.6	4.3	1.2
THPI (3160)	31.3	3.5	166.0	8.9	266.0	9.8	22.7	2.1	5.7	1.5
THPI (1000)	34.3	3.5	170.3	14.8	264.7	5.7	24.3	4.7	6.0	1.0
THPI (316)	45.0	9.8	176.0	7.8	274.3	7.5	23.7	2.1	6.0	1.0
THPI (100)	33.7	4.6	147.0	16.5	274.0	12.8	27.0	1.0	6.3	1.5
DMSO (100 µL/plate)	41.0	1.7	157.3	32.1	275.3	10.1	25.7	2.5	6.7	1.2
2-nitro-fluorene (10)	468.3	16.7	-	-	-	-	-	-	-	-
Sodium azide (10)	-	-	730.3	28.5	-	-	347.3	20.6	-	-
Cumene hydroperoxide (10)	-	-	-	-	1165.0	19.1	-	-	-	-
9-Amino-acridine (100)	-	-	-	-	-	-	-	-	341.0	16.0

Table 3.8.2.16-4 Pre-incubation test with metabolic activation

Test substance (µg/plate)	Number of reverted colonies (mean of three replicates)									
	TA98		TA100		TA102		TA1535		TA1537	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
THPI (5000)	41.0	2.6	144.0	0.0	256.0	2.6	26.3	3.5	4.3	1.2
THPI (3160)	33.3	5.8	127.3	16.9	268.3	6.8	25.0	3.0	6.7	0.6
THPI (1000)	33.0	3.5	154.3	29.3	268.3	10.2	24.3	4.7	6.0	1.7
THPI (316)	36.7	8.1	161.7	3.8	272.0	3.6	27.0	3.5	5.3	1.2
THPI (100)	39.3	6.7	144.7	4.9	276.3	9.1	28.3	5.0	6.0	2.0
DMSO (100 µL/plate)	43.3	10.8	152.3	30.9	274.7	10.0	27.7	1.5	6.7	1.2
2-Amino-anthracene (2)	477.0	18.2	-	-	1154.7	26.5	-	-	363.0	21.4
Cyclophosphamide (1500)	-	-	751.3	37.0	-	-	447.3	21.1	-	-

Conclusions

Under the present test conditions THPI tested up to a concentration of 5000 µg/plate caused no mutagenic effect in the Salmonella typhimurium strains TA 98, TA 100, TA 102, TA 1535 and TA 1537 neither in the plate incorporation test nor in the preincubation test each carried out without and with metabolic activation.

3.8.2.17 Study 15 (2009b)

Reference:	IN VITRO ASSESSMENT OF THE CLASTOGENIC ACTIVITY OF THPI IN CULTURED CHO CELLS
Author(s), year:	Anonymous (2009b)
Report/Doc. number:	LPT Laboratory of Pharmacology and Toxicology GmbH, Hamburg, Rep. No 23732 R-24809
Guideline(s):	OECD 473, EC method B.10 (2000/32/EC)
GLP:	Yes
Deviations to OECD 473 (2016):	Number of examined metaphases was lower than recommended in the guideline (200 instead of 300), but in line with the (old) guideline from 1997. However, as the responses were clear, this is considered a minor deviation.
Acceptability:	Yes.

Summary

Test samples of THPI were assayed in an in vitro cytogenetic study using CHO cell cultures both in the presence and absence of metabolic activation by a rat liver post-mitochondrial fraction (S9 mix) from Aroclor 1254 induced animals. The test was carried out employing 2 exposure times without S9 mix: 4 and 20 hours, and 1 exposure time with S9 mix: 4 hours. The experiment with S9 mix was carried out twice. The harvesting time was 20 hours after starting of exposure. The study was conducted in duplicate.

The concentrations employed were chosen based on the results of a cytotoxicity study. The top concentration employed in the main study was 2500 µg THPI/mL in the experiments without and with metabolic activation (4-h exposure) and 1250 µg/mL in the second experiment without metabolic activation (20-h exposure).
Cytotoxicity was noted at the top concentration of 1250 µg THPI/mL in the second experiment without metabolic activation (20-h exposure). In the first experiment with metabolic activation (4-h exposure) cytotoxicity was noted at the top concentration of 2500 µg THPI/mL. In the experiments without metabolic activation slight test item precipitation was noted at the top concentration of 2500 µg/mL.
Mitomycin C and cyclophosphamide were employed as positive controls in the absence and presence of metabolic activation, respectively.

Tests without metabolic activation (4- and 20-hour exposure)
The mean incidence of chromosomal aberrations (excluding gaps) of the cells treated with THPI at concentrations from 312.5 to 2500 or 78.13 to 625 µg/mL medium (4-h and 20-h exposure, respectively) in the absence of metabolic activation ranged from 0.0% to 1.0%.
The results obtained are considered to be within the normal range of the solvent control where a mean incidence of chromosomal aberrations (excluding gaps) of 0.0% or 1.0% was observed after a 4-hour and 20-hour exposure, respectively. No test item-related polyploidy was noted.

Test with metabolic activation (4-hour exposure)
The mean incidence of chromosomal aberrations (excluding gaps) of the cells treated with THPI at concentrations from 312.5 to 2500 µg/mL medium in the presence of metabolic activation ranged from 0.5% to 2.9%. The results obtained are considered to be within the normal range of the negative control where a mean incidence of chromosomal aberrations (excluding gaps) of 0.5% or 1.0% was observed in the first and second experiment, respectively. No test item-related polyploidy was noted. The range of background-data of cells with aberrations excluding gaps of the vehicle culture of the last 3 experiments was 0.0% to 5.0% (without S9) and 0.0% to 3.0% (with S9).

It is concluded that under the present test conditions, THPI tested up to a concentration of 2500 µg/mL medium, the limit of solubility, in the absence and in the presence of metabolic activation employing two exposure times (without S9) and one exposure time (with S9) revealed no indications of mutagenic properties with respect to chromosomal or chromatid damage.
In the same test, Mitomycin C and cyclophosphamide induced significant damage.

Material and Methods

1. Test Material: THPI (1.2.3.6-Tetrahydrophthalimide)
   - Description: Off-white powder
   - Lot/Batch #: 279-071-01
   - Purity: 98.9%
   - Stability: Expiry date 19.02.2011
   - Storage conditions: Ambient temperature, cool and dry conditions

2. Control Materials:
   - Vehicle and negative control: Dimethyl sulfoxide (DMSO)
   - Positive control without metabolic activation: Mitomycin C
     with metabolic activation: Cyclophosphamide

3. Test System
   - Cell line: Chinese hamster ovary (CHO-K_1) cells, obtained from American Type Culture Collection and stored in polypropylene ampoules at -196°C in 90% MEM and 10% dimethyl sulfoxide
   - Culture medium: MEM (minimum essential medium) supplemented with 10% FCS (fetal calf serum)
   - Growth conditions: 37°C in a humid atmosphere containing 5% carbon dioxide in plastic tissue culture plate (Nunc)
   - Metabolic activation system: Post-mitochondrial fraction (S9 fraction) from rats treated with Aroclor 1254, prepared according to MARON and AMES

TEST PERFORMANCE:
15.01. – 26.03.2009 at LPT Laboratory of Pharmacology and Toxicology GmbH & Co. KG Redderweg, 8, 21147 Hamburg, Germany.

1. Preliminary toxicity test / Exposure concentrations

Cultures of CHO cells in exponential growth were harvested by removing the supernatant medium and the washing the cells in buffer; 0.25% trypsin was then added for 30 seconds. The trypsin solution was removed and the flask incubated at 37°C for 10 minutes: The cells were then resuspended in MEM + 10% FCS and diluted to a yield 5 x 10^4 cells/mL. Aliquots (10 mL) of cells were added to tissue culture plate (ø 10 cm) and the cultures incubated at 37°C in a humid atmosphere containing 5% carbon dioxide.

After 24 hours of incubation 5 mL of culture medium was removed and replaced by 5 mL of S9 mix to one set of cultures followed by adding 100 µL of various dilutions of the test item and of the solvent. To the second set of cultures (i.e. without S9 mix) 100 µL of the dilutions of the test item and of the solvent were added in 5 mL treatment medium. The cultures without S9 mix were also incubated in the presence of the test item for 4 hours at 37°C.

Four hours after addition of the test item the medium containing the test item was carefully removed and replaced with fresh MEM + 10% FCS. The cultures without S9 mix were returned to the incubator for a further 16 hours.

Cultures (one culture for each concentration) were harvested and 1 slide per culture was prepared. 1000 cells per culture were examined at a magnification of x 400; the mitotic index was calculated as the percentage of cells examined which were in mitosis (metaphase). Slides were coded before analysis.

In this preliminary experiment cytotoxicity was noted at a concentrations of 2500 µg THPI/mL in the experiment without metabolic activation (20-h exposure). No signs of cytotoxicity were noted up to the top concentration of 2500 µg THPI/mL in the experiment with metabolic activation (4-h exposure). In addition, slight test item precipitation was noted at the top concentration of 2500 µg THPI/mL.

Hence, the top concentration employed in the main study was 2500 µg THPI/mL in the experiments without and with metabolic activation (4-h exposure) and 1250 µg/mL in the second experiment without metabolic activation (20-h exposure).

2. Main study

Cultures were initiated and maintained as described in Materials and Methods. After 24 hours of incubation 5 mL of culture medium was removed and replaced by 5 mL of S9 mix to one set of cultures followed by adding 100 µL aliquots of various dilutions of the test item. One culture was treated with 100 µL of the solvent control and two with the positive control item cyclophosphamide. The cultures were then incubated at 37°C.

To the remaining set of cultures (i.e. without S9 mix) 100 µL aliquots of the test item were added in 5 mL treatment medium. 100 µL aliquots of the solvent control and two with the positive control item mitomycin C were added in two cultures. The cultures were then incubated at 37°C.

In both experiments carried out without and with metabolic activation 4 hours after addition of the test item the medium containing the test item was carefully removed and replaced with fresh MEM + 10% FCS. The cultures were returned to the incubator for a further 16 hours for recovery until harvest 20 hours after treatment.

In a second set of the experiments a continuous treatment of 20 hours without metabolic activation was carried out and the 4-hour treatment with metabolic activation was repeated.

Duplicate cultures were used per sampling point.

The highest concentration employed in the main study should suppress the mitotic index by 50 - 80%. The lowest dose should be in the region of the solvent control. At least three concentrations of the test item in addition to a solvent and positive control were evaluated both in the presence and absence of metabolic activation.

3. Culture harvesting and slide preparation

Two hours before termination of the 20-hour incubation period, the cell division was arrested by addition of the spindle poison colcemid to each culture at a final concentration of 0.25 µg/mL. After the incubation period the medium was removed and discarded. 3 mL of the 0.25% trypsin solution was then added. After 45 seconds this was removed and placed in a plastic conical centrifuge tube. These cell suspensions were then centrifuged for 10 minutes at 200 x g.

The supernatant was discarded and the cells resuspended in 2.5 mL 0.07 M KCl. After 10 minutes incubation at room temperature, the cell suspensions were centrifuged for 10 minutes at 200 x g. The supernatant was discarded and 4 mL of freshly prepared fixative (3 parts methanol / 1 part glacial acetic acid v/v) added.

The cells were left in fixative for 2-3 hours, then the pellets resuspended by repeated aspiration through a 20 gauge needle, centrifuged at 200 x g for 10 minutes, the supernatant discarded, and the cell pellets resuspended in about 0.5 mL of fresh fixative and 30% glacial acetic acid by repeated aspiration through a Pasteur pipette. Two drops of this cell suspension were dropped onto a cold, pre-cleaned microscope slide. The slides were left to air-dry at room temperature, then stained in 10% Giemsa.

4. Slide evaluation

The slides received code numbers randomly chosen by a computer. The slides were examined under low power (x 100 objective) and those areas judged to be of sufficient technical quality were located and examined under high power (x 1000, oil immersion objective).

For each treatment and culture 100 metaphases were examined, if possible.
Observed aberrations were noted and scored according to J.R.K. SAVAGE (1975).
To examine the toxicity of the test item 1000 cells were scored and the mitotic index was calculated as the percentage of cells in metaphase.

5. Statistical evaluation
The assessment was carried out by a comparison of the number of chromosome aberrations of the samples with those of the solvent control, using the exact test of R.A. FISHER ($p \leq 0.05$) as recommended by the UKEMS guidelines (The United Kingdom Branch of the European Environmental Mutagen Society: Report of the UKEMS subcommittee on guidelines for mutagenicity testing, part III, Statistical evaluation of mutagenicity test data, 1989).

Findings

The concentrations employed were chosen based on the results of a cytotoxicity study from a preliminary experiment.
The top concentration employed in the main study was 2500 µg THPI/mL in the experiments without and with metabolic activation (4-h exposure) and 625 µg/mL (RMS correction; 1250 µg/mL is stated in the study report) in the second experiment without metabolic activation (20-h exposure).
In the main study cytotoxicity was noted at the top concentration of 625 µg/mL (RMS correction; 1250 µg/mL is stated in the study report) THPI/mL in the second experiment without metabolic activation (20-h exposure). In the first experiment with metabolic activation (4-h exposure) cytotoxicity was noted at the top concentration of 2500 µg THPI/mL.
In the experiments without and with metabolic activation test item precipitation was noted at the top concentration of 2500 µg/mL.
Mitomycin C and cyclophosphamide were employed as positive controls in the absence and presence of metabolic activation, respectively.

<u>Tests without metabolic activation (4- and 20-hour exposure)</u>
The mean incidence of chromosomal aberrations (excluding gaps) of the cells treated with THPI at concentrations from 312.5 to 2500 or 78.13 to 625 µg/mL medium (4-h and 20-h exposure, respectively) in the absence of metabolic activation ranged from 0.0% to 1.0%.
The results obtained are considered to be within the normal range of the solvent control. In this study, mean incidences of chromosomal aberrations (excluding gaps) of 0.0% or 1.0% were observed after a 4-hour and 20-hour exposure, respectively.
No test item-related polyploidy was noted.

<u>Test with metabolic activation (4-hour exposure)</u>
The mean incidence of chromosomal aberrations (excluding gaps) of the cells treated with THPI at concentrations from 312.5 to 2500 µg/mL medium in the presence of metabolic activation ranged from 0.5% to 2.9%.
The results obtained are considered to be within the normal range of the negative control. In this study, mean incidences of chromosomal aberrations (excluding gaps) of 0.5% or 1.0% were observed in the first and second experiment, respectively.
No test item-related polyploidy was noted.
The range of background-data of cells with aberrations excluding gaps of the vehicle culture of the last 3 experiments was 0.0% to 5.0% (without S9) and 0.0% to 3.0% (with S9).

Table 3.8.2.17-1 Chromosome analysis in cultured Chinese hamster ovary cells *in vitro* without metabolic activation

Treatment (µg/mL medium)	1st experiment 4-h exposure			2nd experiment 20-h exposure		
	Mitotic index[a]	Number of metaphases scored	% of cells with aberrations excluding gaps	Mitotic index[a]	Number of metaphases scored	% of cells with aberrations excluding gaps
DMSO	1.00	200	0.0	1.00	200	1.0
THPI (78.13)	-	-	-	0.93	200[c]	0.5
THPI (156.3)	-	-	-	0.76	200	0.0
THPI (312.5)	0.97	200	0.5	0.48	200	0.5
THPI (625)	0.98	200	0.5	0.11	181[d]	0.6
THPI (1250)	0.91	200	0.5	-	-	-
THPI (2500)[b]	0.84	200	1.0	-	-	-
Mitomycin C (0.4)	0.61	200	12.0 *	0.59	200	11.5 *

[a] mitotic index: number of metaphases/1000 cells; negative control = 1.00
[b] slight test item precipitation
[c] tetraploidy (excluded from evaluation)
[d] no more metaphases of sufficient quality for evaluation due to cytotoxicity of THPI
* significantly different from negative control ($p \leq 0.05$)

Table 3.8.2.17-2 Chromosome analysis in cultured Chinese hamster ovary cells *in vitro* with metabolic activation

Treatment (µg/mL medium)	1st experiment 4-h exposure			2nd experiment 4-h exposure		
	Mitotic index[a]	Number of metaphases scored	% of cells with aberrations excluding gaps	Mitotic index[a]	Number of metaphases scored	% of cells with aberrations excluding gaps
DMSO	1.00	200	0.5	1.00	200	1.0
THPI (312.5)	1.00	200[c]	1.5	1.04	200	1.0
THPI (625)	0.67	200[c]	1.0	0.90	200[c]	1.0
THPI (1250)	0.58	200[c]	2.5	0.93	200	1.0
THPI (2500)[b]	0.50	200[c, d]	2.9	0.84	200	0.5
Cyclophosphamide (10)	0.64	200[c]	7.0 *	0.63	200[c]	17.0 *

[a] mitotic index: number of metaphases/1000 cells; negative control = 1.00
[b] slight test item precipitation
[c] tetraploidy (excluded from evaluation)
[d] no more metaphases of sufficient quality for evaluation due to cytotoxicity of THPI
* significantly different from negative control ($p \leq 0.05$)

Conclusions

Under the present test conditions, THPI tested up to a concentration of 2500 µg/mL medium, the limit of solubility, in the absence and in the presence of metabolic activation employing two exposure times (without S9) and one exposure time (with S9) revealed no indications of mutagenic properties with respect to chromosomal or chromatid damage.
In the same test, Mitomycin C and cyclophosphamide induced significant damage.

3.8.2.18 Study 16 (2014)

Reference:	Cis-1,2,3,6-Tetrahydrophthalimide: IN VITRO MAMMALIAN CELL GENE MUTATION TEST IN L5178Y TK+/- MOUSE LYMPHOMA CELLS
Author(s), year:	Anonymous (2014)
Report/Doc. number:	C.I.T., Evreux, France No 39770 MLY; report no. R-34855
Guideline(s):	OECD 476
GLP:	Yes
Deviations to OECD 476 (2016):	None
Acceptability:	Yes

Summary

The potential of the test item, cis-1,2,3,6-Tetrahydrophthalimide, to induce mutations at the TK (Thymidine Kinase) locus in L5178Y TK+/- mouse lymphoma cells was investigated.
After a preliminary toxicity test, the test item, cis-1,2,3,6-Tetrahydrophthalimide, was tested in two independent experiments, with and without a metabolic activation system (S9 mix) prepared from a liver microsomal fraction (S9 fraction) of rats induced with Aroclor 1254.
Cultures of 20 mL at 5 x 10^5 cells/mL (3-hour treatment) or cultures of 50 mL at 2 x 10^5 cells/mL (24-hour treatment) were exposed to the test or control items, in the presence or absence of S9 mix (final concentration of S9 fraction 2%). During the treatment period, the cells were maintained as suspension culture in RPMI 1640 culture medium supplemented by heat inactivated horse serum at 5% (3-hour treatment) or 10% (24-hour treatment) in a 37°C, 5% CO_2 humidified incubator. For the 24-hour treatment, flasks were gently shaken at least once.
Cytotoxicity was measured by assessment of Adjusted Relative Total Growth (Adj. RTG), Adjusted Relative Suspension Growth (Adj. RSG) and Cloning Efficiency following the expression time (CE_2). The number of mutant clones (differentiating small and large colonies) was evaluated after expression of the mutant phenotype.
The test item was dissolved in dimethylsulfoxide (DMSO)

Results:
The Cloning Efficiencies (CE_2), the mutation frequencies and the suspension growths of the vehicle controls were as specified in the acceptance criteria. Moreover, the induced mutation frequencies obtained for the positive controls met in each experiment the acceptance criteria specified in the study plan. The study was therefore considered as valid.
Since the test item was found non-toxic and freely soluble (no precipitate observed in the culture medium at the end of the treatment periods) in the preliminary test, the highest dose-level selected for the main experiments was 10 mM, according to the criteria specified in the international guidelines.
Using a treatment volume of 0.5% in culture medium, the selected dose-levels were 0.63, 1.25, 2.5 5 and 10 mM for both experiments (3- and 24-hour treatments).

Experiments without S9 mix
Cytotoxicity:
Following the 3-hour treatment, no toxicity was induced at any of the dose-levels as shown by the absence of any noteworthy decrease in Adj. RTG.
Following the 24-hour treatment, a slight to marked toxicity was noted at dose-levels ≥ 1.25 mM, as shown by a 31 to 67% decrease in Adj. RTG.

Mutagenicity:
Following the 3- and 24-hour treatments, no noteworthy increase in the mutation frequency, which could be considered as biologically relevant or dose-related, was noted at any of the tested dose-levels in comparison to the vehicle controls. Thus, these results did not meet the criteria for a positive response.

Experiments with S9 mix
Cytotoxicity:
In the first and second experiments, no noteworthy decrease in Adj. RTG was observed at any of the tested dose-levels, except a non-related slight decrease in Adj. RTG observed at 0.63 mM in the first experiment.

Mutagenicity:
No noteworthy increase in the mutation frequency, which could be considered as biologically relevant or dose related, was induced in any experiment, in comparison to the vehicle controls. Thus these results did not meet the criteria of a positive response.

Conclusion:
Under the experimental conditions of this study, the test item, cis-1,2,3,6-Tetrahydrophthalimide, did not show any mutagenic activity in the mouse lymphoma assay, in the presence or in the absence of a rat metabolizing system.

Material and Methods

1. Test Material:	cis-1,2,3,6-Tetrahydrophthalimide (THPI)
Description:	White powder
Lot/Batch #:	919100
Purity:	99%
Stability:	Expiry date 31.07.2017
Storage conditions:	Room temperature

2. Control Materials:

Vehicle and negative control: Dimethyl sulfoxide (DMSO)

Positive control without metabolic activation: Methylmethane sulfonate (MMS)
with metabolic activation: Cyclophosphamide (CPA)

3. Test System

Cell line: L5178Y TK+/- cells, stored in a cryoprotective medium [10% horse serum and 10% dimethylsulfoxide (DMSO)] at -80°C

Culture medium: RPMI 1640 culture medium containing L-Glutamine (2mM), penicillin (100 U/mL), streptomycin (100 µg/mL) and sodium pyruvate (200 µg/mL) sup plemented by heat inactivated horse serum at 10%, v/v

Growth conditions: 37 °C, 5% carbon dioxide humidified incubator

Metabolic activation system: S9 fraction was purchased from Moltox (Molecular Toxicology, INC, Boone, NC 28607, USA) and obtained from the liver of rats treated with Aroclor 1254 (500 mg/kg) by the intraperitoneal route.

TEST PERFORMANCE:
11.03. – 08.07.2013 at CiToxLAB France, BP 563, 27005 Evreux, France

1. Preliminary toxicity test

To assess the cytotoxicity of the test item, six dose-levels (one culture/dose-level) were tested both with and without metabolic activation.
Treatments of 3 hours (with and without S9 mix) and 24 hours (without S9 mix) were performed using the selected final concentrations and conditions described under 2. Main experiments. At the end of treatment, the cells were rinsed, and then cell concentrations were adjusted to 2 x 10^5 cells/mL and cultured for 2 days, as for the mutagenicity experiments.
At the end of the 2-day mutant phenotype expression period, the cultures were adjusted in order to seed an average of 1.6 cells per well in 96-well microtiter plates.
Approximately one week after incubation at 37°C in a humidified atmosphere of 5% CO_2/95% air, the clones were counted to determine the cloning efficiency.

2. Main experiments

In two independent experiments, five dose-levels of the test item (two cultures/dose-level) were tested, both with and without metabolic activation, as follows:

- Without S9 mix: first experiment of 3h-treatment, second experiment of 24h-treatment since the results of first experiment were negative
- With S9 mix: first and second experiments of 3h-treatment

In each experiment, the following controls (at least two cultures/control) were included:

- vehicle controls: cultures treated with the vehicle,
- positive controls: cultures treated with: MMS, in the absence of S9 mix or CPA, in the presence of S9 mix.

For the 3-hour treatment, cultures of 20 mL at 5 x 10^5 cells/mL in RPMI 5 were treated in 50-mL tubes with the test or control items for 3 hours, in a 37°C, 5% CO_2 humidified incubator.
For the 24-hour treatment, cultures of 50 mL at 2 x 10^5 cells/mL in RPMI 10 were treated in flasks with the test or control items for 24 hours, in a 37°C, 5% CO_2 humidified incubator (flasks were gently shaken at least once).
At the end of the treatment period, the cells were rinsed. After centrifugation and removal of the supernatant, the pellets were suspended in RPMI 10 and the cells were counted using a hemocytometer.
When sufficient cells survived, the cell concentrations were adjusted to 2 x 10^5 cells/mL and then, to enable the expression of the mutant phenotype, the cells were incubated in RPMI 10 medium for 48 hours, in a 37°C, 5% CO_2 humidified incubator.
During the mutant phenotype expression period, the cultures were maintained at the density of approximately 2 x 10^5 cells/mL, whenever possible.
At the end of this expression period, the cell density of each culture was determined using a hemocytometer and the cells were seeded after serial dilutions as follows:

Viability plates
To define the number of viable cells (CE_2: Cloning Efficiency at the end of the expression period), an average of 1.6 cells/well were seeded in two 96-well plates/culture (four plates/dose-level). After at least 7 days of incubation in a 37°C, 5% CO_2 humidified incubator, the clones were counted.

Mutant plates
To select the TFT (trifluorothymidine resistant) mutant cells (for the determination of CE_{mutant}), 2000 cells/well were seeded in four 96-well plates/culture (eight plates/dose-level). After 11-12 days of incubation in a 37°C, 5% CO_2 humidified incubator in the presence of 4 µg TFT/mL of culture medium, the clones were counted, differentiating small and large colonies:

- size of small colonies: < 25% of the diameter of the well,
- size of large colonies: > 25% of the diameter of the well.

For scoring of colonies in mutant plates, the following parameters were considered:

- well containing mutant colony (small or large),
- well not containing mutant colony,
- when both small and large colonies are present in the same well both mutant colonies were counted (one small and one large).

3. Evaluation of the results

Treatment of viability plate results
Data from the viability plates (empty wells) were used to calculate CE_2 (Cloning Efficiency at the end of the expression period) and RCE_2 (viability relative to vehicle controls at the end of the expression period). These values indicated the viability of the cell populations at the end of the expression period.
For evaluation of cytotoxicty, the adjusted relative total growth was calculated (Adj. RTG, please refer to the study report for details in the calculation).

Treatment of mutant plate results
Data from the mutant plates (empty wells) were used to calculate CE_{mutant} (Cloning Efficiency in selective medium). This value is an indicator of the absolute mutant frequency. CE_{mutant} is calculated from the zero term of the Poisson distribution (only mutant clones are able to grow in TFT containing medium).
The relative Mutant Frequency (MF) and the Induced Mutation Frequency (IMF) were calculated.

Statistical analysis of the results
A trend test was performed, if needed, to assess the linear trend between the mutation frequency and the dose. This statistical analysis was performed using SAS Enterprise Guide software.
Only individual mutation frequencies obtained from cultures showing an Adj. RTG ≥ 10% were used in this analysis.

Evaluation criteria
IWGT recommendations (Moore et al., 2003; Moore et al., 2006; Moore et al., 2007) were followed for the determination of a positive result, which should fulfill the following criteria:

- at least at one dose-level the mutation frequency minus the mutation frequency of the vehicle control (IMF) equals or exceeds the Global Evaluation Factor (GEF) of 126 x 10^{-6},
- a dose-related trend is demonstrated by a statistically significant trend test.

Unless an effect is considered as clearly positive, the reproducibility of a positive effect should be confirmed.
Noteworthy increases in the mutation frequency observed only at high levels of cytotoxicity (Adj. RTG lower than 10%), but with no evidence of mutagenicity at dose-levels with Adj. RTG between 10 and 20%, are not considered as positive results.
A test item may be considered as non-mutagenic when there is no culture showing an Adj. RTG value between 10 and 20% if (Moore et al., 2002):

- there is at least one negative data point between 20 and 25% Adj. RTG and no evidence of mutagenicity in a series of data points between 100 and 20% Adj. RTG,
- there is no evidence of mutagenicity in a series of data points between 100 and 25% and there is also a negative data point between 10 and 1% Adj. RTG.

Findings

Preliminary toxicity test

Using a test item concentration at 302.2 mg/mL in the vehicle (dimethylsulfoxide) and a treatment volume of 0.5% in culture medium (i.e. 100 µL/20 mL of culture medium for the 3-hour treatments and 250 µL/50 mL of culture medium for the 24-hour treatment), the highest recommended dose-level of 10 mM (which corresponds to 1511 µg/mL) was achievable. Therefore, the dose-levels selected for treatment of the preliminary test were 0.02, 0.2, 1, 2, 5 and 10 mM.
At the highest tested dose-level of 10 mM, the pH was approximately 7.1 (as for the vehicle control) and the osmolality was equal to 369 mOsm/kg H_2O (374 mOsm/kg H_2O for the vehicle control). The top dose of 10 mM was therefore suitable for treatment of the cells.
At the end of the 3- and 24-hour treatment periods, no precipitate was observed in the culture medium.
Following the 3-hour treatments with and without S9 mix, no toxicity was induced at any of the tested dose-levels as shown by the absence of any noteworthy decrease in the Adjusted Relative Total Growth (Adj. RTG). Following the 24-hour treatment without S9 mix, a slight to moderate toxicity was induced at dose-levels ≥ 5 mM, as shown by a 35 to 59% decrease in the Adj. RTG.

Main experiments

The Cloning Efficiencies (CE_2), the mutation frequencies and the suspension growths of the vehicle controls were as specified in the acceptance criteria. Moreover, the induced mutation frequencies obtained for the positive controls met in each experiment the acceptance criteria specified in the study plan. The study was therefore considered as valid.
Since the test item was found non-toxic and freely soluble (no precipitate observed in the culture medium at the end of the treatment periods) in the preliminary test, the highest dose-level selected for the main experiments was 10 mM, according to the criteria specified in the international guidelines.
Using a treatment volume of 0.5% in culture medium, the selected dose-levels were 0.63, 1.25, 2.5, 5 and 10 mM for both experiments (3- and 24-hour treatments).

Experiments without S9 mix
Cytotoxicity:
Following the 3-hour treatment, no toxicity was induced at any of the dose-levels as shown by the absence of any noteworthy decrease in Adj. RTG.
Following the 24-hour treatment, a slight to marked toxicity was noted at dose-levels ≥ 1.25 mM, as shown by a 31 to 67% decrease in Adj. RTG.

Mutagenicity:
Following the 3- and 24-hour treatments, no noteworthy increase in the mutation frequency, which could be considered as biologically relevant, was noted at any of the tested dose-levels in comparison to the vehicle controls. Moreover, in both experiments, a statistical analysis performed on the individual mutation frequencies of total induced mutants obtained at dose-levels up to 10 mM did not demonstrate a linear trend between the mutation frequency and the dose ($p > 0.05$). Thus, in the absence of S9 mix, these results did not meet the criteria for a positive response.

Experiments with S9 mix
Cytotoxicity:
In the first and second experiments, no noteworthy decrease in Adj. RTG was observed at any of the tested dose-levels, except a non-dose related slight decrease in Adj. RTG observed at 0.63 mM in the first experiment which was not reproduced in the second experiment.

Mutagenicity:
No noteworthy increase in the mutation frequency, which could be considered as biologically relevant, was induced in any experiment, in comparison to the vehicle control. Moreover, a statistical analysis was performed on the individual mutation frequencies of total induced mutants obtained in the first and second experiments at dose-levels up to 10 mM. This statistical analysis showed a linear trend between the mutation frequency and the dose in the first experiment ($p < 0.05$). But since this result was not reproduced in the second experiment where no linear trend was demonstrated and since the induced mutation frequencies obtained in both experiments never reached the global evaluation factor, these results were considered to meet the criteria for a negative response.

Conclusions

Under the experimental conditions of this study, the test item, cis-1,2,3,6-Tetrahydrophthalimide, did not show any mutagenic activity in the mouse lymphoma assay, in the presence or in the absence of a rat metabolizing system.

3.8.3 Human data

No relevant studies

3.8.4 Other data

Reference:	Genetic toxicology of folpet and captan
Author(s), year:	Arce, G.T., Gordon, E.B., Cohen, S.M., Singh, P. (2010)
Report/Doc. number:	Critical Reviews in Toxicology, 2010; 40(6): 546-574
Guideline(s):	Not applicable (review article)
GLP:	Not applicable
Deviations:	Not applicable
Acceptability:	Yes

Abstract

Folpet and captan are fungicides whose genotoxicity depends on their chemical reaction with thiols. Multiple mutagenicity tests have been conducted on these compounds due to their positive activity *in vitro* and their association with gastrointestinal tumors in mice. A review of the collective data shows that these compounds have *in vitro* mutagenic activity but are not genotoxic *in vivo*. This dichotomy is primarily due to the rapid degradation of folpet and captan in the presence of thiol-rich matrices typically found *in vivo*. Genotoxicity has not been found in the duodenum, the mouse tumor target tissue. It is concluded that folpet like captan presents an unlikely risk of genotoxic effects in humans.

Summary and discussion

Table 3.8.4-1 Summary of captan genotoxicity

6. Study type	7. Conditions	8. Genotoxic activity
Gene mutation *in vitro*		
Microbial	-S9	+++
	+S9	++
	+Thiols	-
Mammalian	-S9	+
	+S9	-
Gene mutation *in vivo*		
Drosophila SLRL		-
Mouse-specific locus		-
Chromosomal - mammalian *in vitro*		
Somatic cells	-S9	±
	+S9	nt
Chromosomal - mammalian *in vivo*		
Somatic cells		-
Germ cells		-
Duodenum crypt cell—mouse		
Micronuclei		-
DNA adduct		-

+++, highly positive; ++, positive; +, weak positive; ±, equivocal; −,negative; nt, not tested.

A tiered Weight Of Evidence (WOE) approach has been used to evaluate the available database of captan genotoxicity studies. In this WOE, more weight was placed on the results from the higher tier *in vivo* studies.

Although the lower tier studies are intentionally designed to optimize sensitivity, the higher tier studies are more relevant to determine the potential for somatic and heritable mutations in humans.

Captan induces gene mutation in microbial systems, and the mutation frequency is greatly diminished or eliminated by the addition of thiol containing components. *In vitro* mammalian test systems for both gene mutation and chromosomal damage show mixed results overall in the absence of S9 and little or no activity when S9 is present.

The collective *in vivo* tests indicate the absence of genotoxicity. In particular, captan does not induce clastogenic changes in the carcinogenic target tissue, the mouse duodenum. The WOE conclusion is that captan is not an *in vivo* genotoxicant.

Captan does not produce mutagenic or clastogenic effects following oral ingestion *in vivo*. If allowed access to DNA, as in *in vitro* test systems, captan and/or thiophosgene have the ability to induce mutagenic effects in prokaryotic and eukaryotic cells. The presence of thiol-containing small molecules and macromolecules in the whole animal neutralize captan before it can induce DNA damage.

3.9 Carcinogenicity

3.9.1 Animal data

3.9.1.1 Study 1 (1982)

Reference:	2-year oral toxicity/carcinogenicity study of captan in rats
Author(s), year:	Anonymous (1982a)
Report/Doc. number:	International Research and Development Corporation, unpublished report No. 153-097 (Company file: R-9282/TMN-0768)
Guideline(s):	In-house method
GLP:	Yes; some parts of the study were conducted prior to 20 June 1979 (the effective date of GLP Regulation), however, it does not affect the validity of the study.
Deviations to OECD 453 (2009):	The study met the essential criteria of the test guideline.
Acceptability:	Yes

Material and Methods

In a 2-year carcinogenicity/toxicity feeding study with captan (purity 89%; batch no. SX-944), CD strain rats were administered test substance at dosage levels of 0, 25, 100 and 250 mg/kg bw/day (70/sex/dose), based on the most recent group mean food intake and body weight data. Interim sacrifices were conducted with 10 rats/sex/dose at 12 and 18 months of the study. At study termination all surviving animals were sacrificed and necropsied.
Parameters evaluated were a determination of the concentration of captan in the diet, clinical signs, behaviour and mortality (twice daily), body weight and food intake (pre-test, weekly through to week 14, and once every two weeks thereafter), ophthalmoscopic examination (pre-test and at 6, 12, 18 and 24 months), haematology, clinical chemistry and urinalysis (pre-test, at 6, 12 and 18 months in 10 rats/sex/dose, and at 24 months in 20 rats/sex/dose), gross pathology, organ weights at scheduled sacrifices (heart, kidneys, liver, spleen, brain, testes, ovaries, pituitary, thyroid/ parathyroids and adrenals) and histopathology.

Findings

Levels of captan in the diet: Test diets sampled during the study contained from 63 to 131% of the target concentrations (average of duplicate samples). The average recovery over the study period was 98 to 99% at all dose levels. Analysis of the diet indicated that the distribution of captan was sufficiently homogeneous, being within 10% of the respective mean concentration for that batch of diet. Test diets stored for 7 days at room temperature contained 83 to 131% of the initial captan concentration. The test article was considered to be stable under laboratory conditions.

General observations: There were no treatment-related effects on general appearance, behaviour, survival or food consumption. Throughout the study, the survival was considered generally similar for the control and treated rats. Group mean body weights were significantly lower than the control in the 100 and 250 mg/kg bw/day dose groups in males (Figure 3.9.1.1-1) and females (Figure 3.9.1.1-2) throughout the treatment period, with terminal body weights 12 to 20% lower than controls. Slight decreases in group mean body weights were noted at 25 mg/kg bw/day. These slight decreases were statistically significant for males at week 25 and females at weeks 66 and 80, but not considered to be biologically significant (Table 3.9.1.1-2). The group mean body weights at week 104 are shown in Table 3.9.1.1-1.

Figure 3.9.1.1-1 Oral carcinogenicity/toxicity study in the rat: body weight in males (males)

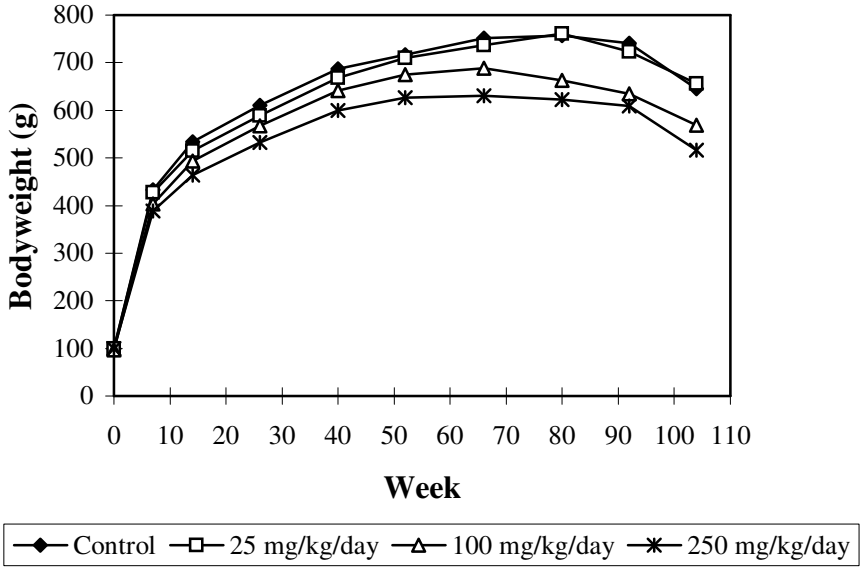
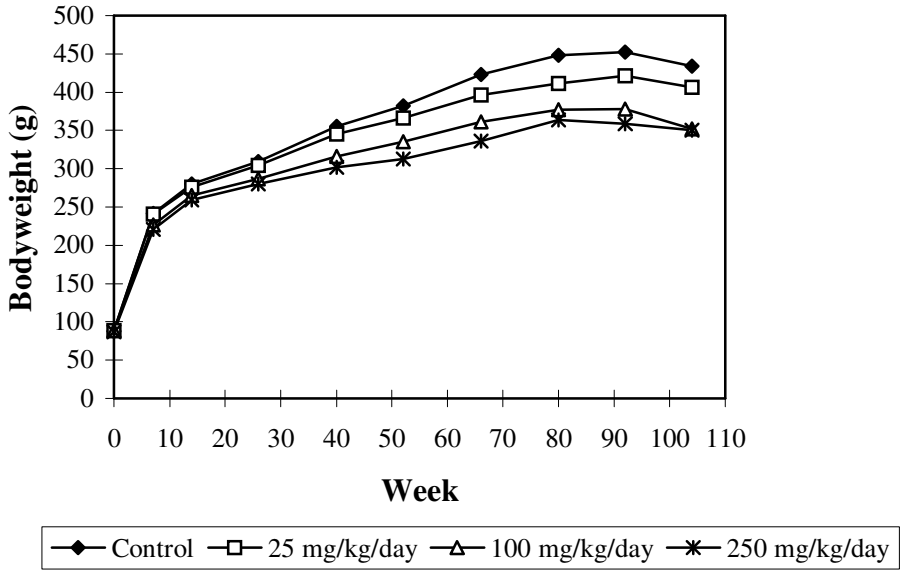


Figure 3.9.1.1-2 Oral carcinogenicity/toxicity study in the rat: body weight in females (mean)



Although statistically significant decreases in food consumption were noted for all treated groups throughout the study, these decreases were slight and not considered biologically significant. The group mean food consumption values through 104 weeks of study were as follows.

Table 3.9.1.1-1 Oral carcinogenicity/toxicity study in the rat: Group mean food consumption values through 104 weeks of study and group mean body weights at the end of the study (week 104)

Dosage level (mg/kg bw/day)	**Mean food consumption (g/rat/day) (% difference from control)**		**Mean body weight (g) (% difference from control)**	
	Males	**Females**	**Males**	**Females**
0 (control)	25.3	18.7	646	434
25	25.7 (+1.6)	18.1 (-3.2)	656 (+1.5)	406 (-6.5)
100	24.7 (-2.4)	18.0 (-3.7)	569 (-11.9)	352 (-18.9)
250	24.0 (-5.1)	17.8 (-4.8)	516 (-20.1)	350 (-19.4)

Table 3.9.1.1-2 Oral carcinogenicity/toxicity study in the rat: Group mean (with % difference from control in brackets), standard deviation (SD), number of animals (N) and statistical significance for body weights at selected weeks of the study

Week of study		**Dosage level (mg/kg bw/day)**			
		0 (control)	**25**	**100**	**250**
Males					
26	Mean (%)	610	**589*** (-3.4)	**567**** (-7.0)	**533** (-12.6)**
	SD	61.0	56.0	51.7	61.6
	N	68	67	67	67
66	Mean (%)	752	737 (-2.0)	**688**** (-8.5)	**630** (-16.2)**
	SD	92.8	89.9	82.9	82.9
	N	54	56	56	54
80	Mean (%)	757	751 (-0.8)	**663** (-12.4)**	**623** (-17.7)**
	SD	108.7	96.8	95.0	81.8
	N	43	43	44	38
104	Mean (%)	646	656 (+1.5)	**569* (-11.9)**	**516** (-20.1)**
	SD	132.5	139.4	77.4	88.0
	N	28	24	20	24
Females					
26	Mean (%)	309	304 (-1.6)	**287**** (-7.1)	**280**** (-9.4)
	SD	48.7	31.2	27.5	29.6
	N	69	70	70	68
66	Mean (%)	428	**396*** (-7.5)	**361** (-15.7)**	**336** (-21.5)**
	SD	81.8	68.0	55.0	54.6
	N	57	55	57	53
80	Mean (%)	448	**411*** (-8.3)	**377** (15.8)**	**364** (-18.7)**
	SD	76.1	79.9	62.4	65.0
	N	47	41	46	39
104	Mean (%)	434	406 (-6.5)	**352** (-18.9)**	**350** (-19.4)**
	SD	74.8	93.5	77.5	92.4
	N	28	26	29	31

* Significantly different from the control group mean ($p < 0.05$).
** Significantly different from the control group mean ($p < 0.01$).

Ophthalmoscopy: There were no treatment-related ophthalmological changes.

Haematology, clinical chemistry and urinalysis: There were no treatment-related effects on haematological or clinical chemistry parameters. The lower LDH values noted for the mid and high dose rats at 12 months of the study were not seen at any other interval of analysis and were therefore not considered to be related to the test substance. Urinanalysis values were similar for control and treated rats.

Gross pathology, organ weights, histopathology: No toxicologically significant macroscopic changes related to the administration of captan were observed during necropsy of rats in this study at the 12 and 18 months interim sacrifices, terminal sacrifice or in rats that died during the entire study period.
Statistically significant increases in mean absolute and relative liver and kidney weights were observed at the 18-month sacrifice in 250 mg/kg bw/day males (Table 3.9.1.1-3). There were no toxicologically significant changes in absolute or relative organ weights at terminal sacrifice. Any other statistically significant increases in relative organ weights at interim or terminal sacrifice were considered to be mostly due to decreases in body weight or spurious in nature, and therefore not toxicologically relevant.
The increased liver weight in 18-month interim sacrifice 250 mg/kg bw/day males was related to the significant hepatocellular hypertrophy of a centrilobular, focal, multifocal or diffuse nature observed microscopically in the 250 mg/kg bw/day group. This lesion was present in all 10 males and 8 of the 10 females, compared to zero incidence in other treatment groups and the controls. Hepatocellular hyperthrophy was not evident in animals at the 12 month interim sacrifice. At terminal sacrifice and in animals that died between 12 months to termination, hepatocellular hyperthrophy was observed in few animals at all dose levels and in the control group.
No microscopic changes were noted in the kidney. There were no other treatment-related microscopic findings. The incidence of microscopic neoplastic and non-neoplastic lesions was comparable between treatment groups and the controls. There were no statistically or toxicologically significant increases in any tumour type, total tumours, total benign tumours or total malignant tumours.

Table 3.9.1.1-3 Oral carcinogenicity/toxicity study in the rat: absolute and relative liver and kidney weights in male rats (18 month interim sacrifice)

Tissue	Dose group (mg/kg bw/day)			
	Control	25	100	250
Liver:				
Absolute (g)	22.42 ± 2.611	23.30 ± 4.922	25.84 ± 2.220	30.40 ± 4.475**
Relative (%)	3.23 ± 0.296	3.28 ± 0.545	3.62 ± 0.475	4.74 ± 0.356**
Kidney				
Absolute (g)	2.50 ± 0.397	2.66 ± 0.385	2.98 ± 0.687	3.21 ± 0.405**
Relative (%)	0.36 ± 0.024	0.38 ± 0.054	0.41 ± 0.088	0.51 ± 0.062**

** Significantly different to the control ($p < 0.01$).

Table 3.9.1.1-4 Oral carcinogenicity/toxicity study in the rat: absolute and relative (% body weight) mean organ weights in rats – extended table

Dose groups (mg/kg bw/day)	Males				Females			
	Control	25	100	250	Control	25	100	250
12-month Interim Sacrifice								
Body weight (g)	755	686	653	607	355	354	321	308
Liver, absolute (g)	25.73	22.92	23.77	23.26	12.31	12.09	10.93	13.22
Liver, relative (%)	3.39	3.36	3.65	3.87	3.45	3.43	3.41	4.29**
Adrenal, absolute (mg)	51	69*	58	65	75	78	82	88
Adrenals, relative (% x 10^2)	0.67	1.01**	0.90*	1.08**	2.15	2.23	2.54	2.85*
Kidney, left, absolute (g)	2.43	2.60	2.72	2.69	1.40	1.37	1.43	1.52
Kidney, left, relative (%)	0.32	0.38*	0.42**	0.45**	0.40	0.39	0.45	0.50**
Kidney, right, absolute (g)	2.47	2.62	2.70	2.79	1.48	1.42	1.47	1.55
Kidney, right, relative (%)	0.33	0.38	0.42*	0.47**	0.42	0.41	0.46	0.50*
Testes, absolute (g)	4.13	4.11	3.81	3.88	-	-	-	-
Testes, relative (%)	0.55	0.61	0.58	0.65*	-	-	-	-
Heart, absolute (g)	1.72	1.85	1.76	1.69	1.00	1.15	1.09	1.16
Heart, relative (%)	0.23	0.27**	0.27**	0.28**	0.31	0.33	0.34	0.38**
Brain, absolute (g)	2.23	2.33	2.23	2.16	2.02	2.04	2.01	2.00
Brain, relative (%)	0.30	0.34*	0.34*	0.36**	0.58	0.58	0.63	0.66
18-month Interim								

Sacrifice								
Body weight (g)	695	714	720	640	394	424	381	330
Liver, absolute (g)	22.42	23.30	25.80	30.40**	14.15	14.33	14.43	13.73
Liver, relative (%)	3.23	3.28	3.62	4.74**	3.59	3.42	3.85	4.19*
Adrenal, absolute (mg)	68	78	82*	190	86	101	123	96
Kidney, left, absolute (g)	2.48	2.64	2.97	3.45**	1.50	1.59	1.50	1.51
Kidney, left, relative (%)	0.36	0.37	0.41	0.54**	0.40	0.38	0.40	0.47
Kidney, right, absolute (g)	2.50	2.66	2.98	3.23**	1.51	1.63	1.58	1.55
Kidney, right, relative (%)	0.36	0.38	0.41	0.51**	0.41	0.39	0.42	0.48
Heart, absolute (g)	1.87	2.05	2.19*	2.04	1.32	1.39	1.35	1.29
Heart, relative (%)	0.27	0.29	0.30	0.32**	0.36	0.33	0.36	0.40
Terminal Sacrifice								
Body weight (g)	640	648	563	504	430	405	341	345
Liver, absolute (g)	23.15	21.11	21.13	22.34	15.42	14.17	14.37	15.93
Liver, relative (%)	3.68	3.37	3.82	4.46**	3.62	3.48	4.23*	4.59**
Adrenal, absolute (mg)	113	126	94	87	150	119	159	127
Kidney, left, absolute (g)	2.78	2.92	3.40*	3.21	1.70	1.56	1.70	1.70
Kidney, left, relative (%)	0.46	0.48	0.62*	0.65**	0.41	0.39	0.52**	0.51**
Kidney, right, absolute (g)	2.81	2.89	3.33	3.18	1.77	1.57*	1.75	1.76
Kidney, right, relative (%)	0.46	0.47	0.60*	0.64**	0.42	0.40	0.53*	0.53**
Testes, absolute (g)	3.22	3.48	3.22	3.13	-	-	-	-
Testes, relative (%)	0.50	0.56	0.58	0.61**	-	-	-	-
Heart, absolute (g)	2.16	2.15	2.12	2.11	1.61	1.51	1.43*	1.39**
Heart, relative (%)	0.35	0.35	0.38	0.43**	0.38	0.38	0.43	0.42
Thyroid/Parathyroid, absolute (mg)	47	58*	49	50	39	43	41	48
Thyroid/Parathyroid, relative (% x 10^2)	0.75	0.95	0.88	1.01*	0.93	1.10	1.23**	1.50*
Brain, absolute (g)	2.25	2.30	2.21	2.15	2.06	2.03	1.99	2.00
Brain, relative (%)	0.37	0.37	0.40	0.44**	0.49	0.52	0.61**	0.61**

* Significantly different to the control ($p < 0.05$)
** Significantly different to the control ($p < 0.01$)

Table 3.9.1.1-5 Oral carcinogenicity/toxicity study in the rat: Summary of key histopathology findings (non-neoplastic) at 24 months in males

Organ and Finding	**Dosage (mg/kg bw/day)**			
	0	**25**	**100**	**250**
Liver (Number Examined)	43	45	44	36
Cytoplasmic Vacuolation, Hepatocytes	21	21	29	21
Sinusoidal Congestion	32	30	26	24
Bile Duct Proliferation	19	15	20	17
Dilated Bile Duct	1	0	0	0
Bile Duct Hyperplasia	0	0	0	1
Angiectasis	1	0	0	0
Focal Fibrosis	0	1	0	0
Peritonitis	0	1	0	0
Periportal/sinusoidal Lymphoid Infiltrates	33	30	33	33
Adhesions	1	0	0	1
Focal Necrosis	4	5	6	3
Focal Degeneration	7	11	7	8
Hepatocellular Hypertrophy	2	2	8	0
Hepatocellular Hyperplasia	0	2	0	0
Nodular Hyperplasia	1	0	3	1
Centrilobular hypertrophy	0	0	0	12

Cyst(s)	1	3	0	1
Kidneys (Number Examined)	43	45	44	36
Mineralized Debris, Pelvis	0	2	2	2
Mineralization	0	0	0	0
Chronic Nephritis	43	45	44	36
Pyelonephritis	0	0	0	1
Proteinaceous Tubular Cysts	11	13	22	27
Pyelitis, acute, diffuse, bilateral, slight	1	5	0	1
Pelvic Dilation	4	3	5	7
Periarteritis	2	0	2	0
Hyaline droplet change	1	0	0	0
Arteriosclerosis	1	1	0	0
Hemorrhage	1	0	0	3
Abscessation	0	1	0	0
Adrenals (Number Examined)	43	45	44	36
Haematopoeisis	0	0	0	0
Congestion	8	15	3	5
Medullary cell hyperplasia, local, unilateral	8	5	7	2
Cortical Nodule	1	1	0	0
Cortical cell hyperplasia	1	0	0	1
Cytoplasmic Vacuolation, Cortical Cells	22	27	22	20
Heart (Number Examined)	43	45	44	36
Arteriosclerosis	4	4	1	0
Pararteritis	5	4	6	4
Pericarditis	0	1	0	0
Arterial Thrombosis	0	0	1	1
Endocarditis	0	2	0	0
Interstitial Lymphoid Infiltrates	4	1	2	2
Focal Necrosis	0	1	0	0
Focal anganation	29	27	26	17
Thyroid/Parathyroid (Number Examined)	43	45	44	36
Focal C-Cell Hyperplasia	0	1	1	0
Parathyroid Hyperplasia	9	5	10	11
Periarteritis	0	0	1	0
Cystic follicle(s)	4	3	1	2

Table 3.9.1.1-6 Oral carcinogenicity/toxicity study in the rat: Summary of key histopathology findings (non-neoplastic) at 24 months in females

Organ and Finding	**Dosage (mg/kg bw/day)**			
	0	**25**	**100**	**250**
Liver (Number Examined)	47	40	46	38
Cytoplasmic vaculation	31	24	29	26
Perihepatitis	0	0	0	1
Adhesions	1	0	0	0
Sinusoidal Congestion	34	18	22	17
Bile Duct Proliferation	12	14	9	7
Bile Duct Hyperplasia	0	0	0	1
Angioectasia	0	0	0	1
Periportal/sinusoidal Lymphoid Infiltrates	29	23	21	22
Focal Necrosis	5	5	3	6
Cholangiofibrosis	0	0	0	1
Hepatocellular Hypertrophy	7	5	8	1
Hepatocellular Hyperplasia	1	1	1	1
Nodular Hyperplasia	2	1	0	0
Centrilobular hypertrophy	0	0	0	4
Cyst(s)	1	0	1	1

Extramedullary Haematopoiesis	4	2	2	1
Kidneys (Number Examined)	47	40	46	39
Hyperplasia, pelvic epithelium, focal, unilateral, slight	11	11	9	7
Mineralized Debris, Pelvis	16	19	18	9
Mineralization	0	1	0	0
Chronic Nephritis	46	37	43	36
Pyelonephritis	0	0	1	1
Tubular Nephrosis	1	1	0	0
Proteinaceous Tubular Cysts	4	1	4	1
Pyelitis	1	0	2	0
Pelvic Dilation	6	6	4	0
Hyaline droplet change	0	1	2	0
Arteriosclerosis	1	0	0	0
Congestion	1	0	0	0
Haemorrhage	1	1	0	0
Haematopoeisis	1	0	0	0
Embolic Pyelonephritis	1	1	0	0
Adrenals (Number Examined)	46	40	46	39
Haematocyst	0	1	0	1
Focal Necrosis	0	0	1	0
Congestion	41	37	41	34
Medullary cell hyperplasia, local, unilateral	5	3	3	0
Haematopoeisis	1	0	1	0
Cytoplastic Vacuolation, Cortical Cells	31	21	25	24
Heart (Number Examined)	47	40	46	39
Arteriosclerosis	2	0	0	1
Periarteritis	0	0	2	0
Pericarditis	0	0	0	1
Interstitial Lymphoid Infiltrates	4	1	1	0
Focal Necrosis	0	1	0	0
Thyroid/Parathyroid (Number Examined)	47	40	46	39
Congestion	0	0	1	0
Focal C-Cell Hyperplasia	2	0	0	2
Parathyroid Hyperplasia	3	1	0	0
Cystic follicle(s)	0	0	5	3

Table 3.9.1.1-7 Oral carcinogenicity/toxicity study in the rat: summary of neoplastic lesions and tumor incidences

Dose groups (mg/kg bw/day)	**Males**				**Females**			
	Control	**25**	**100**	**250**	**Control**	**25**	**100**	**250**
Summary of neoplastic lesions								
No. of animals	70	70	70	70	70	70	70	70
Animals with tumors (n)	34	28	31	27	48	43	50	50
Animals with tumors (%)	49%	40%	44%	38%	69%	61%	71%	71%
Total no. of tumors	46	32	41	34	91	68	99	84
Total no. of benign tumors	38	28	35	30	81	61	86	75
Total no. of malignant tumors	8	4	6	4	9	7	13	9
Animals with benign tumors (n)	29	25	26	24	44	41	47	48
Animals with benign tumors (%)	41%	36%	37%	34%	63%	59%	67%	69%
Animals with malignant tumors (n)	8	4	6	4	9	7	13	8

Animals with malignant tumors (%)	11%	6%	9%	6%	13%	10%	19%	11%
Tumor incidences								
Abdominal cavity, cortical cell carcinoma	-	1	-	-	-	-	-	-
Abdominal cavity, lipoma	-	-	-	-	-	1	-	-
Adrenal, pheochromocytoma	9	2	3	2	-	-	-	-
Adrenal, cortical cell carcinoma	-	-	-	-	1	-	-	-
Adrenal, cortical cell adenoma	-	-	-	-	-	-	1	1
Aorta, malignant aortic body tumor	-	-	-	-	-	1	-	-
Brain, gliosarcoma	1	-	-	-	-	-	-	-
Brain, astrocytoma	-	1	-	-	-	-	-	-
Brain, glioma	-	1	-	1	-	-	-	-
Cervix, polyp	-	-	-	-	-	-	2	1
Duodenum, adenoma	1	-	-	-	-	-	-	-
Duodenum, leiomyoma	1	-	-	-	-	-	-	-
Ear, squamous cell carcinoma, Zymbal´s gland	-	-	-	1	-	-	1	-
Foot, osteoma	-	-	-	-	1	-	-	-
Heart, haemangioma	-	-	-	1	-	-	-	-
Haemolymphoreticular system, lymphosarcoma / malignant lymphoma	1	-	2	1	2	-	1	3
Haemolymphoreticular system, malignant fibroblastocytoma	1	-	-	-	-	1	2	-
Haemolymphoreticular system, haemangiosarcoma	1	-	-	-	-	-	1	-
Haemolymphoreticular system, reticulum cell sarcoma	-	-	-	-	-	1	-	-
Haemolymphoreticular system, sarcoma undifferentiated	-	-	-	-	-	-	1	-
Harderian gland, adenoma	-	-	-	-	1	-	-	-
Harderian gland, adenocarcinoma	-	-	-	-	-	1	-	-
Kidney, cortical cell adenoma /tubular adenoma	1	-	2	3	-	-	-	-
Kidney, renal cell carcinoma / tubular adenocarcinoma	-	1	1	1	-	-	-	-
Kidney, liposarcoma	1	-	1	-	-	-	-	-
Kidney, mesenchymal tumor	-	-	-	-	-	-	-	1
Lip, papilloma	-	-	1	-	-	-	-	-
Liver, hepatocellular adenoma	-	-	1	-	-	-	-	-

Liver, hepatocellular carcinoma	-	-	-	-	-	-	1	-
Mammary gland, fibroadenoma	-	-	-	-	21	16	23	19
Mammary gland, intraductal papilloma	-	-	-	-	2	1	2	1
Mammary gland, cystademona, papillary / adenoma	-	-	-	-	4	5	10	6
Mammary gland, adenocarcinoma, papillary	-	-	-	-	1	3	3	5
Mammary gland, adenoma	1	-	1	-	-	-	-	-
Mouth, fibroma	-	1	-	-	-	-	-	-
Ovary, luteal cell adenoma	-	-	-	-	-	1	-	-
Ovary, granulosa cell adenoma	-	-	-	-	-	-	1	-
Ovary, thecal cell adenoma	-	-	-	-	-	-	-	1
Paranasal cavity, squamous cell carcinoma	1	-	-	-	-	-	2	-
Parathyroid, adenoma	-	1	1	-	-	-	-	-
Pancreas, islet cell adenoma	3	2	3	1	5	1	1	3
Pancreas, acinar cell adenoma	-	-	-	-	-	1	-	-
Pituitary, adenoma	16	13	18	15	38	31	36	35
Pituitary, carcinoma	-	1	-	-	-	-	-	-
Skeletal muscle, rhabdomyoma	1	1	-	-	-	-	-	-
Seminal vesicles, adenocarcinoma	-	-	1	-	-	-	-	-
Skin, papilloma	1	-	1	-	-	-	-	1
Skin, fibrosarcoma	2	-	-	-	-	-	-	-
Skin, keratoacanthoma	-	-	1	1	-	-	1	-
Skin, squamous cell carcinoma	-	-	1	1	2	-	-	-
Skin, basal cell adenoma	-	-	-	-	1	-	-	-
Spleen, haemangioma	-	-	-	-	-	1	-	-
Stomach, fibrosarcoma	-	-	-	-	1	-	-	-
Subcutis, lipoma	-	1	1	-	1	1	-	-
Subcutis, fibroma	-	1	1	1	-	1	-	-
Subcutis, fibrosarcoma	-	1	-	-	-	-	-	1
Testes, interstitial cell adenoma	1	2	-	1	-	-	-	-
Thyroid, follicular cell adenoma	2	-	-	2	-	-	-	1
Thyroid, c-cell adenoma	1	2	-	2	1	-	1	2
Thymus, thyoma	-	-	-	-	-	-	-	1
Urinary bladder, transitional cell adenoma	-	-	1	-	-	-	-	-
Urinary bladder, transitional cell papilloma	-	-	-	-	-	-	-	1

Uterus, polyp	-	-	-	-	4	2	6	1
Uterus, leiomyosarcoma	-	-	-	-	1	-	1	-
Uterus, adenomcarcinoma	-	-	-	-	1	-	-	-
Vagina, polyp	-	-	-	-	2	-	1	-
Vagina, leiomyoma	-	-	-	-	-	-	1	-

Conclusions

The NOAEL in the 2-year toxicity/carcinogenicity feeding study in rats was 25 mg/kg bw/day in males and females based on significant body weight decreases, larger than 10% compared to the controls, in the 100 mg/kg bw/day dose group and above. Captan administered in the diet was not oncogenic in the rat at levels equivalent of up to 250 mg/kg bw/day.

3.9.1.2 Study 2 (1983)

Reference:	Life-span oral carcinogenicity study of Merpan in rats
Author(s), year:	Anonymous (1983)
Report/Doc. number:	TNO Division for nutrition and food reserach, The Netherlands; unpublished report No. V 83.233/200153 (Company file: R-3608)
Guideline(s):	In-house method (equivalent to EC method 87/302/EEC part B)
GLP:	Yes
Deviations to OECD 453 (2009):	duration of study 130 weeks instead ot 104 weeks, measurements of food consumption and food efficiency should be made at least monthly after the first 13 weeks, clinical biochemistry and urinalysis were not performed, ophthalmosopy scheduled for week 0 and week 13 inadvertently not performed
Acceptability:	Yes

Material and Methods

In a 130-week carcinogenicity feeding study with captan (purity and batch no. not specified in report), SPF (Cpb:WU; Wistar random) strain rats were administered test substance at dietary concentrations of 0, 125, 500 and 2000 ppm, (50/sex/dose). Parameters evaluated were a determination of the concentration of captan in the diet, clinical signs (daily), palpable tumours (weekly), mortality (daily, then twice daily from 18 months onward), ophthalmoscopic examination (pretest and at weeks 27, 52, 77 and 104), body weight (pretest and weekly until week 12, once every two weeks thereafter), food intake (weekly from week 1 to 12, and at weeks 23/24, 35/36, 47/48, 59/60, 71/72, 83/84, 95/96, 107/108, 119/120 and 129/130) and food efficiency (over the first 12 weeks), haematology (at weeks 13, 27, 53, 79 and 105), gross pathology, organ weights (heart, kidneys, liver, spleen, brain, testes, ovaries, pituitary, thyroid and parathyroids and adrenals) and histopathology.

Findings

Levels of captan in the diet: Analysis of the diet indicated that the distribution of captan was sufficiently homogeneous (coefficient of variation was < 5%). On average the levels of captan in the diet were 124, 504 and 2083 ppm for the 125, 500 and 2,000 ppm diets, respectively. Storage of diet for five weeks resulted in considerable loss of captan, with losses amounting to 66, 41 and 28% for the low-, mid- and high- doses, respectively. Losses over a two to four week period amounted to 40, 10 and 5%, respectively. Diets in the present study were stored for less than three weeks and the levels in diets consumed by rats were approximately 20, 5 and 2.5% less than the intended dose, equivalent to approximately 0, 5, 24 and 98 mg/kg bw/day for the low-, mid- and high-dose groups, respectively.

General observations: There were no treatment-related clinical signs. Survival rate was not affected by treatment. Body weight was decreased in the 2000 ppm male (Figure 3.9.1.2-1) and female (Figure 3.9.1.2-2) dose groups throughout the study. The difference was significantly different to the control during the first two years of the study in males and, for the majority of the study, in females. The overall decrease in body weight at 2000 ppm was about 10% for both males and females.

Figure 3.9.1.2-1 Oral carcinogenicity study in the rat: group mean body weights in males

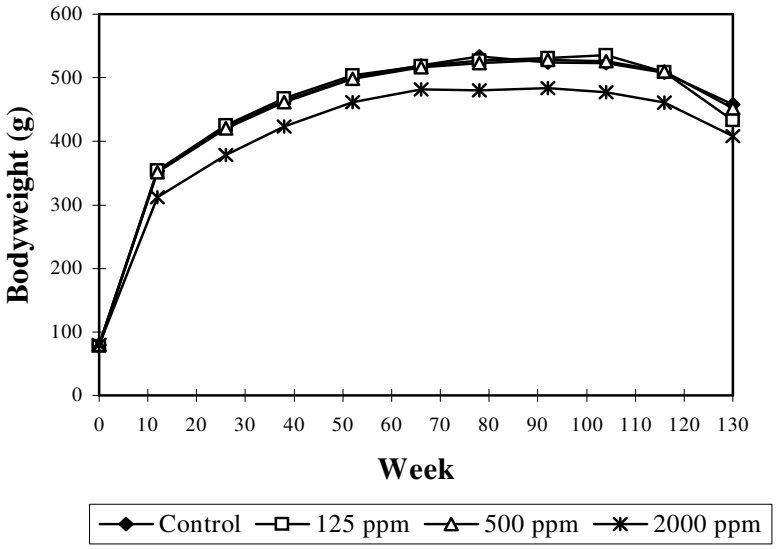
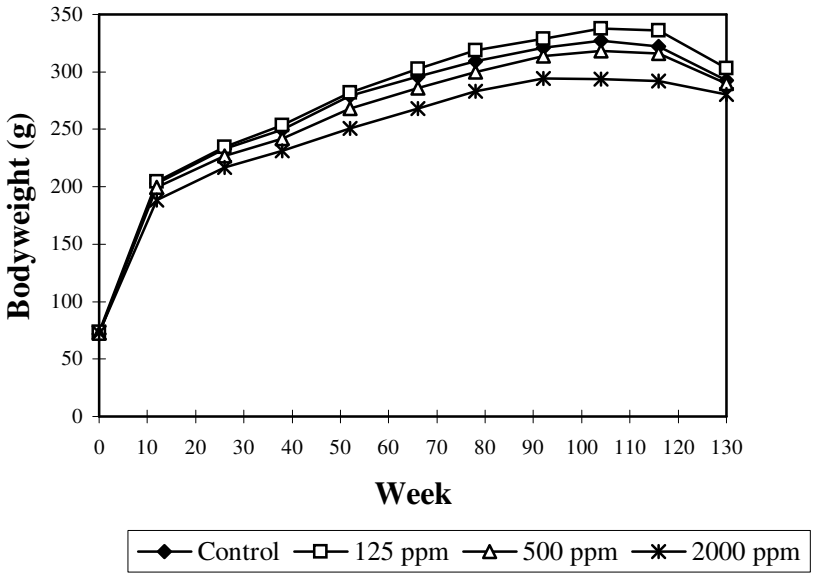


Figure 3.9.1.2-2 Oral carcinogenicity study in the rat: group mean body weights in females



Food intake was slightly diminished in 2000 ppm males (Figure 3.9.1.2-3) and females (Figure 3.9.1.2-4) for the first two years of the study, however intakes during the last half year of the study were comparable to the controls. Differences with controls were statistically significant at nearly all stages in males as well as in females during the first year.
At 2000 ppm, food efficiency was reduced, however not statistically significantly, in the first week of the study in males (0.293 g bodyweight gain/g food/week in the 2000 ppm group compared to 0.416 g gain/g food/week in the control) and females (0.286 g gain/g food/week in the 2000 ppm group 0.371 g gain/g food/week in the control). For the majority of the other timepoints measured, food efficiency was either comparable or even increased at 2000 ppm compared with the controls.

Figure 3.9.1.2-3 Oral carcinogenicity study in the rat: food intake in males (mean)

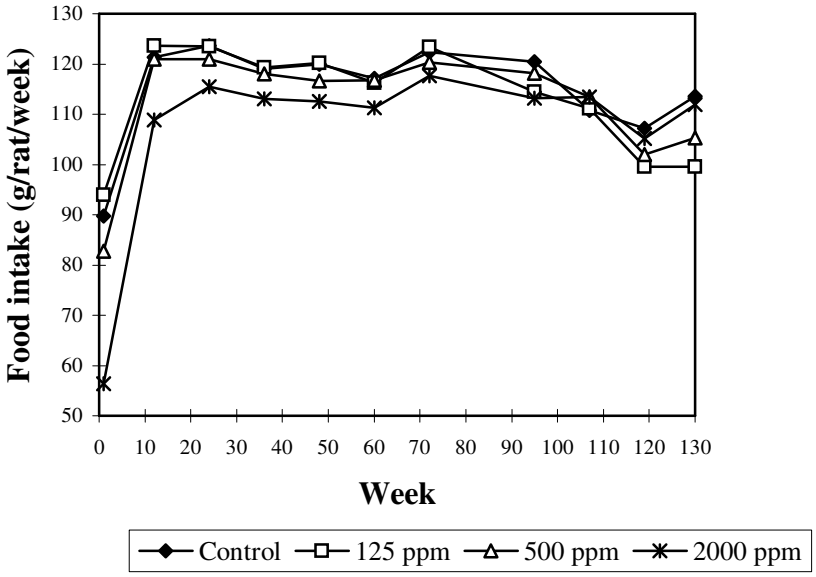
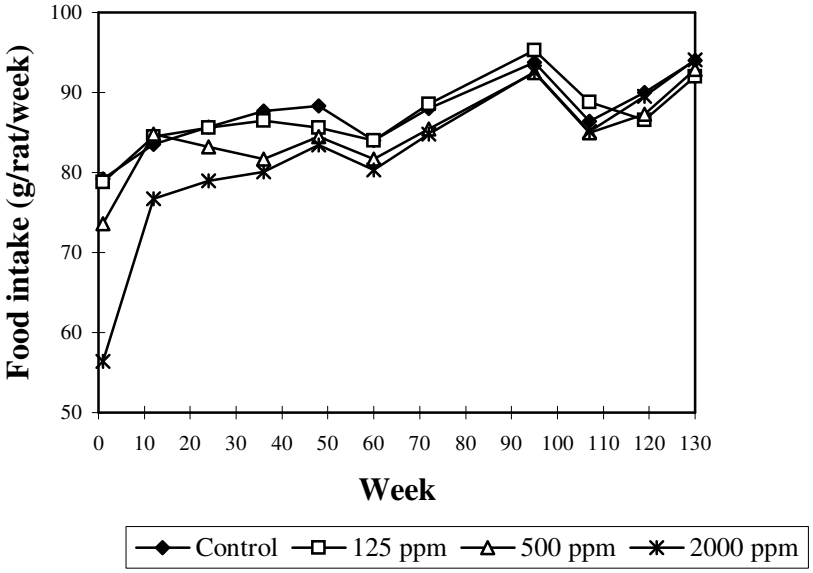


Figure 3.9.1.2-4 Oral carcinogenicity study in the rat: food intake in females (mean)



Ophthalmoscopy: There were no treatment-related ophthalmological changes.

Haematology: There were no treatment-related effects on haematological parameters.

Gross pathology, organ weights, histopathology: The incidence of kidneys with a granular appearance was increased in 2000 ppm males compared to the controls (Table 3.9.1.2-1). However, this finding was not confirmed microscopically and is therefore probably of little toxicological significance. No other treatment related effects were observed upon gross examination.
Microscopic examination revealed an increased incidence of brown pigment accumulated in the lungs of females in the 2000 ppm dose group compared to the control. A decrease in the frequency of hepatocellular vacuolation in males and of periarteritis and fatty atrophy in the pancreas of females was observed in the 2000 ppm groups compared to the control.

However, the incidence of all of these microscopic lesions was considered to be consistent with background data of other recent life-span studies carried out at the same institute with the same strain of rat. Furthermore the incidence of these lesions is considered to be variable in rats of this age and therefore the effect is not considered to be toxicologically significant.

Table 3.9.1.2-1 Oral carcinogenicity study in the rat: macroscopic changes - incidence of granular surface to kidney in males

Dose group			
Control	**125 ppm**	**500 ppm**	**2000 ppm**
10/50[1]	12/50	18/50	25/50

[1] Number of animals affected/number observed.

There was no treatment-related increase in the incidence of hyperplastic or preneoplastic lesions.
Neoplasms observed most frequently were fibroadenomas of the mammary gland and polyps of the uterus in females and adenomas of the pituitary in males and females. A few tumour types, not common in the strain of rats used, were found in a small number of animals in one group only i.e. sarcoma of the uterus in the top-dose group and leiomyosarcomas of the small intestine in males of the mid-dose group. Also lymphosarcomas were observed in a few animals. However, there was no statistically significant difference in the incidence of any tumour type between test groups and the controls, and no relationship between dose and tumour incidence was observed. No association between the occurrence of tumours and the presence of hyperplastic or pre-neoplastic changes was visible.

Table 3.9.1.2-2 Oral carcinogenicity study in the rat: summary of non-neoplastic changes in males (number of observations/investigated animals)

Histopathological findings	**Dose Levels**			
	0	**125**	**500**	**2000**
Adrenals				
Medullary Basophilic Focus	9/50	10/50	6/49	2/49
Ganglion-Cell Proliferation	-	2/50	1/49	1/49
Small cortical Small Cell Focus	-	-	1/49	-
Haemorrhagic Cortical Degeneration	5/20	4/50	1/49	2/20
Cortical Vacuolation	5/20	21/50	19/49	4/20
Sinusoidal Dilation	-	-	1/49	-
Kidneys				
Nephrosis				
Very Slight	1/19	NE	NE	1/20
Slight	7/19	NE	NE	5/20
Moderate	3/19	NE	NE	6/20
Severe	1/19	NE	NE	4/20
Very Severe	3/19	NE	NE	1/20
Mononuclear-cell Infiltrate	6/19	NE	NE	4/20
Periolomerular Sclerosis	-	NE	NE	1/20
Pelvic Mineralisation	1/19	NE	NE	3/20
Liver				
Bile Duct Proliferation	16/49	24/49	25/50	15/50
Focus of Cellular Alteration: Clear Cell Type	6/49	4/49	3/50	5/50
Focus of Cellular Alteration: Basophilic Type	-	1/49	-	-
Bile Duct Sclerosis	9/19	6/49	13/50	6/20
Mononuclear-cell Infiltrate	2/19	3/49	7/50	2/20
Hepatocellular Vacuolation	6/19	5/49	8/50	1/20
Aggregates of RES Cells and Necrotic Hepatocytes	-	3/49	4/50	1/20
Congestion	-	1/49	-	-
Hepatocellular Necrosis	-	2/49	1/50	1/20
Focal Brown Pigment Accumulation	-	-	1/50	-

Pancreas				
Periarteritis	2/20	NE	NE	2/20
Fatty Atrophy	1/20	NE	NE	2/20
Change of Acini Into Duct Like Structures	3/20	NE	NE	-
Loss of Zymogen Granules	-	NE	NE	1/20
Small Intestines				
Hyperplasia of Patches of Peyer	-	NE	NE	1/20
Spleen				
Extra Medullary Haematopoiesis				
Very slight	-	1/50	1/50	1/20
Slight	5/20	5/50	6/50	3/20
Moderate	-	1/50	1/50	1/20
Severe	-	2/50	1/50	-
Brown Pigment Accumulation				
Very Slight	-	-	2/50	-
Slight	1/20	2/50	6/50	1/20
Moderate	1/20	-	-	-
Occasional Granuloma	-	1/50	-	-
Macrophage Accumulation	-	-	1/50	-
Periarteritis	-	-	-	1/20
Stomach				
Epithelial Dysplasia	-	NE	NE	1/50
Basal-cell Hyperplasia	-	NE	NE	1/50
Fundic Glandular Dilatation	5/20	NE	NE	-
Mononuclear-cell Infiltrate	1/20	NE	NE	-
Submucosal Mineralisation	1/20	NE	NE	-
Submucosal Oedema	-	NE	NE	1/20

NE = Not evaluated

Table 3.9.1.2-3 Oral carcinogenicity study in the rat: summary of non-neoplastic changes in females (number of observations/investigated animals)

Histopathological findings	**Dose Levels**			
	0	**125**	**500**	**2000**
Adrenals				
Zona Glomerulosa Light-Cell Foci	18/49	14/47	17/50	15/49
Medullary Basophilic Focus	4/49	2/47	2/50	2/49
Ganglion-Cell Proliferation	1/49	4/47	5/50	1/49
Small Cortical Basophilic Focus	2/49	-	1/50	-
Small Cortical Light-cell Focus	1/49	1/47	-	1/49
Haemorrhagic Cortical Degeneration	4/19	21/47	11/50	2/19
Cortical Vacuolation	6/19	5/47	11/50	3/19
Sinusoidal Dilation	3/19	9/47	12/50	2/19
Thrombus	2/19	1/47	1/50	4/19
Extramedullary Haematopoiesis	1/19	3/47	-	2/19
Mononuclear-cell Infiltrate	-	2/47	-	1/19
Cortical Hypertrophic Cell Focus	-	1/47	2/50	-
Kidneys				
Urothelial Hyperplasia in Pelvis	-	-	-	1/49
Nephrosis				
Very Slight	10/18	NE	NE	10/19
Slight	6/18	NE	NE	2/19
Moderate	1/18	NE	NE	-
Severe	-	NE	NE	-
Very Severe	-	NE	NE	-
Mononuclear-cell Infiltrate	10/18	NE	NE	9/19
Brown Pigment Accumulation	11/18	NE	NE	11/19

Corticomedullary Mineralisation	8/18	NE	NE	2/19
Periolomerular Sclerosis	5/18	NE	NE	2/19
Pelvic Mineralisation	7/18	NE	NE	2/19
Medullary Mineralisation	-	NE	NE	3/19
Hydronephrosis	2/18	NE	NE	-
Periarteritis	1/18	NE	NE	-
Liver				
Bile Duct Proliferation	6/50	8/49	9/50	1/49
Focus of Cellular Alteration: Clear Cell Type	2/50	-	1/50	1/49
Small Cell Focus	2/50	2/49	2/50	2/49
Focus of Cellular Alteration: Basophilic Type	-	2/49	-	-
Bile Duct Sclerosis	3/20	2/49	5/50	1/20
Mononuclear-cell Infiltrate	9/20	8/49	8/50	8/20
Hepatocellular Vacuolation	7/20	10/49	11/50	5/20
Single Cell Necrosis	3/20	7/49	6/50	3/20
Multiple Cysts	2/20	2/49	6/50	3/20
Aggregates of RES Cells and Necrotic Hepatocytes	8/20	10/49	10/50	7/20
Congestion	-	1/49	1/50	1/20
Increased Number of Kupffer Cells	-	1/49	1/50	2/20
Hepatocellular Necrosis	-	2/49	2/50	2/20
Focal Fatty Degeneration	1/20	1/49	-	-
Bile Duct dilation	-	1/49	1/50	-
Sinusoidal Dilation	-	1/49	2/50	-
Focal Fibrosis	1/20	-	-	-
Focus of Cellular Alteration	-	-	1/50	1/20
Pancreas				
Brown Pigment Accumulation	6/19	NE	NE	3/18
Periarteritis	5/19	NE	NE	-
Fatty Atrophy	6/19	NE	NE	-
Focal Mononuclear-cell Infiltrate	4/19	NE	NE	4/18
Change of Acini Into Duct Like Structures	2/19	NE	NE	1/18
Spleen				
Extra Medullary Haematopoiesis				
Very slight	5/19	15/40	10/50	5/18
Slight	4/19	14/40	21/50	6/18
Moderate	4/19	5/40	1/50	2/18
Severe	1/19	3/40	1/50	2/18
Brown Pigment Accumulation				
Very Slight	2/19	12/40	11/50	3/18
Slight	10/19	22/40	29/50	11/18
Moderate	4/19	11/40	9/50	3/18
Lymphoid Hyperplasia	-	-	-	1/18
Polymorphonuclear Leukocytic Infiltration	-	1/40	-	-
Stomach				
Fundic Glandular Dilatation	9/18	NE	NE	5/18
Uterus				
Glandular Endometrial Hyperplasia	2/40	NE	NE	4/50
Cystic Endometrial hyperplasia	2/40	NE	NE	1/50
Glandular Proliferation	1/40	NE	NE	1/50
Glandular Dilatation	2/19	NE	NE	3/20
Atypia	1/19	NE	NE	-
Inflammatory Cell Infiltrate	1/19	NE	NE	1/20

NE = Not evaluated

Table 3.9.1.2-4 Oral carcinogenicity study in the rat: summary of neoplastic lesions and incidences of selected tumors

Dose groups	Males				Females			
	Control	125 ppm	500 ppm	2000 ppm	Control	125 ppm	500 ppm	2000 ppm
No. of animals	50	50	50	50	50	50	50	50
No. of animals with tumors	32	28	34	28	42	43	40	47
Total no. of tumors	51	41	48	48	75	79	70	76
Total no. of benign tumors	38	27	34	36	58	58	55	51
Total no. of malignant tumors	13	14	14	12	17	21	15	25
Total no. of metastatic tumors	-	-	-	-	-	1	-	2
No. of animals with benign tumors	27	21	24	24	35	37	33	36
No. of animals with malignant tumors	12	14	14	12	17	20	14	19
Mammary gland (examined)	(48)			(48)	(49)			(48)
Mammary gland, fibroadenoma	-	-	-	-	19	16	13	13
Mammary gland, adenocarcinoma	-	1	-	-	4	5	3	2
Uterus (examined)	-	-	-	-	(40)	(49)	(50)	(50)
Uterus, fibromatous polyp	-	-	-	-	7	11	12	10
Uterus, endometrial stromal sarcoma	-	-	-	-	-	-	-	2
Uterus, poorly differentiated sarcoma	-	-	-	-	-	-	-	1
Uterus, unclassified sarcoma	-	-	-	-	-	-	-	1
Pituitary (examined)	(46)	(45)	(49)	(50)	(48)	(49)	(49)	(46)
Pituitary, haemorrhagic tumor	8	7	3	10	15	16	15	14
Pituitary, solid tumor	1	3	2	1	1	3	2	1
Pituitary, malignant large haemorrhagic tumor	-	-	-	-	-	1	-	-
Pituitary, metastasizeing large haemorrhagic tumor	-	-	-	-	-	1	-	-
Small intestine (examined)	(50)			(50)	(46)			(48)
Small intestine, leiomyosarcoma	-	-	3	-	-	-	-	-
Small intestine, fibrosarcoma	-	-	-	-	-	-	1	1
Haematopoietic system, lymphosarcoma	-	-	-	1	-	1	1	2

None of the absolute organ weights showed a dose-related or significant deviation from the control value. The relative weight of the liver was slightly, but statistically significantly increased (+15%) in the 2000 ppm males (Table 3.9.1.2-5). However, this was not accompanied by any treatment-related histopathological changes and is considered to be due to the decreased body weights in the top dose males. There were no other treatment-related effects.

Table 3.9.1.2-5 Oral carcinogenicity study in the rat: relative liver weight (g/kg bw) in males (mean ± sem)

	Dose group			
	Control	**125 ppm**	**500 ppm**	**2000 ppm**
Body weight (g)	457.7 ± 13.4	432.9 ± 16.2	452.4 ± 18.1	407.7 ± 12.0
Absolute liver weight (g)	14.86 ± 0.41	14.15 ± 0.41	15.31 ± 0.70	15.25 ± 0.68
Relative liver weight (g/kg bw)	32.7 ± 0.9	33.4 ± 1.3	34.0 ± 1.1	37.6 ± 1.6*

* Significantly different to the control ($p < 0.05$); Anova + Dunnett tests (two sided).

Conclusions

The NOAEL in this 130-week life-span feeding study was 500 ppm in male and female rats (equivalent to 24 mg/kg bw/day), based on significant body weight reduction at 2000 ppm (equivalent to 98 mg/kg bw/day). Captan administered in the feed at dietary levels of up to 2000 ppm was not oncogenic in the rat.

3.9.1.3 Study 3 (1981)

Reference:	Lifetime oncogenic feeding study of captan technical (SX-944) in CD-1 mice (ICR derived)
Author(s), year:	Anonymous (1981)
Report/Doc. number:	Chevron Chemical Company, unpublished report No. SOCAL 1150 (Company file: R-8292/TMN-0767)
Guideline(s):	In-house method
GLP:	No
Deviations to OECD 453 (2009):	Limit dose of 1000 mg/kg bw/day is exceeded, small dose spacing (interval approx. 1.6), ophthalmological examination not conducted, no clinical biochemistry, no urinalysis
Acceptability:	Yes

Material and Methods

In a life-time oncogenicity feeding study captan (purity 90.7%, batch number SX-944) was administered to CD-1 strain mice at dietary concentrations of 2,000, 6,000 and 10,000 ppm for the first four weeks. From week 5, the concentrations were increased to 6,000, 10,000 and 16,000 ppm, respectively, until week 113 (80 animals/sex/dose). This alteration was made as a consequence of a report of captan administration to B6C3F1 mice which indicated an association between the occurrence of duodenal tumours and captan administration at 8,000 ppm and 16,000 ppm (National Cancer Institute (NCI) oncogenicity report). To confirm this finding, the dietary concentrations of captan were increased in the fifth week to achieve comparable exposure levels.
Control mice received untreated diet (80/sex).
Parameters evaluated were analysis of dietary concentrations, clinical signs, mortality (one to three times/day), palpation for tissue masses (weekly), body weight (weekly until week 8 and then every four weeks), food consumption (weekly until week 5 and then every four weeks), haematology (at week 56, 8/sex/dose, and at study termination on survivors previously bled at week 56 plus additional animals, if necessary, to give 8/sex/dose), gross pathology, histopathology.

Findings

Levels of captan in the diet: Weekly means demonstrated that the levels of captan in the diet were in most cases within 10% of nominal. Overall means, with only one exception, were within 5% of nominal. Diet stored for seven days contained 3% less active substance than fresh diet. Diet sampled from hoppers at the end of the weekly feeding period contained 6% less active substance than fresh diet.

General observations: An overall summary of the survival of control and treated animals is given in Table 3.9.1.3-1. Mortality over the duration of the study was increased at 16,000 ppm compared to the controls. However, survival was at least 90% in all groups at week 52. Adequate numbers of males were at risk from late appearing tumours as 83%, 79%, 75% and 68% survived in the control, 6,000 ppm, 10,000 ppm and 16,000 ppm dose groups until week 75, respectively. Survival in females at week 75 was slightly higher with 85, 89, 85 and 74%, respectively. A significantly higher incidence

of mortality in the 16,000 ppm dose group compared to the control was observed in males from week 84 to the end of the study and in females at weeks 84 and 96.

Table 3.9.1.3-1 Oral carcinogenicity study in the mouse: summary of survival

	Number of animals			
	0 ppm	**6,000 ppm**	**10,000 ppm**	**16,000 ppm**
Females				
Start of study	80	80	80	80
Found dead	37	34	35	50
Killed *in extremis*	15	14	12	11
Total deaths	52	48	47	61
Terminal sacrifice	28	32	33	19
Males				
Start of study	80	80	80	80
Found dead	34	34	45	53
Killed *in extremis*	12	14	9	17
Total deaths[3]	46	48	54	70
Terminal sacrifice	33[1]	32	25[2]	10

[1] One animal killed accidentally.
[2] One animal missing - presumed cannibalised.
[3] Total deaths (excluding accidental death) prior to terminal sacrifice.

The cause of death, as determined by histopathology was frequently not apparent or definite. However, when the condition could be determined, the cause of death in these animals was attributed to lymphosarcoma/myeloproliferative disease or duodenal neoplasms. Amyloidosis in multiple organs was also a prominent cause of death in females.

Animals in the 16,000 ppm dose group appeared thin/small, weak, emaciated and had distended puffy abdomens after week 60 and these signs were attributed to reduced food intake. Males in the 10,000 and 16,000 ppm dose groups were less aggressive, had lower incidences of penis/scrotum swellings or injuries, preputial gland swellings, bite marks and scabs. Hyperactivity in 16,000 ppm males was increased. The 10,000 ppm and 16,000 ppm animals had a reduced incidence of respiratory abnormalities, darkened eyes and exophthalmos. The 16,000 ppm females had an unkempt appearance and areas of alopecia, most frequently around the eyes.

There was no treatment-related increase in the number of palpable masses among the treated groups compared to the controls.

Body weights in males (Figure 3.9.1.3-1) and females (Figure 3.9.1.3-2) were significantly lower than the control in all dose groups throughout the study. Lower body weights were observed after one week of dietary exposure to captan and persisted as a dose-related effect through 104 weeks, Average body weight gain revealed that the lower body weights in the treatment groups resulted from effects induced within the first 20 weeks of the study, with the most significant decrease occurring in week 1. After week 20, no consistent effects on body weight gain were observed.

Food consumption was significantly lower in all treated groups compared to the controls from week 1 to weeks 84 to 104 in females and from week 1 to weeks 64 to 80 in males. The effect was dose related and generally reflected the reduction in average body weight. In females, food intake was significantly lower within the first 12 weeks at 10,000 and 16,000 ppm. For 6,000 ppm females and all male treatment groups, food consumption was reduced primarily during the week 1 and week 5 (when the higher doses of captan were introduced).
Apparently, food intake was initially low in all treatment groups due to unpalatability of the captan diets.

The time-weighted average captan consumption was calculated for the individual dose groups. Accordingly, 6000, 10000 and 16000 ppm are equivalent to 599, 1030 and 1980 mg/kg bw/day in males and to 634, 1080 and 1880 mg/kg bw/day in females.

Figure 3.9.1.3-1 Oral carcinogenicity study in the mouse: body weights in females (mean)

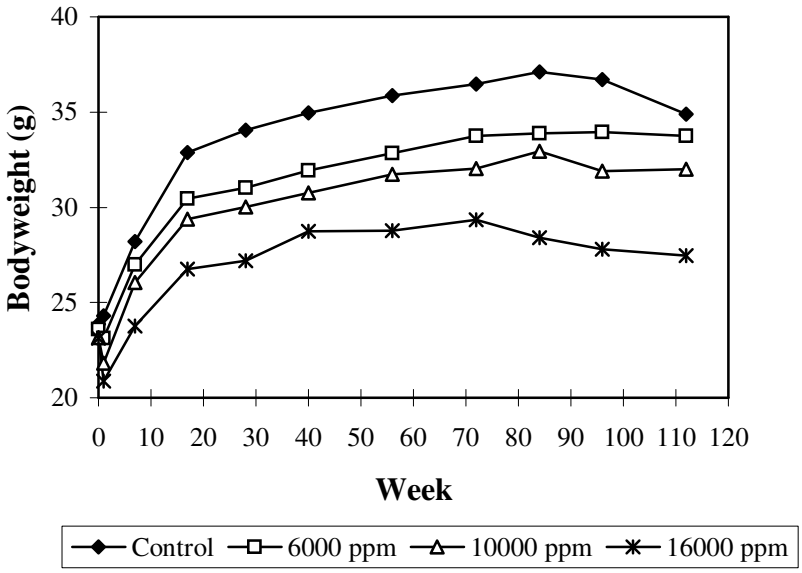
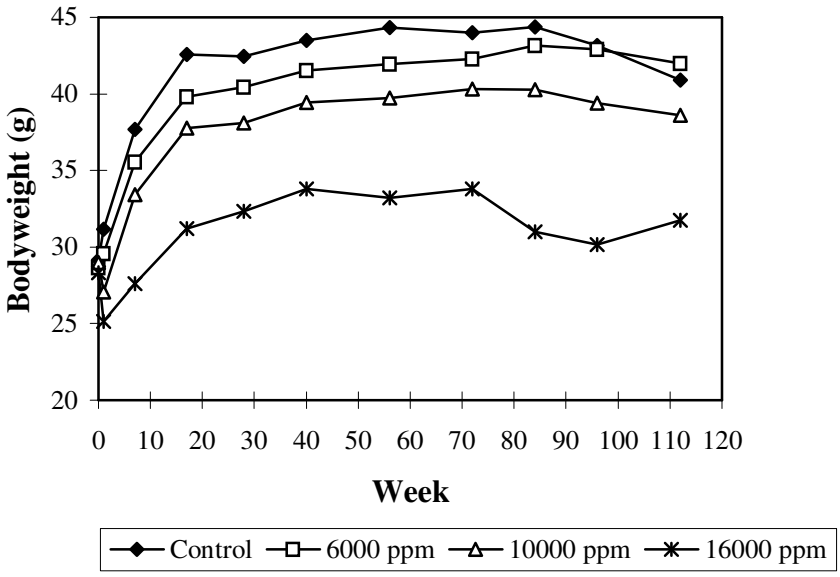


Figure 3.9.1.3-2 Oral carcinogenicity study in the mouse: body weight in males (mean)



Haematology: There were no treatment-related haematological effects.

Gross pathology, histopathology: Gross abnormalities (masses, nodular lesions, thickenings) were primarily located in the proximal 3 cm of the duodenum and were observed in the duodenum of treated animals and were evident as early as week 56. The 16,000 ppm males displayed earlier evidence of duodenal masses than other test groups and displayed a greater number of multiple duodenal masses which were evident after week 82. There was a positive correlation between the administration of captan and the incidence of duodenal tumours in males and females. A summary of the incidence of neoplasms is given in Table 3.9.1.3-2.

Table 3.9.1.3-2 Oral carcinogenicity study in the mouse: summary of incidence of neoplasms

	Total animals			
	Examined	**Neoplasms**	**Benign neoplasms**	**Malignant neoplasms**
Females				
Control	80	46	27	27
6,000	80	64**	43*	43*
10,000	80	56	25	43*
16,000	80	57	27	40
Males				
Control	80	53	40	26
6,000	80	51	37	26
10,000	79[1]	45	27	29
16,000	80	54	25*	40*

Significantly different from the control * $p < 0.05$, ** $p < 0.01$.
[1] one animal was missing.

There was a significant increase ($p < 0.001$) in the total number of duodenal neoplasms in each of the captan treatment groups. The primary neoplasms were identified as duodenal adenocarcinomas and adenomas (Table 3.9.1.3-3).

Table 3.9.1.3-3 Oral carcinogenicity study in the mouse: summary of incidence of duodenal neoplasms

	Dose (ppm)			
	Control	**6,000**	**10,000**	**16,000**
Females				
Number examined[1]	72	78	76	76
Number duodenal neoplasms	2	24***	19***	29***
Adenocarcinoma[2]	0	17***	14***	20***
Adenoma[2]	2	10*	8	12*
Undifferentiated sarcoma	0	0	0	0
Males				
Number examined[1]	74	73	72	75
Number duodenal neoplasms	2	20***	21***	39***
Adenocarcinoma[2]	1	10***	14***	30***
Adenoma[2]	1	11*	7	11*
Undifferentiated sarcoma	0	1	0	0

[1] Excluding severely autolysed and missing tissues.
[2] Tabulated by total number of neoplasms; some had multiple (i.e. both benign and malignant) neoplasms.
Significantly different from the control * $p < 0.05$, *** $p < 0.001$.

The 16,000 ppm males generally showed proliferative changes of the duodenum earlier than other test groups. All dose groups demonstrated a significantly higher incidence ($p > 0.001$) of duodenal adenocarcinomas than the controls. Of the total number of malignant duodenal tumours only six metastasised to other organs (in three mid-dose and one high-dose male, and in one low-dose and one mid-dose female). The metastases reached only one secondary site (mesenteric lymph node, liver, pancreas, colon or diaphragm). Probably because of the limited occurrence of metastasis, animals with duodenal neoplama did not show a shorter lifespan than animals without duodenal neoplasm.
The incidence of duodenal neoplasms was generally similar in both sexes. A positive dose related trend in the incidence of adenocarcinomas was observed in both sexes ($p < 0.005$). The incidence of duodenal adenomas was significantly increased in low- and high-dose males and females ($p < 0.05$).

Captan treatment at all doses statistically increased ($p < 0.01$) mucosal hyperplasia of the duodenum in both males and females compared to controls (Table 3.9.1.3-4). Mucosal hyperplasia of the jejunum was also increased in mid-dose females ($p < 0.02$) compared to the control. Jejunal neoplasia was evident in all test groups but at a very low incidence (3% female controls, and 1 to 4% in treated female dose groups). The incidence of jejunal neoplasia was significantly higher ($p < 0.05$) in mid-dose males (10%) compared to the controls (1%). Mucosal hyperplasia in the stomach was significantly higher in the stomach of low- and mid-dose animals (31 and 26%, respectively, $p < 0.05$) compared to the control (10%). The changes were inversely related to dose in both sexes.

Table 3.9.1.3-4 Oral carcinogenicity study in the mouse: summary of incidence of duodenal hyperplasia

	Dose (ppm)			
	Control	**6,000**	**10,000**	**16,000**
Males				
Number examined[1]	74	73	72	75
Mucosal hyperplasia[2]	3	39**	36**	24**
Submucosal gland hyperplasia[2]	0	1	3	1
Females				
Number examined[1]	72	78	76	76
Mucosal hyperplasia[2]	6	33**	37**	34**
Submucosal gland hyperplasia[2]	0	2	1	2

[1] Excluding severely autolysed and missing tissues.
[2] Tabulated by total number of lesions.
Significantly different from the control ** $p < 0.01$.

The incidence of lung and liver tumours in males was found to be negatively correlated with captan administration. This trend is considered to be attributable to the control and lower dose group males surviving longer than the high dose males. However, sufficient numbers of male mice were at risk for the development (and assessment) of these late appearing tumours. The incidence of liver and lung tumours was not significantly associated with tumours of the duodenum. The number of thymic lymphosarcomas was slightly increased ($p < 0.09$) in high-dose females (4/26) compared to the control (0/30).

Administration of captan was associated with reductions in the appearances of various infectious, degenerative or inflammatory diseases. There was no apparent association between the occurrence of duodenal neoplasms and the occurrence of non-neoplastic lesions.

Conclusions

Administration of captan in the feed at dietary concentrations of 6,000, 10,000 and 16,000 ppm (equivalent to 599, 1030 and 1980 mg/kg bw/day in males and to 634, 1080 and 1880 mg/kg bw/day in females) induced both benign and malignant duodenal tumours in CD-1 mice. Under the conditions of the study, captan was, therefore, found to be considered to be oncogenic in the mouse. High dose animals had significantly lower survival than controls. All captan treated groups had significantly lower average body weights and average food consumption than the controls. The lowest dose level tested of 6,000 ppm is therefore concluded to be a LOAEL.

3.9.1.4 Study 4 (1983)

Reference:	A lifetime oral oncogenicity study of captan in mice
Author(s), year:	Anonymous (1983)
Report/Doc. number:	Bio/dynamics Inc., unpublished report No. 80-2491 (Company file: R-7995/TMN-0765)
Guideline(s):	In-house method
GLP:	No
Deviations to OECD 453 (2009):	Large dose spacing between 800 and 6000 ppm (7.5 fold), no ophthalmological examination, body weights and food consumption should be measured at least weekly for the first 13 weeks, no haematology and no clinical biochemistry, no urinalysis, only selected tissues were examined histopathologically (sections of gastrointestinal tissues, all grossly abnormal tissues and all tissues with lestions were examined for all animals; sections of lungs and mesenteric lymph nodes were examined from animals in which gastrointestinal lesions were observed)
Acceptability:	Yes

Material and Methods

In a life-span carcinogenicity/toxicity feeding study with captan (purity 89%; batch no. SX-1086), CD-1 (ICR derived) strain mice were administered test substance at dietary concentrations of 0, 100, 400, 800 and 6000 ppm (100/sex/dose). Diets were administered continuously for approximately 22-months. Parameters evaluated were a determination of the concentration of captan in the diet (see addendum to this study by Leary, 1982), mortality and gross signs of toxicological

or pharmacological effects (three times/day), detailed examination for signs of local or systemic toxicity, pharmacologic effects and palpation for tissue masses (weekly), body weight and food intake (pretest, weekly through to week 8 and every two weeks thereafter), gross pathology and histopathology (sections of gastrointestinal tissues, all grossly abnormal tissues and all tissues with lesions were examined for all animals; sections of lungs and mesenteric lymph nodes were examined from animals in which gastrointestinal lesions were observed). Statistical analysis of body weight and food consumption values was performed.

Findings

Test substance intake: Mean values of test substance intake over the period of the study were 15.1, 60.9, 122.8 and 924.8 mg/kg bw/day (males) and 17.7, 70.4, 141.9 and 1042.7 mg/kg bw/day (females) in the 100, 400, 800 and 6000 ppm dose groups, respectively.

General observations: The incidence of mortality for the 6000 ppm males (35%) was higher than control males (15%) during the first fourteen months of the study. Mortality rates of males from the other treated groups were comparable to control. Mortality rates in the female control and treatment groups were comparable.
At 6000 ppm, pronounced alopecia, primarily around the eyes, was observed in males and females in the first month of treatment through to study termination. This was considered related to the administration of captan. The incidence of other clinical signs was comparable between controls and treated groups.

Mean body weights of 100, 400 and 800 ppm males were slightly reduced (3 to 7%) after one week of the study compared to the control. However, the mean body weights for males in these groups were unremarkable during the remainder of the study. The body weight of 6000 ppm males was reduced compared to the controls throughout the study (Figure 3.9.1.4-1). During the first year, the decrease averaged 5% and was statistically significant at the majority of time points. Decreases from the control during the second year were of lesser magnitude and not statistically significant. The body weight of 6000 ppm females was reduced throughout the study by an average of 7% over the period of the study (Figure 3.9.1.4-2). For the most part the difference was statistically significant from weeks 1 to 72. In other female treated groups, there were no patterns evident which were suggestive of a treatment-related effect on body weight.

Food consumption exhibited a high degree of variability from week to week. This is commonly seen in chronic mouse studies. However, there was a trend towards increased food consumption in treated males, most notably in the 6000 ppm group, during the first three months of the study (Figure 3.9.1.4-3). Food consumption in females was not affected by treatment.

Figure 3.9.1.4-1 Oral carcinogenicity study in the mouse: body weight in males (mean)

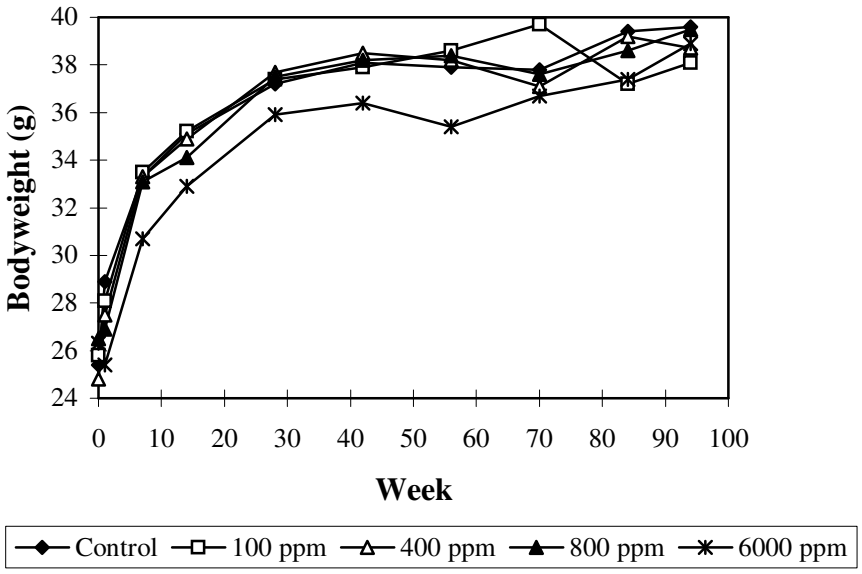


Figure 3.9.1.4-2 Oral carcinogenicity study in the mouse: body weight in females (mean)

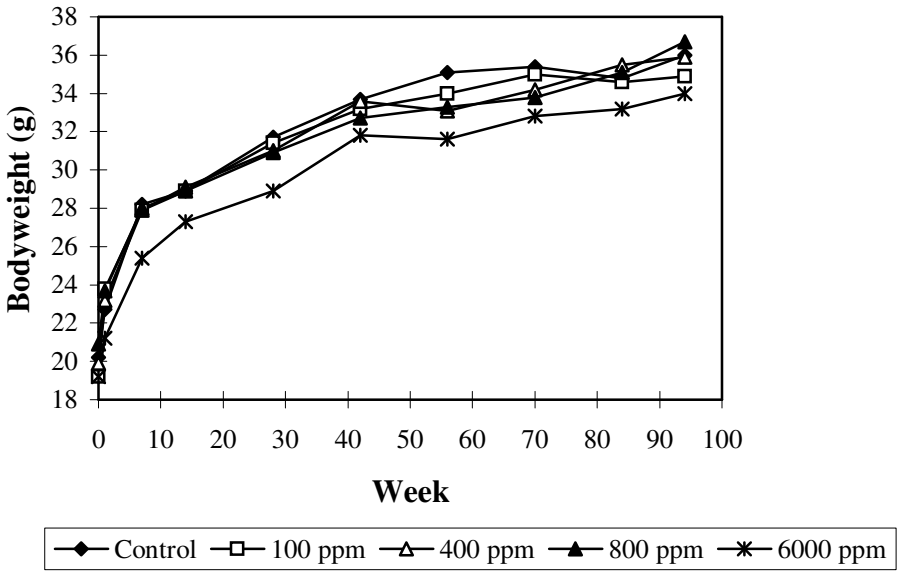
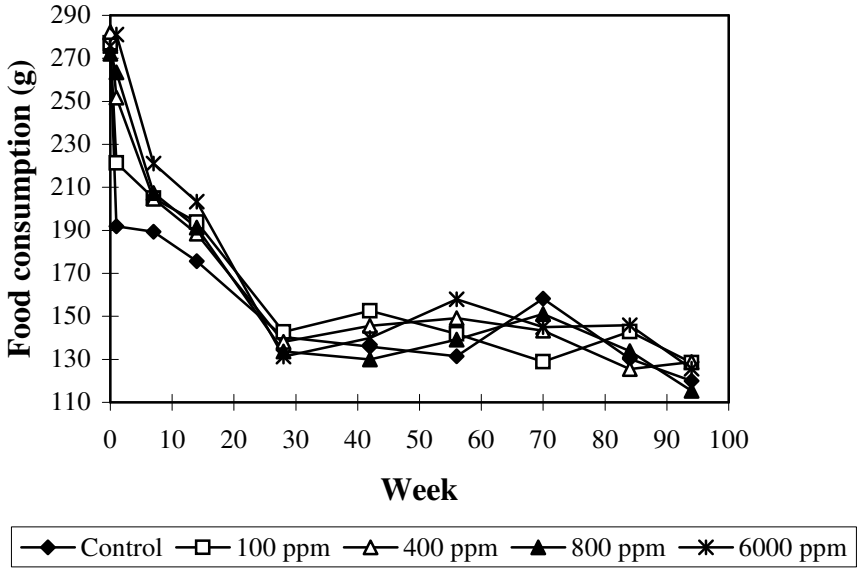


Figure 3.9.1.4-3 Oral carcinogenicity study in the mouse: food consumption in males (mean)



Gross pathology, organ weights, histopathology: The results of the gross post-mortem revealed an increase in the incidence of lesions of the small intestine (masses, nodules, raised areas) in female mice from the 800 and 6000 ppm groups compared to controls.

Microscopic examination revealed an increased incidence of small intestinal proliferative (non-neoplastic and neoplastic) lesions compared to controls. A summary of the number of animals with proliferative lesions (focal hyperplasia of the mucosa, benign and malignant neoplasms) in various parts of the gastro-intestinal tract (stomach, duodenum, jejunum/ileum, cecum and colon) are given in Table 3.9.1.4-1 (hyperplastic lesions) and Table 3.9.1.4-2 (neoplastic lesions).

Table 3.9.1.4-1 Oral carcinogenicity study in the mouse: Summary of the incidence of animals with hyperplastic lesions in the gastrointestinal tract

Tissue	Lesion	Dose (ppm)				
		0	100	400	800	6,000
Males						
Duodenum:	Number examined	84	79	90	89	85
	Focal hyperplasia	1	1	0	0	4
Jejunum/ileum	Number examined	81	78	77	74	79
	Focal hyperplasia	0	0	0	0	0
Females						
Duodenum:	Number examined	85	83	89	82	93
	Focal hyperplasia	2	2	0	3	8[1]
Jejunum/ileum	Number examined	85	81	78	83	87
	Focal hyperplasia	1	1	0	1	0

[1] One animal with two duodenal lesions (one hyperplasia and one polyp).

Table 3.9.1.4-2 Oral carcinogenicity study in the mouse: Summary of animals with neoplastic lesions in the gastrointestinal tract

Tissue	Lesion	Dose (ppm)				
		0	100	400	800	6,000
Males						
Stomach	Number examined	97	95	98	92	97
	adenoma/polyp(s)	0	2	0	1	2
	carcinoma (primary)	0	1[1]	0	0	0
	squamous cell carcinoma (primary)	0	0	0	0	0
Duodenum	Number examined	84	79	90	89	85
	adenoma/polyp(s)	0	3	0	0	3
	carcinoma (primary)	0	0[3]	0	0	1
Jejunum/ileum	Number examined	81	78	77	74	79
	adenoma/polyp(s)	0	1	1	0	0
	carcinoma (primary)	0	0	0	0	0
Cecum and colon	Number examined	75	87	87	77	72
	adenoma/polyp(s)	0	0	0	0	0
	carcinoma	0	0	0	0	0
	leiomyosarcoma	0	0	0	0	0
Females						
Stomach:	Number examined	97	96	99	96	97
	adenoma/polyp(s)	0	0	1	1[2]	2
	carcinoma (primary)	0	0	0	0	0
	squamous cell carcinoma (primary)	0	0	0	0	1
Duodenum	Number examined	85	83	89	82	93
	adenoma/polyp(s)	0	0	1[4]	1[2]	3[4,5]
	carcinoma (primary)	0	0	0	0	1
Jejunum/ileum	Number examined	85	81	78	83	87
	adenoma/polyp(s)	0	1	2[4]	2	2[4]
	carcinoma (primary)	0	0	0	0	1
Cecum and colon	Number examined	85	85	85	84	87
	adenoma/polyp(s)	0	0	0	0	1
	carcinoma	1	0	0	0	0
	leiomyosarcoma	0	0	0	1	0

[1] One animal metastatic to duodenum.
[2] One animal with polyps in two organs (stomach and duodenum).
[3] One animal metastatic from stomach (for primary tumour see stomach).
[4] One animal with polyps in two organs (duodenum and jejunum/ileum).
[5] One animal had two duodenal lesions (one hyperplasia and one polyp).

The incidence of duodenal hyperplasia was higher in the 6,000 ppm males and females compared to controls. The number of animals with benign and malignant duodenal neoplasms was also increased at the high dose compared to controls. Four animals of each sex developed duodenal neoplasms at high dose (three benign and one malignant in each group). These lesions are considered rare in the mouse and historical data from the same laboratory show that one male mouse of 718 developed duodenal carcinoma. No other duodenal neoplasms were present in 718 male and 737 female control mice.
The incidence of benign duodenal neoplasms in 100 ppm males was 3 out of 79. However, the biological significance of this result is unclear as males from the 400 and 800 ppm dose groups developed no duodenal neoplasms. No compound-related neoplasms were reported in organs other than the small intestine. An overall summary of the neoplasms by classification is given in Table 3.9.1.4-3. There was no treatment-related effect on the incidence of non-neoplastic lesions.

Table 3.9.1.4-3 Oral carcinogenicity study in the mouse: Overall summary of primary neoplasms by classification

	Dose (ppm)				
Neoplastic classification summary	**0**	**100**	**400**	**800**	**6,000**
Males					
Total primary neoplasms	22	26	19	25	19
Percentage of animals with one or more (%)	22	24	18	22	18
Total benign neoplasms	2	10	6	5	8
Percentage of animals with one or more (%)	2	9	6	5	8
Total malignant neoplasms	20	16	13	20	11
Percentage of animals with one or more (%)	20	15	13	19	11
Total metaplastic neoplasms	19	5	3	20	7
Percentage of animals with one or more (%)	6	4	1	4	2
Females					
Total primary neoplasms	23	26	31	24	34
Percentage of animals with one or more (%)	19	22	29	21	32
Total benign neoplasms	5	3	10	8	15
Percentage of animals with one or more (%)	5	3	9	6	14
Total malignant neoplasms	18	23	21	16	19
Percentage of animals with one or more (%)	17	20	20	15	19
Total metaplastic neoplasms	29	26	77	7	33
Percentage of animals with one or more (%)	6	7	14	2	13

Conclusions

In this oral carcinogenicity study in mice, males dosed with 6000 ppm of captan exhibited a higher incidence of mortality than controls during the first fourteen months of the study. Furthermore, at 6000 ppm, mean body weights of males and females were slightly reduced throughout the study and alopecia, primarily around the eyes, was noted in both sexes. Food consumption was highly variable in control and treated animals, but showed a trend towards increased food consumption in treated males, most notable in the high dose group during the first 3 months of the study.
At dose levels of 800 and 6000 ppm an increase in lesions of the small intestine was observed in females. Captan administered in the diet at 6000 ppm was associated with an increased incidence of non-neoplastic and neoplastic lesions in the small intestine of male and female mice. Captan is therefore considered to be oncogenic in the mouse under the conditions of the study. (Note: All slides of the duodenum from this study were re-examined by light microscopy in the study by Robinson, 1994)
The NOAEL was 400 ppm (equivalent to 60.9 and 70.4 mg/kg bw/day in males and females, respectively) based on gross abnormalities in the small intestine in females at 800 ppm (equivalent to 141.9 mg/kg bw/day).

3.9.1.5 Study 4 (1982)

Reference:	Addendum to lifetime oral oncogenic hazard assessment in mice with Chevron Captan Technical (SX-1086) Biodynamics project No. 80-2491. Diet analyses.
Author(s), year:	Anonymous (1982)
Report/Doc. number:	Chevron Chemical Company, unpublished report No. 721.11/S-1837 (Company file: R-7995 / TMN-0765)
Guideline(s):	In-house method
GLP:	No
Deviations:	Not applicable
Acceptability:	Yes

Experimental diets from the oral carcinogenicity study in mice by Anonymous (1983) were analysed by acetone extraction and gas chromatography utilizing an electron capture detection.
Samples of diets were taken in the Daly study weekly for the first 20 weeks and monthly thereafter. The following samples were taken from each batch of feed: (i) freshly prepared diet (day 1), (ii) pooled sample of diets remaining in the feeders of five or more animals on day 3, and (iii) pooled sample of diets remaining in the feeders of five or more animals on day 8.
Furthermore, prior to the initiation of the study, nine samples per dose level (3 each of the top, middle and bottom of each batch) were taken for homogeneity analysis.

Levels of captan in the diet:

Levels of captan in freshly prepared diets (on day 1) were in accordance with nominal concentration. Freshly prepared diet contained on average 94.8 to 101% (coefficient of variation 4.2 to 19%) of the nominal concentration of captan.
At days 3 and 8, considerable degradation was observed. Diet analysed from animal feeders after 2 days (on day 3) contained 73.4 to 90% of nominal (coefficient of variation 10 to 16%) and from feeders on day 8, 60.2 to 86.1% of nominal (coefficient of variation 12 to 22%). Degradation was more pronounced at lower dietary levels.

Results show that the diet mixing procedure was adequate to obtain a homogeneous distribution of captan in the diet with values of 98.0 to 104% (coefficient of variation 3.3 to 6.8%).

3.9.1.6 Study 4 (1994)

Reference:	Captan: A CTL re-examination of duodenal sections from a lifetime oral oncogenicity study in mice (Bio/dynamics project no. 80-2491)
Author(s), year:	Anoynmous (1994)
Report/Doc. number:	Zeneca Central Toxicology Laboratory, UK; unpublished report No. CTL/T/2829 (Company file: R-9775a / TMN-0765)
Guideline(s):	In-house method
GLP:	Yes
Deviations:	Not applicable
Acceptability:	Yes

Material and Methods

All slides of the duodenum from the life-time oral oncogenicity study conducted in the mouse by Bio/dynamics (Anonymous, 1983) were re-examined by light microscopy. Proliferative lesions were graded for hyperplasia, adenoma, adenoma with atypia and adenocarcinoma.

Findings

With respect to proliferative lesions, there was an increase in benign neoplasms (adenoma and adenoma with atypia) in 800 and 6000 ppm females and possibly in 6000 ppm males. The incidence of adenocarcinoma was increased at 6000 ppm in both males and females. Although adenoma was present at a low incidence in control males and females, adenocarcinoma was absent from these groups. Focal mucosal hyperplasia was increased in 6000 ppm males and females. In addition, the incidence of some non-proliferative lesions was altered by treatment with captan. Lymphoid infiltration was more prominent in 6000 ppm males and 800 and 6000 ppm females compared to control. Amyloidosis was high in all groups including the controls. In males this was slightly reduced in the 800 and 6000 ppm dose groups.

There were no significant differences in the incidence of proliferative and non-proliferative lesions between the 100 and 400 ppm dose groups and the controls.
A summary of the significant findings is given in Table 3.9.1.6-1.

Table 3.9.1.6-1 Oral carcinogenicity study in the mouse (re-examination of duodenal sections): Microscopic findings in the duodenum of mice

	Dose (ppm)				
	0	**100**	**400**	**800**	**6000**
Males					
Number examined	91	83	93	87	84
Number missing	9	17	7	13	16
Adenocarcinoma (malignant)	0	0	0	0	2
Adenoma (benign)	2	3	0	1	4
Focal mucosal hyperplasia	4	2	7	6	12
Adenoma with atypia (benign)	0	0	0	0	0
Lymphoid proliferation	9	8	10	12	23
Amyloidosis	60	61	75	54	49
Females					
Number examined	85	82	83	81	91
Number missing	15	18	17	19	9
Adenocarcinoma (malignant)	0	0	0	0	1
Adenoma (benign)	3	1	1	7	3
Focal mucosal hyperplasia	11	9	8	13	20
Adenoma with atypia (benign)	0	0	0	0	3
Lymphoid proliferation	10	13	14	18	18
Amyloidosis	52	51	47	45	55

Conclusions

Based on the re-evaluation of the duodenal slides, the NOAEL level for microscopic changes to the duodenum is considered to be 400 ppm, equivalent to 60.9 and 70.4 mg/kg bw/day in males and females, respectively.

Overall assessment
Captan induces gastrointestinal tumors (predominantly in the duodenum) in mice but not rats. The mode of action has been elucidated as 1) causing cell loss of the duodenal villi due to the irritative properties of captan; 2) increased proliferation of the crypts in an effort to replace these lost cells; and, 3) eventual development of polyps, adenomas and carcinomas. This mode of action does not have a mutagenic component. Human risk assessment, based on exposure estimates, concludes captan is unlikely to cause tumors in humans.

3.9.2 Human data

3.9.2.1 Wagner Palshaw, M. (1980 and 1987)

Reference:	An epidemiologic study of mortality within a cohort of Captan workers
Author(s), year:	Wagner Palshaw, M.(1980)
Report/Doc. number:	Stauffer Chemical Company, R-4641/TMN-0881
Guideline(s):	Not applicable
GLP:	Not applicable
Deviations:	Not applicable
Acceptability:	Yes

Reference:	An epidemiologic study of mortality within a cohort of Captan workers. Addendum I
Author(s), year:	Wagner Palshaw, M.(1987)
Report/Doc. number:	Stauffer Chemical Company, R-4641/TMN-0881
Guideline(s):	Not applicable
GLP:	Not applicable
Deviations:	Not applicable
Acceptability:	Yes

A report was submitted which describes a retrospective cohort mortality study conducted on 134 employees potentially exposed to technical grade Captan and its analogue, Folpet, at the Calhio Chemical Company plant in Perry, Ohio, USA. The cohort consisted of all male employees (including terminated, retired or deceased employees) who had worked in production or maintenance for a minimum of three months during the period from 1 January 1954 through to 31 December 1976. The addendum to this report describes the deaths among the original cohort which occurred during an extended folbsw up period from 1 January 1977 through to 31 December 1983. An additional four workers who met the criteria for inclusion in the original cohort were considered in the addendum. Analysis of the combined data is presented in the addendum.

A total of 25 deaths occurred among the 134 workers during the period from 1954 to 1983 in contrast with 18.7 expected deaths (based on US death rates). The standardised mortality ratio (SMR) for all causes of death was 134. The excess deaths were primarily from circulatory disease and external causes. The average age of death was 54 years. There was no statistically significant excess of any death. Two deaths from cancer (pancreas and mediastinum) were observed in contrast with 4.1 expected. Cancer (adenocarcinoma of the colon - cause of death fracture of right hip; adenocarcinoma at gastro-oesophageal junction - cause of death cerebral infarct) was listed on the death certificate as significant contributing factor in the cause of death of two workers, but not related to terminal disease. No duodenal cancers were observed.

No excess deaths were observed in persons starting work before the age of 35 or among persons who died since 1975. There was no evidence that persons who worked for longer periods of time had excess mortality in comparison to those who worked for fewer than five years. Workers categorised as having moderate exposure had a greater excess of deaths from 'all deaths' and from cancer than workers who were categorised as having high exposure.

There was no evidence to suggest that Captan, or its analogue Folpet, was carcinogenic to humans at the levels of exposure experienced by the workers. No duodenal cancers were observed. These data, while limited, support the conclusion that Captan is not a human carcinogen.

3.9.3 *In vitro* data (e.g. in vitro germ cell and somatic cell mutagenicity studies, cell t ransformation assays, gap junction intercellular communication tests)

No relevant studies.

3.9.4 Other data (e.g. studies on mechanism of action)

3.9.4.1 Study 5 (1991)

Reference:	Captan: DNA binding study in the mouse
Author(s), year:	Anonymous (1991)
Report/Doc. number:	ICI Central Toxicology Laboratories, unpublished report No. CTL/P/3380 (Company file: R-5548/TMN-0853)
Guideline(s):	In-house method
GLP:	Yes
Deviations:	Not applicable
Acceptability:	Yes

Material and Methods

CD-1 strain male mice (100 animals/dose group) were administered single doses of either [^{35}S]-captan (radiochemical purity > 98%, specific activity 888 MBq/mM) by oral gavage at a dose rate of 900 mg/kg bw, [^{14}C]-1-methyl-1-nitrosourea (positive control) by oral gavage at a dose rate of 80 mg/kg bw or a vehicle control (Carboxymethylcellulose). Earlier studies have shown that when [^{14}C]-captan was administered to rats, approximately 25% of the radioactivity is recovered as expired $^{14}CO_2$. Equally, it is possible that the labelled carbon may be capable of entering DNA by normal carbon metabolism, and this could not be distinguished from the covalently bound chemical without extensive extraction and coelutification procedures. Use of [^{35}S]-captan allows a more simple procedure (although it does not preclude incorporation of [^{35}S] into endogenous sulphur containing compounds such as cysteine).
Six hours post-dose, animals were sacrificed and the stomach, small intestine, liver and bone marrow were sampled and DNA extracted and purified from each of the tissues. The radioactivity associated with DNA extracts was measured by liquid scintillation counting (LSC). Further purification of liver, jejunum and duodenum was carried out using caesium chloride gradient ultracentrifugation and analysed for radioactivity by LSC and UV spectrophotometry.

Findings

Association of radioactivity with extracted DNA:
Radioactivity was associated with DNA extracted from all tissues derived from animals dosed with [^{35}S]-captan or [^{14}C]-1-methyl-1-nitrosourea. The covalent binding indices (= µmole chemical bound per mole nucleaotides/mmole chemical administered per kg animal) for these are given in Table 3.9.4.1-1.

Table 3.9.4-1 DNA binding study in the mouse: summary of covalent binding indices

Tissue	Covalent binding index	
	[^{35}S]-captan	[^{14}C]-1-methyl-1-nitrosourea
Stomach	38	164
Duodenum	46	249
Jejunum	91	282
Liver	38	1729
Bone marrow	2.8	480

Caesium chloride fractionation of DNA extracts:
Analysis of DNA from liver, jejunum and duodenum from mice dosed with [^{35}S]-captan using caesium chloride gradients resulted in separation of the radioactive peak from the main concentration of DNA. In contrast radioactivity from 1-methyl-nitrosourea mice was contained within the main DNA fraction. Dialysates collected after ultracentrifugation of the DNA from [^{35}S]-captan treated mice were largely devoid of radioactivity, indicating that radioactivity was associated with large molecular weight molecules.

Conclusions

The results from the study indicate that captan was associated with the DNA fraction from all tissues examined. However, the results from the caesium chloride fractionation failed to confirm that radioactivity was actually covalently bound to DNA. It cannot therefore be concluded there is a covalent interaction between captan and DNA.

3.9.4.2 Study 6 (1993)

Reference:	First revision to the potential of captan to react with DNA
Author(s), year:	Anonymous (1993)
Report/Doc. number:	Zeneca Central Toxicology Laboratory, unpublished report No. CTL/R/1131 (Company file: R-7106/TMN-0855)
Guideline(s):	In-house method
GLP:	No
Deviations:	Not applicable
Acceptability:	Yes

Material and Methods

Groups of *in vitro* and *in vivo* studies were carried out to assess the chemical degradation of captan and the potential of captan (chemical purity 99.9%, radiochemical purity ≥ 95%) to react with DNA and to characterise the products.

In the chemical degradation studies, a series of incubations were performed. Typically, [^{14}C]-trichloromethyl captan (specific activity 5.5 KBq/µmol, 2 mL: 1.75 mM in 50% methanol), Tris buffer (25 mM pH=7), and glutathione (final concentration = 1.75 mM) were incubated at 20°C.

In vitro DNA binding studies were carried out in which [^{14}C]-trichloromethyl captan (specific activity 52.5 - 59.2 KBq/µmol), at a final concentration 0.1 to 0.2% v/v, was incubated with calf thymus DNA (0.48 mg/mL) and GSH in buffer for 30 minutes. DNA was precipitated off and removed by centrifugation. Aliquots of supernatants were removed for liquid scintillation counting (LSC). Precipitates were dried and then counted in Optiphase MP. In another *in vitro* study, to assess the potential of captan to react with deoxynucleosides, 1.75 mM captan was incubated with nucleosides in the presence and absence of glutathione. Deoxynucleosides were incubated in pairs: 2'deoxyadeonsine (dA) and 2'thymidine (T); 2'deoxycytidine (dC) and 2'deoxyguanosine (dG) at a concentration of 3.5 mM. In buffered systems Tris was present and 2'deoxynucleosides were present at a concentration of 1.75 mM. To increase the sensitivity of the assay, higher concentrations of deoxynucleosides were incubated with [^{14}C]-trichloromethyl captan for 22 hours in the presence and absence of glutathione. In experiments involving bases their solubility was restricted to 0.175 mM and glutathione was present at 0.5 mM. All incubation products were analysed by HPLC with UV detection and LSC of eluent products.

In the *in vivo* studies, 6 to 12 male CD-1 strain mice were administered a single dose of [^{35}S]-N-acetyl cysteine, 2-oxo-[^{35}S]-thiazolidine-4-carboxylate or 2-thioxo-[^{35}S]-thiazolidine-4-carboxylate by oral gavage (3 mmol/kg in vehicle; 10 mL/kg bw). Four to 12 mice received vehicle only. The compound 2-thioxo-[^{35}S]-thiazolidine-4-carboxylate has been identified as a product of captan metabolism *in vivo* and it is considered that it and N-acetyl cysteine and 2-oxo-[^{35}S]-thiazolidine-4-carboxylate will be efficiently delivered into the cell where incorporation into glutathione and protein synthesis *de novo* may occur. Six hours post-dose animals were sacrificed, the livers removed and the DNA isolated. Samples of DNA (1 mg/mL) were fractionated by ultracentrifugation on a continuous caesium chloride gradient and fractions were analysed by LSC.

Findings

In vitro studies:

Chemistry of captan in incubations: Captan even in simple aqueous solution was found to be extremely unstable. Degradation was found to be increased by increasing pH and temperature and by the addition of glutathione and buffer salts. The chemical and metabolic breakdown of captan was complex. Qualitatively, the metabolites of captan were the same in the absence or presence of glutathione, although quantitatively glutathione increased the rate of the reaction. It was suggested that the influence of pH, temperature, glutathione and buffer salts may account for the discrepancies and variable results seen in mutagenicity assays. It is also unlikely that *in vitro* conditions reproduce the environment that exists in the mouse duodenum and this may account for the poor correlation between *in vitro* and *in vivo* data.

Characterisation of captan-DNA binding: When [^{14}C]-captan was incubated with calf thymus DNA, approximately 0.3% of the radioactivity was associated with the DNA. In the absence of glutathione this association was present at time zero and did not increase with time. This suggested that the material was loosely bound, unextracted material rather than covalently bound material. In the presence of glutathione the radioactivity associated with DNA initially increased but rapidly reached a constant level which was independent of time. Levels of associated radiolabel were not affected by pH.

Characterisation of captan-deoxynucleoside/base interactions: No reaction was observed in the presence or absence of glutathione.

In vivo studies:

Association of radioactivity with DNA extracts: Radioactivity was associated with the DNA extracts from mice dosed with [^{35}S]-N-acetyl cysteine, 2-oxo-[^{35}S]-thiazolidine-4-carboxylate and 2-thioxo-[^{35}S]-thiazolidine-4-carboxylate. The hepatic covalent binding indices (CBI) for these chemicals and the values reported for [^{35}S]-captan by Study 5 (1991), section 3.9.4.1, which uses the same methodology as reported in this study, are summarised in Table 3.9.4.2-1. Examinations of DNA purity in extracts by direct ultraviolet spectroscopy gave $A_{260:280}$ ratios od 1.8 – 2.0, corresponsing to < 0.5 to 10% protein by Micro-Bradford estimation.

Table 3.9.4.2-1 Potential for captan to react with DNA: summary of covalent binding indices

Compound	$A_{260:280}$ ratio	[DNA] mg/mL	Protein (mg/mL)	[^{35}S] bound DNA fraction (dpm/mg)	CBI
Captan[a]	1.66	3.65	<0.0013	1185	38
N-acetyl cysteine	2.01	3.0	<0.0013	333	144
2-oxothiazolidine-4-carboxylate	1.79	1.53	0.14	3973	130
2-thioxothiazolidine-4-carboxylate	1.88	0.95	0.048	557	20

[a] Results of the *in vivo* study with captan were taken from the study of Study 5 (1991), section 3.9.4.1; not tested in this study.

Caesium chloride fractionation of DNA extracts: Analysis of DNA from mice dosed with either [^{35}S]-N-acetyl cysteine or 2-oxo-[^{35}S]-thiazolidine-4-carboxylate, using caesium chloride gradients in each case, resulted in separation of the radioactive peak from the main concentration of DNA. This was also found for captan by Study 5 (1991), section 3.9.4.1. Due to low amounts of DNA, caesium chloride gradient fractionation was not performed on 2-thioxo-[^{35}S]-thiazolidine-4-carboxylate.

Conclusions

In vitro methodologies failed to demonstrate any clear evidence for a reaction of captan or its breakdown products with DNA, its deoxynucleosides or free bases.
For *in vivo* results, the present study showed that radiolabelled cysteine precursors resulted in similar radiolabelling associated with hepatic DNA as reported for captan (Study 5). Therefore, it is concluded that the high covalent binding indices found for captan can be attributed to sulphur exchange and incorporation into trace amounts of contaminating protein always present in DNA samples. Furthermore, when DNA samples were fractionated using a caesium chloride gradient, radioactivity was separated from the main DNA peak. This, together with the absence of covalent binding *in vitro*, is consistent with the lack of convincing genotoxicity *in vivo*.
There is no clear indication of a reaction between captan and DNA bases or deoxynucleosides, suggesting that captan is a weak- or non-genotoxin *in vivo*. Consequently, the duodenal tumours seen in mice may arise from a mechanism other than one involving a direct interaction of captan and DNA.

3.9.4.3 Study 7 (1996)

Reference:	Captan: A time course study of induced changes in the small intestine and stomach of the male CD1 mouse
Author(s), year:	Anonymous (1996)
Report/Doc. number:	Zeneca Central Toxicology Laboratories, unpublished report No. CTL/P/4893 (Company file: R-8923/TMN-0763)
Guideline(s):	In-house method
GLP:	Yes
Deviations:	Not applicable
Acceptability:	Yes

Material and Methods

In a study carried out to investigate the time course of pathological changes in the small intestine and stomach, captan (purity 89.4%, batch number WRC14264-13-1) was administered to male CD-1 mice in the diet at 3000 ppm (25 males). Animals were sacrificed after 1, 3, 7, 14 or 28 days (five mice per time point). Two controls were run, control group 1 (25 males) and control group 2 which was a pair fed control group (25 males) to account for anticipated reductions in food consumption and body weight gain in mice fed 3000 ppm captan. Control group 1 mice and captan treated mice were fed the appropriate diet ad libitum, whilst the amount offered to pair fed controls (control group 2) was approximately equal to the average food consumption of the captan treated mice. Both control group mice were sacrificed after 1, 3, 7, 14 or 28 days (five mice/group/timepoint). Parameters evaluated were captan levels in the diet, clinical signs (daily), body weights (day 1 (pre-treatment), days 2 to 7, and days 15, 22 and 29), food consumption (recorded continuously), gross pathology and microscopic examination of duodenum, ileum, jejunum and stomach.

Findings

Levels of captan in the diet:
The mean achieved concentration of captan in the diet was 101% of nominal. The homogeneity of captan was good with percent deviations within 0.1% of the overall mean. Captan was found to have satisfactory stability in the diet. After storage for 42 days the concentration had fallen by less than 5%.

General observations:
Two mice were killed in extremis on day 4, one with excessive body weight loss in the 3000 ppm group and one with excessive body weight loss and adverse clinical findings in the pair fed control. Body weights of mice in the 3000 ppm group were lower than the group 1 control group with terminal body weights approximately 11% lower. Group 2 controls were also lower than the group 1 controls due to the restricted diet with terminal body weights approximately 7% lower. Food consumption by 3000 ppm captan mice was lower than the controls throughout the study.

Gross pathology:
Distension of the duodenal lumen was observed in 3000 ppm male mice on day 1 (4/5 mice), day 3 and 7 (2/5 mice) and day 14 (5/5 mice). Distension was not observed on day 28. No abnormalities were observed in the stomach, ileum or jejunum in 3000 ppm mice at any timepoint.

Histopathology:
Treatment-related findings in the duodenum of mice fed captan in the diet at 3000 ppm were observed from day 3 of treatment and comprised crypt cell hyperplasia, shortening of villi and general disorganisation of villus enterocytes. From day 7, immature cells were additionally seen at villus tips. The incidence and severity of these findings were maintained from day 7 to day 28. A summary of the histopathological findings in the duodenum is given in Table 3.9.4.3-1. The incidence of treatment-related findings in other gastrointestinal tissues was lower than in the duodenum. On day 3 and 7, gastritis was observed in the glandular portion of the stomach (1/5 mice) and on day 28 focal parakeratosis in the stomach (1/4 mice). No treatment-related findings were observed in the jejunum and ileum of captan treated mice at any timepoint. In the pair fed control, duodenal villus height increase was observed on days 1, 3 and 7 only (1/5 mice). No changes were observed in the stomach, jejunum or ileum.

Table 3.9.4.3-1 A time course study of induced changes in the small intestine and stomach: summary of histopathological findings in the duodenum

	Time (days)				
	1	**3**	**7**	**14**	**28**
Control group 1					
Number examined	5	5	5	5	4
No abnormalities detected	2	2	1	3	2
Oedema - lamina propria	3	3	4	2	2
Control group 2 (pair fed)					
Number examined	5	5	5	4	5
No abnormalities detected	3	0	2	3	2
Oedema - lamina propria	2	5	2	1	3
Increased villus height	1	1	1	0	0
Captan - 3000 ppm					
Number examined	5	5	5	5	4
No abnormalities detected	3	0	0	0	0
Oedema - lamina propria	2	1	0	0	0
Crypt cell hyperplasia	0	4	5	5	4
Disorganisation - villus enterocytes	0	2	5	5	4
Immature villus enterocytes	0	0	5	5	4
Focal gastric metaplasia	0	0	1	1	0
Villus shortening	0	3	5	5	4

Conclusions

The results obtained are consistent with an irritant mode of action of captan on the duodenal villus epithelium, resulting in an increased loss of villus cells and concomitant chronic hyperplasia of the crypt epithelium. Such chronic stimulation of crypt cell replication in the mouse supports a non-genotoxic mechanism for the formation of adenoma and ultimately adenocarcinoma in the duodenum of the mouse.

3.9.4.4 Study 8 (1995)

Reference:	Investigation of duodenal hyperplasia in mice
Author(s), year:	Anonymous (1995)
Report/Doc. number:	Zeneca Central Toxicology Laboratory, unpublished report No. CTL/P/4532 (Company file: R-8193/TMN-0770)
Guideline(s):	In-house method
GLP:	Yes
Deviations:	Not applicable
Acceptability:	Yes

Material and Methods

In a study carried out to investigate duodenal hyperplasia, captan (purity 89.4%, batch number WRC14264-13-1) was administered to male CD-1 mice in the diet at 0, 400, 800, 3000 or 6000 ppm (5 animals/sex/dose) for a period of 56 days. At termination, mice were given a single intraperitoneal injection of bromodeoxyuridine (BRDU) at a dose level of 24 mg/animal, approximately one hour prior to sacrifice. Parameters evaluated were the level and homogeneity of captan in the diet, clinical signs (daily), body weight (daily during week 1, thereafter weekly) and food consumption (daily during week 1, thereafter weekly). At termination duodenal hyperplasia was evaluated using visual examination of stained sections for histopathological changes, assessment of the mean number of cells in the crypt cell population, measurement of bromodeoxyuridine (BRDU) labelling index (% cells labelled with BRDU as a ratio to the total number of cells in crypts) and measurement of villus height to crypt height. The stomach, jejunum and ileum were evaluated for histopathological changes only.

Findings

Levels of captan in the diet:
The achieved dietary concentrations of captan were within 9% of nominal. Homogeneity in diet containing nominal concentrations of 400 and 6000 ppm was within 7% of the overall mean. Captan was sufficiently stable in diet stored at room temperature and at -20°C for over 24 and 73 days, respectively.

General observations:
There were no treatment-related clinical signs. Body weight gains in 3000 and 6000 ppm mice were lower than the controls throughout the study, although these changes were mainly confined to the first week of treatment, when a fall in body weight was observed. In the 400 and 800 ppm groups a slight decrease in body weight gain was observed during the first few days of treatment. Food consumption was reduced in males and females in the 3000 and 6000 ppm groups, especially during week 1. No consistent effect at 400 and 800 ppm was observed, although slightly reduced values were observed during day 1 and 2. The results are summarized in Table .

Table 3.9.4.4-1 Bodyweight data (g) of male animals, mean ± SD

Captan in diet (ppm)	0	400	800	3000	6000
Males					
Body weight at study start	34.4 ± 1.8	34.7 ± 2.0 +0.9%	34.6 ± 1.6 +0.6%	35.2 ± 1.7 +2.3%	33.8 ± 1.9 -1.7%
Body weight at day 29	41.7 ± 2.8	39.9 ± 1.5 -4.3%	42.2 ± 2.1 +1.2%	39.6 ± 1.3 -5.0%	37.8 ± 3.0 -9.4%
Final body weight (day 57)	48.2 ± 5.3	44.1 ± 3.5 -8.5%	47.6 ± 3.8 -1.2%	**41.4 ± 2.4*** **-14.1%**	**41.8 ± 2.3*** **-13.3%**
Body weight gain	13.8 ± 4.1	9.5 ± 1.8	13.0 ± 2.5	6.2 ± 1.3	8.1 ± 2.5
Bodyweight gain /100g food eaten (days 1-7)	4.0	3.5	4.9	-3.1	-9.7
Bodyweight gain /100g Weekly (weeks 2-8)	3.7	2.9	3.3	2.5	3.7
Females					
Body weight at study start	25.1 ± 1.3	25.2 ± 1.4	25.7 ± 1.5	25.6 ± 0.8	25.6 ± 1.2
Body weight at day 29	29.0 ± 1.4	29.8 ± 1.4	31.7 ± 2.2	29.8 ± 0.7	29.6 ± 1.0
Final body weight (day 57)	32.1 ± 4.9	34.5 ± 2.3 -8.5%	33.7 ± 2.7 -1.2%	31.3 ± 2.4 -14.1%	30.7 ± 2.0 -13.3%
Body weight gain	7.0 ± 4.0	9.3 ± 2.0	7.9 ± 1.6	5.7 ± 2.1	5.2 ± 1.4
Bodyweight gain /100g food eaten (days 1-7)	2.7	4.2	5.0	2.5	-0.7
Bodyweight gain /100g Weekly (weeks 2-8)	2.1	2.6	2.0	1.9	2.2

* Significantly different from the control ($p < 0.05$).

Gross pathology:
No treatment-related macroscopic changes were observed.

Microscopic changes:
Captan induced morphologically discernible, diffuse hyperplasia of crypt cells, which was localised to the first 7 cm of the duodenum after the pylorus of the stomach at 3000 ppm and 6000 ppm in males and 800, 3000 and 6000 ppm in females. Increased crypt cell mitoses and villous shortening were also observed in the 3000 and 6000 ppm males and 800, 3000 and 6000 ppm in females. These changes were accompanied by a mononuclear inflammatory cell response in the lamina propria. Mild crypt cell hyperplasia in the jejunum was seen in 3000 and 6000 ppm males. Mild hyperplasia of the forestomach epithelium with hypertrophy of the gastric pits of the glandular portion and dilatation of the gastric gland of the stomach in 3000 ppm males and 6000 ppm males and females was observed. A summary of the histopathological changes observed in mice is given Table 3.9.4.4-2.

Table 3.9.4.4-2 Summary of histopathological findings

	Dose (ppm)				
	0	**400**	**800**	**3000**	**6000**
Duodenum:					
Males					
Examined	5	5	5	5	5
No abnormalities detected	5	4	4	0	0
Increased mitotic figures	0	0	0	5	4
Crypt hyperplasia	0	1	0	5	5
Decreased villous height	0	0	0	5	4
Inflammation lamina propria	0	0	1	5	3
Females					
Examined	5	5	5	5	5
No abnormalities detected	5	5	0	0	0
Increase mitotic figures	0	0	0	3	4
Crypt hyperplasia	0	0	5	5	5
Decreased villous height	0	0	1	3	4
Inflammation lamina propria	0	0	4	4	4
Jejunum:					
Males					
Examined	5	5	5	5	5
No abnormalities detected	5	5	5	1	1
Crypt cell hyperplasia	0	0	0	4	4
Females					
Examined	5	5	5	5	5
No abnormalities detected	5	5	5	5	4
Crypt cell hyperplasia	0	0	0	0	1
Stomach:					
Males					
Examined	5	5	5	5	5
No abnormalities detected	5	5	3	0	1
Hyperplasia of non-glandular epithelium	0	0	0	3	1
Dilatation of gastric gland	0	0	0	2	1
Hypertrophy of gastric pits/atrophy	0	0	2	2	3
Females					
Examined	5	5	5	4	5
No abnormalities detected	3	4	2	3	2
Hyperplasia of non-glandular epithelium	0	0	0	0	2
Dilatation of gastric gland	0	0	0	0	1
Hypertrophy of gastric pits/atrophy of gastric gland	2	1	3	1	1

There was a significant increase in crypt cell labelling index at 3000 and 6000 ppm in both males and females and an increase in the crypt cell population at dietary levels of 800 ppm and above in males and females. Although the number of crypt cells was increased in 400 ppm males, this was not statistically significant and was due to one animal. The result in this animal was considered to be spurious. In addition, there was a decrease in the villous to crypt height ratio in males and females at 3000 and 6000 ppm. A summary of the mean number of crypt cells, BRDU labelling index (LI %) and villus to crypt height ratio in the duodenum is given in Table 3.9.4.4-3.

Table 3.9.4.4-3 Duodenal hyperplasia in mice: summary of mean number of crypt cells, BRDU labelling index (LI %) and villus to crypt height ratio in the duodenum

Dose (ppm)	No. crypt cells	LI %	Villus: crypt ratio
Males			
0	59.45 ± 4.8	31.3 ± 4.9	5.4 ± 0.4
400	74.2 ± 21.3	30.8 ± 5.6	5.0 ± 1.6
	(65.0 ± 6.1)[1]		
800	**71.4 ± 9.6***	30.0 ± 6.7	5.3 ± 0.5
3000	**153.2 ± 26.1****	**40.3 ± 3.5***	**1.4 ± 0.5****
6000	**111.0 ± 30.1****	**45.4 ± 6.1****	**1.9 ± 0.9****
Females			
0	68.2 ± 3.3	30.1 ± 3.0	5.9 ± 0.5
400	69.3 ± 1.7	30.2 ± 1.3	6.0 ± 0.6
800	**79.2 ± 9.8***	31.0 ± 2.7	5.9 ± 0.4
3000	**102.3 ± 23.4****	**47.2 ± 3.4****	**2.6 ± 0.8****
6000	**127.6 ± 36.0****	**46.4 ± 4.1****	**1.4 ± 0.5****

[1] Value in parenthesis excludes animal no. 8.
* Significantly different from the control ($p < 0.05$).
** Significantly different from the control ($p < 0.01$).

Conclusions

Feeding male and female mice captan at dietary concentrations of 800 ppm and above for 56 days produced inflammation and hyperplasia of the duodenum. The hyperplasia was restricted to the first 7 cm after the pylorus of the stomach. Hyperplasia of the jejunum and forestomach was observed in mice fed captan at dietary concentrations of 3000 ppm and above. Quantitative changes reflecting the duodenal hyperplasia were seen at doses of 800 ppm and above. The NOAEL for pathological change, including duodenal inflammation and hyperplasia was 400 ppm in males and females.

3.9.4.5 Study 9 (1985)

Reference:	Identification of a preneoplastic alteration following dietary administration of captan technical to CD-1 mice
Author(s), year:	Anonymous (1985)
Report/Doc. number:	Stauffer Chemical Company, unpublished report No. T-11007 (Company file: R-8271/TMN-0766)
Guideline(s):	In-house method
GLP:	No
Deviations:	Not applicable
Acceptability:	Yes, but limited as only one dose level was used.

Material and Methods

In a study designed to identify the early histological changes in the small intestine, male mice (CD-1 strain, Charles River Breeding Laboratories, Kingston, NY) were administered captan (purity 89.1%, batch number EHC 0355-27; WRC-4912-26-12) in the diet at doses of 0 or 6000 ppm for various periods of time according to the following regimen (Table 3.9.4.5-1). In previous studies, 6000 ppm has been associated with duodenal neoplasms in mice. Of the animals in groups 1 and 2 scheduled for sacrifice at 3, 6, 9 and 12 months (35/timepoint/group), 10 were scheduled for pre-sacrifice intraperitoneal (ip) injection of metabolised iron, 10 for pre-sacrifice ip injection of tritiated thymidine (^{3}H-thymidine), 10 for histological studies of intestinal enzymes (alkaline phosphatase [AP], glucose-6-phosphate dehydrogenase [G6PDH] and gamma-glutamyl transpeptidase [GGT]) and 5 mice were scheduled for gastrointestinal reduced sulphydryls determination. Interim and terminal sacrifice animals were starved prior to sacrifice. Parameters evaluated were clinical signs, body weight and food consumption (every two weeks for first 56-weeks and then monthly) and gross pathology. Designated animals were examined for haematoxylin and eosin (H & E) morphology; mucins morphology; iron accumulation, morphology; radioautography, morphology; histochemical (AP; G6PDH; GGT) and non-protein bound reduced sulphydryls.

Table 3.9.4.5-1 Experimental design

Group	Dietary concentration (ppm)	Number of animals	Sacrifice intervals (months)
1	0	35	3
		35	6
		35	9
		35	12
		35*	18
		Remaining	20
2	6000	35	3
		35	6
		35	9
		35	12
		35*	18
		Remaining	20
3A	6000 for 6 months; followed by 0 for 7-12 months or 7 - 18 months	10	12
3B		Remaining	18
4A	6000 for 12 months; followed by 0 for 13-18 months or 13 - 20 months	10	18
4B		Remaining	20

* 15 animals in each of groups 1 and 2 were used to replace animals that died spontaneously during the first 12-months. Although 35 animals in each of group 1 and 2 were scheduled for sacrifice at 18-months only 10 in each group were sacrificed. The remaining animals were sacrificed at 20-months.

Findings

Levels of captan in the diet:
Measured concentrations of captan in the diet ranged from 90 to 118% of nominal.

General observations:
Administration of captan did not adversely affect survival of mice. Clinical findings were not considered related to treatment. Administration of captan for 6, 12 and 18 months produced a significant reduction in weight gain. The diminished weight was apparent from week 1 and remained significantly lower than the control throughout the study, only slightly recovering during the designated recovery period (Table). Food consumption was generally reduced for all dose groups throughout the study. The average consumption of captan is summarised in Table . The pattern of consumption was similar for all three groups.

Table 3.9.4.5-2 Identification of pre-neoplastic lesions in the mouse: body weight changes

Dose	Percent of initial weight		
	week 31	week 53	week 75
Control	147	150	147
6000	130	133	140
6000 (recovery group A)	130	137	133
6000 (recovery group B)	126	132	132

Table 3.9.4.5-3 Identification of pre-neoplastic lesions in the mouse: average captan consumption (mg/kg bw/day)

Interval (weeks)	Intervals measured	Dose group (ppm)		
		6000	6000A	6000B
1 - 25	13	715	712	719
1 - 51	26	678	-	689
1 - 83	21*	660	-	-

* Monthly consumption measurements.

Gross pathology and histopathology (continuous captan treatment):
Pathological changes were observed in the stomach and small intestine. In the small intestine the first few cm of the organ was primarily affected. Significant pathological changes to the stomach were limited to the first three months of

treatment and were limited to the non-glandular portion of the forestomach and included necrotising (ulcers and/or erosions) and proliferative (epithelial hyperplasia) changes. Significant pathological changes were seen at all timepoints in the small intestine. At 3 months sacrifice, the incidence of ulcers and/or erosions was 20% and epithelial hyperplasia 57%. Dilatation of the small intestine (predominantly the proximal segment) was seen in mice treated for 6, 9, 12 and 18+ months at incidences of 80, 97, 89 and 43%, respectively. Intestinal dilatation was seen infrequently in the control and recovery groups. The highest incidence in recovery group mice was 21% (12 months captan treatment).

The incidence of epithelial lesions of the small intestine is summarised in Table . The incidence of epithelial lesions in the small intestine was significantly increased in captan treated animals. The most common small intestine lesion was focal epithelial hyperplasia, the incidence of which was significantly increased above the controls. The incidence of diffuse epithelial hyperplasia was less frequent and was not seen in the controls. There was an increase of epithelial lesions, epithelial hyperplasia (any hyperplasia) and focal epithelial hyperplasia of the small intestine with increasing age and continuous captan treatment ($p < 0.01$). These trends with increasing age were also seen in the control ($p < 0.01$). There was a significant trend toward decreasing incidence of diffuse epithelial hyperplasia with increasing age and continuous captan treatment ($p < 0.05$). Focal hyperplasia developed in areas of diffuse hyperplasia, although the latter was not a prerequisite for the development of focal hyperplasia. Mice exhibited multiple foci of focal hyperplasia and the average number of focal hyperplasia increased with age and treatment. Focal hyperplasia was only seen in the control at 12 and 18 months.

Table 3.9.4.5-4 Identification of pre-neoplastic lesions in the mouse: incidence of hyperplastic and neoplastic epithelial lesions in the small intestine

	Treatment												
	Lifetime without captan					**Lifetime with captan**					**Part-time with captan**		
Dose level	**0 ppm**					**6000 ppm**					**6000 ppm**		
Treatment period (month)	**0-3**	**0-6**	**0-9**	**0-12**	**0-18+**	**0-3**	**0-6**	**0-9**	**0-12**	**0-18+**	**0-6[a]**	**0-6[b]**	**0-12[c]**
Study age at death (month)	**3**	**6**	**9**	**12**	**18+**	**3**	**6**	**9**	**12**	**18+**	**12**	**18**	**18+**
No. animals	**20**	**20**	**20**	**20**	**26**	**20**	**20**	**20**	**18**	**22**	**10**	**16**	**24**
No. animals with lesions	0	0	0	5	6	15**	20**	18**	18**	22**	3	4	16
No. with hyperplasia:	0	0	0	5	5	15**	20**	17**	18**	22**	1	2	7
Diffuse	0	0	0	0	0	7	8	3	3	3	0	0	0
Microfocal	0	0	0	2	1	0	0	0	0	1	1	2	3
Focal	0	0	0	3	5	14**	19**	17**	17**	22**	1	2	6
No. with neoplasia	0	0	0	0	1	0	2	5**	3	8**	2	3	10
Benign	0	0	0	0	0	0	2	5	3	4	2	1	5
Malignant	0	0	0	0	1	0	0	0	0	6	0	2	7

[a] Recovery period 7 through to 12 months.
[b] Recovery period 7 through to 18 months.
[c] Recovery period 13 through to 18+ months.
** Significantly different from the control group ($p < 0.01$).
*** Significantly different from the control ($p < 0.025$).

Focal epithelial hyperplasia tended to be more widely distributed in the intestine of control mice compared to the captan treated mice. In lifetime treated mice 95% of focal hyperplasias were in the proximal 7 cm and 99% in the proximal 14 cm of the small intestine compared to 28 and 83%, respectively in the controls.

Adenomas and carcinomas also developed in the small intestine (all neoplasms were in the proximal 7 cm). Neoplasms and hyperplasias appeared to arise from the same type of crypt epithelial cell and both neoplastic and non-neoplastic epithelial lesions shared structural similarities. These included extensive lengthening of intestinal glands, hyperplasia of the tunica muscularis, dilatation of the submucosal arteries and veins, fibrosis of the submucosa and lymphocyte and/or plasma cell infiltration of the lamina propria, submucosa and/or tunica muscularis. Adenomas and adenocarcinomas were first recognised 9 and 12 months after treatment.

There was a significant trend, with age and continuous captan treatment, towards increasing incidence of epithelial lesions, epithelial hyperplasia (any hyperplasia) and focal epithelial hyperplasia of the small intestine ($p < 0.01$). The trend with

increasing age was also seen in the control ($p < 0.01$). There was a trend, with age and continuous captan treatment, toward increasing incidence of epithelial neoplasia ($p < 0.01$). This trend was not observed in the control. There was also a significant trend toward decreasing incidence of diffuse epithelial hyperplasia with increasing age and continuous captan treatment ($p < 0.05$), but not in the control.

Gross pathology and histopathology (part-time captan treatment):
Removal of captan from the diet resulted in a reduction in the incidence of focal epithelial hyperplasia below the incidence of the concurrent lifetime treated mice to a level no greater than the concurrent control mice. Histological appearance of some focal epithelial hyperplasias in recovery mice differed significantly from those in lifetime treated mice. The lesion in recovery mice exhibited muscular hyperplasia, submucosal fibrosis and ecstasia of the submucosal vessels, but with much less epithelial hyperplasia. This appeared to be due to resolving foci of focal hyperplasia in which the extent of epithelial repair (cessation of epithelial proliferation) exceeded the repair of mesenchymal tissues. The incidence of neoplasia in recovery group mice was not significantly different from lifetime treated mice, but the incidence of neoplasia in two recovery groups (6/6 and 12/6) was greater than the control mice. However, the incidence of neoplasia in the recovery group with the shortest exposure (6 months) and longest recovery (12 months) was not significantly different to that in the concurrent control. Both adenomas and adenocarcinomas were seen in recovery group animals. A summary of the epithelial lesions in the small intestine of recovery animals is given in Table above.

Conclusions

Lifetime or part-time exposure of mice to captan in the diet at 6000 ppm had no adverse effect on survival but produced significant reductions in weight gain. Lifetime exposure of mice to captan in the diet resulted in dilatation of the proximal small intestine, diffuse hyperplasia of columnar epithelium of the proximal small intestine, an increased incidence of focal hyperplasia and neoplasia of columnar epithelium of the small intestine. Part-time exposure of mice to captan did not produce an increase in the incidence of focal hyperplasia of columnar epithelium of the small intestine. The increased incidence of neoplasia was observed in mice exposed for 6 months with 6 months recovery and in mice exposed for 12 months with 6-8 months recovery. However, there was not an increased incidence of neoplasia in mice exposed for 6 months and allowed to recover for 12 months.

3.9.4.6 Study 10 (1982)

Reference:	The effect of captan on duodenal sulfhydryl levels in rats
Author(s), year:	Anonymous (1982)
Report/Doc. number:	Stauffer Chemical Company, unpublished report No. T-10832 (Company file: R-4685/TMN-0772)
Guideline(s):	In-house method
GLP:	No
Deviations:	Not applicable
Acceptability:	Yes

Material and Methods

Male and female Sprague-Dawley CD rats were administered captan (batch number EHC 0065-46) dissolved in corn oil by oral gavage at a single dose of 0, 20, 200 or 2,000 mg/kg bw in a volume of 10 mL/kg (35 animals/sex/dose level). The animals were 8-10 weeks old. Five animals per sex were sacrificed after 2, 4, 24, 48, 72, 120 and 240 hours. The upper 4 cm section of small intestine was removed from each animal, homogenised in trichloroacetic acid and centrifuged to analyse non-protein bound reduced sulfhydryls by measuring the absorbance of the supernatant at 405 nm using a spectrophotometer. The absorbance was measured against a standard curve.

Findings

Males treated with captan at 200 mg/kg bw showed significantly higher mean sulfhydryl levels compared to untreated after two hours, and all treated male groups showed higher levels after 4 hours to 24 hours. The mid and high dose groups showed significantly increased levels at 48 hours and 72 hours, while only the high dose group (2,000 mg/kg bw) remained significantly high after 120 hours. After 240 hours, sulfhydryl levels were similar to untreated.

Females treated with captan at 200 mg/kg bw showed significantly higher mean sulfhydryl levels compared to untreated after 4 hours and all treated female groups showed higher levels after 24 hours. This effect persisted for up to 24 hours.

At 48 hours, both the mid dose and high dose groups were significantly increased compared to control, while after 72 hours only the high dose group was significantly elevated. After 120 hours and 240 hours, sulfhydryl levels were similar to untreated. The results are summarised in Table 3.9.4.6-1.

Table 3.9.4.6-1 Mean duodenal sulfhydryl levels in rats (± SD)

Dose (mg/kg bw)	Mean sulfhydryl levels (µg/g)						
	2 hr	4 hr	24 hr	48 hr	72 hr	120 hr	240 hr
Males							
0	1085 ±66	1059 ±81	1134 ±84	1011 ±27	1006 ±82	1004 ±55	1078 ±32
20	1159 ±110	**1361*** ±98	**1402*** ±72	1075 ±79	1067 ±47	969 ±42	1084 ±28
200	**1275*** ±122	**1376*** ±79	**1672*** ±141	**1630*** ±130	**1256*** ±137	1028 ±70	1070 ±32
2,000	1198 ±62	**1281*** ±51	**1672*** ±160	**1437*** ±141	**1757*** ±239	**1172*** ±30	1079 ±81
Females							
0	1153 ±89	1204 ±70	1080 ±27	1035 ±52	992 ±91	1024 ±82	1052 ±50
20	1288 ±103	1408 ±93	**1339*** ±215	1038 ±57	1124 ±67	949 ±49	1022 ±62
200	1369 ±138	**1533*** ±153	**1609*** ±73	**1364*** ±142	1131 ±78	955 ±74	1099 ±75
2,000	1388 ±209	1218 ±195	**1384*** ±187	**1446*** ±171	**1744*** ±189	1032 ±142	1123 ±9

* Significantly greater than untreated, $p < 0.05$.

Conclusions

Rats showed increased duodenal sulfhydryl levels following administration of captan. The onset, magnitude and duration of the effect were similar to that recorded in a separate study in mice. Therefore, increased duodenal sulfhydryl levels do not provide additional information to explain the oncogenicity recorded in mice but not rats.

3.9.4.7 Study 11 (1980)

Reference:	Effect of dietary captan on soluble thiol content of liver and duodenal tissues
Author(s), year:	Anonymous (1980)
Report/Doc. number:	Stauffer Chemical Company, unpublished report No. MRC-B-109/MRC-80-04 (Company file: TMN-0771)
Guideline(s):	In-house method
GLP:	No
Deviations:	Not applicable
Acceptability:	Yes

Material and Methods

Female Swiss Webster mice were administered captan (batch number 60106 Lot 4) in the diet at doses of 0, 4,000, 8,000 or 16,000 ppm for 35 days (Group 1) or 0, 500, 1,000, 2,000, 4,000, 8,000 or 16,000 ppm for up to 213 days (Group 2). At various times after the onset of administration, two mice were sacrificed and the left hepatic lobe and the duodenum from the pyloric sphincter to the crossover of the colon were removed. Samples of the tissues were separately homogenised in EDTA solution, mixed with trichloroacetic acid and centrifuged. Aliquots of the supernatant were mixed with 5,5'-dithio-bis-2-nitrobenzoic acid and absorbance measured at 412 nm with a spectrophotometer. The concentration of soluble thiols was measured by comparing the absorbance against a standard curve.

Findings

Elevated soluble thiol levels in duodenal sections were recorded in Group 1 animals administered captan in the diet for up to 35 days at 4,000, 8,000 and 16,000 ppm compared to untreated. Levels in the liver were similar to, or slightly to moderately below, untreated levels. The effects became apparent after one day of administration. Similar results were recorded in Group 2 animals which were administered captan at higher doses over a longer period. Levels in the duodenum reached a maximum after 84 days of administration and then decreased slightly. Levels of soluble thiols in the duodenum in the Group 2 animals showed a dose response to captan up to 16,000 ppm but there was a slight dose-related depression on levels of soluble thiols in the liver.

The results are summarised in Table (Group 1) and Table (Group 2).

Table 3.9.4.7-1 Mean soluble thiol levels as a percentage of untreated values (Group 1 – 35 days treatment)

Days on diet	Soluble thiol levels (% of untreated)					
	4,000 ppm		8,000 ppm		16,000 ppm	
	duodenum	liver	duodenum	liver	duodenum	liver
1	153	92	146	88	152	82
2	150	64	190	71	164	43
5	140	81	209	98	145	43
9	193	81	196	86	205	90
14	215	122	227	110	205	84
21	210	92	213	92	253	56
35	193	91	191	98	185	79
Mean	179 ± 31	89 ± 18	196 ± 26	92 ± 12	187 ± 38	68 ± 20

Table 3.9.4.7-2 Mean soluble thiol levels as a percentage of untreated values (Group 2 – 213 days treatment)

Days on diet	Soluble thiol levels (% of untreated)											
	500 ppm		1,000 ppm		2,000 ppm		4,000 ppm		8,000 ppm		16,000 ppm	
	duo[1]	liver	duo	liver	duo	liver	duo	liver	duo	liver	duo	liver
35	145	102	190	93	182	109	201	95	239	90	217	78
49	116	80	153	82	156	100	150	85	164	81	204	82
84	167	96	159	100	197	97	197	91	202	90	258	78
213	131	101	133	101	163	112	173	108	192	88	195	78
Mean	140 ± 22	95 ± 10	159 ± 24	94 ± 9	175 ± 19	105 ± 7	180 ± 24	95 ± 10	199 ± 31	87 ± 4	219 ± 28	79 ± 2

[1] Duodenum.

Conclusions

The administration of captan in the diet at 500 up to 16,000 ppm to mice led to increased levels of soluble thiols in the duodenum, while a slight decrease was observed in the liver. This may reflect an adaptive response of duodenal tissue to exposure to compounds requiring glutathione for detoxification.

3.9.4.8 Study 12 (1996)

Reference:	The bioavailability of captan to the duodenum of CD-1 mice following dietary administration
Author(s), year:	Anonymous (1996)
Report/Doc. number:	Central Toxicology Laboratory, unpublished report No. CTL/R/1260 (Company file: R-8746/TMN-0852A)
Guideline(s):	In-house method
GLP:	No
Deviations:	Not applicable
Acceptability:	Yes

Material and Methods

In a first study, male CD-1 mice (4-6 weeks old, 28-30 g) were administered captan (purity 89.4%; batch number 14264-13-1) in the diet at 0, 400 or 3,000 ppm (30 animals per dose group) for eight days. After this (and at the start of the 12 hr dark phase of the animals' artificial light cycle), the 400 and 3,000 ppm treated diets were substituted by diets containing [1,2 ^{14}C] cyclohexane captan (radiochemical purity 99.1%; specific activities 46.1 and 6.4 MBq/mmoles for the 400 and 3,000 ppm doses, respectively). After 6, 12, 18, 24 and 30 hours, six animals per group were sacrificed, the gastrointestinal tract was removed and blood and urine samples were taken.
In a second study, mice dosed with unlabelled captan in the same way at the same doses also received radiolabelled diets containing [1,2 ^{14}C] cyclohexane captan (specific activities 9.8 and 1.4 MBq/mmoles for the 400 and 3,000 ppm diets, respectively). Five animals per dose group were sacrificed after 12 and 24 hours and blood, stomach, stomach contents, duodenal sections and duodenal section contents were processed. Tissues were analysed for total radiolabel and captan/metabolite content by HPLC with UV and radiochemical detection.

Findings

Following 400 and 3,000 ppm, during the first 6 and 12 hours of radiolabelled administration (i.e. during the first 12-hour dark period), radiolabel was concentrated in the stomach and in the caecum. After 18 and 24 hours (i.e. during the first light period), there was lower radiolabel in the stomach suggesting reduced food intake. During the 24-30 hour period (i.e. the second dark period), the radioactivity peak in the stomach was again observed. The concentration of radiolabel in the duodenum (i.e. the first 10 cm of intestine) and in the next 30 cm of intestine, the concentration of radiolabelled material remained constant during the 30 hour administration indicating that a steady state concentration of captan and/or metabolites was maintained. The concentrations found were in proportion to the doses administered: approximately 7-fold higher material was present from duodenum to caecum in the high dose group compared to the low dose group.

The majority of radiolabel in the stomach and duodenum appeared to be associated more with the contents than the tissue. Due to technical difficulties to completely separate duodenal contents from duodenal tissue, the results are likely to underestimate that proportion.

Captan was not found in the duodenum of any animal and only a small amount of metabolite was detected. Captan was detected in the stomach of three animals following 3,000 ppm (one following 12-hour administration of radiolabelled captan; two following 24 hour administration of radiolabelled captan). Metabolites were also detected in the stomach. The only metabolite identified in the gastrointestinal tract was THPI, which was found in the stomach.

Small amounts of radiolabel were detected in the blood. Urinary metabolites were present in higher amounts than in the gastrointestinal tract. No captan was detected in blood or urine.

Conclusions

Radiolabel reached a low, and dose dependent, steady state level in the duodenum following dietary administration of captan. The radiolabel found was associated with the duodenal contents rather than the tissue. There was no accumulation of radiolabel in the gastrointestinal tract. Captan was detected in the stomach in a few animals. No parent captan was detected in the duodenum, blood and urine. The report concludes that captan was largely degraded in the stomach before reaching the duodenum.
However, the pH in the duodenum is higher than that in the stomach and captan is more stable under acidic conditions. Furthermore, some captan was found in the stomach whereas only metabolites were found in the duodenum. Therefore, although a part of the degradation of captan may have already occurred in the stomach, the major degradation site is considered to be in the duodenum where degradation occurred very rapidly, prior to the first analysis six hours after administration of the last captan dose.

3.9.4.9 Study 13 (1992)

Reference:	Captan: autoradiography studies in the mouse
Author(s), year:	Anonymous (1992)
Report/Doc. number:	ICI Central Toxicology Laboratory, unpublished report No. CTL/P/3474 (Company file: R-7105/TMN-0769)
Guideline(s):	In-house method
GLP:	Yes
Deviations:	Not applicable
Acceptability:	Yes

Material and Methods

Six male Crl: CD (ICR)BR mice were orally dosed with 900 mg/kg bw of [^{35}S] captan (radiochemical purity ≥ 99%, specific activity 888 MBq/mmoles). After 6, 24 and 120 hours of dosing, two animals were sacrificed. One animal was prepared for whole body radiography and from the other, portions of stomach, duodenum and ileum were taken and dissected for light microscopic autoradiography.

Findings

Whole body radiography:

After six hours administration, the majority of the radiolabel was seen in the stomach contents, with high levels also present in the oesophagus and intestinal contents. Moderate levels were detected in the kidney. Levels in the salivary glands were variable. Low concentrations were found in the liver, blood, skin and bone. After 24 hours the distribution of radiolabel was similar, though moderate levels were then found in the skin, trachea cartilage and bone. After 120 hours, no radiolabel was detected in the stomach or oesophagus and only low levels were found in the intestines. Moderate levels were found in the skin, trachea cartilage and bone.

Tissue radiography:
In the stomach, the majority of radiolabel was present in the squamous layers of the epithelium. In the duodenum and ileum, the majority of the radiolabel was located at the tips of the villi.

Conclusions

Whole body autoradiography following administration of captan suggests that captan is largely excreted via the gastrointestinal and urinary tracts. The majority of radiolabel had been excreted after five days. The radiolabel found in the bone and tracheal cartilage was thought to represent the utilisation of radioactive sulphur rather than incorporation of captan or a metabolite. There was no evidence of long-term retention of captan in the duodenum, demonstrating its rapid degradation. In addition, the radioactivity in the duodenum was located at the tips of the villi, which is the major site of interaction with the compound resulting in its irritation effect to the villi tips.

3.9.4.10 Becker et al. (2015)

Reference:	Increasing Scientific Confidence in Adverse Outcome Pathways: Application of Tailored Bradford-Hill Considerations for Evaluating Weight of Evidence
Author(s), year:	Becker, R A, Ankley, G T, Edwards, S W, Kennedy, S W, Linkov, I, Meek, B, Sachana, M, Segner, H, Van Der Burg, B, Villeneuve, D L, Watanabe, H and Barton-Maclaren, T S. (2015)
Report/Doc. number:	Regul Toxicol Pharmacol, 72, 514-37.
Guideline(s):	N/A published review article.
GLP:	N/A
Deviations	Not applicable
Acceptability:	Reliable

Background: The publication is an article discussing, and attempting to formalise, a transparent standardised approach to using the evolved Bradford Hill (BH) considerations with the accompanying guidance outlined in the OECD handbook for developing and assessing adverse outcome pathways (AOPs). It does this using case examples to clearly assign levels of confidence to key events (KEs) and key event relationships (KERs) and attempts to promote consistency in evaluation within and across AOPs. The article discusses the major lessons learned in the hope that a better understanding of the nature and form of documentation required to generate robust AOPs and increase the confidence in the application of AOPs to aid regulatory decision making. The reference list is extensive and only a limited set of references are included with this summary. Should the reader require more information they are referred to the original publication.

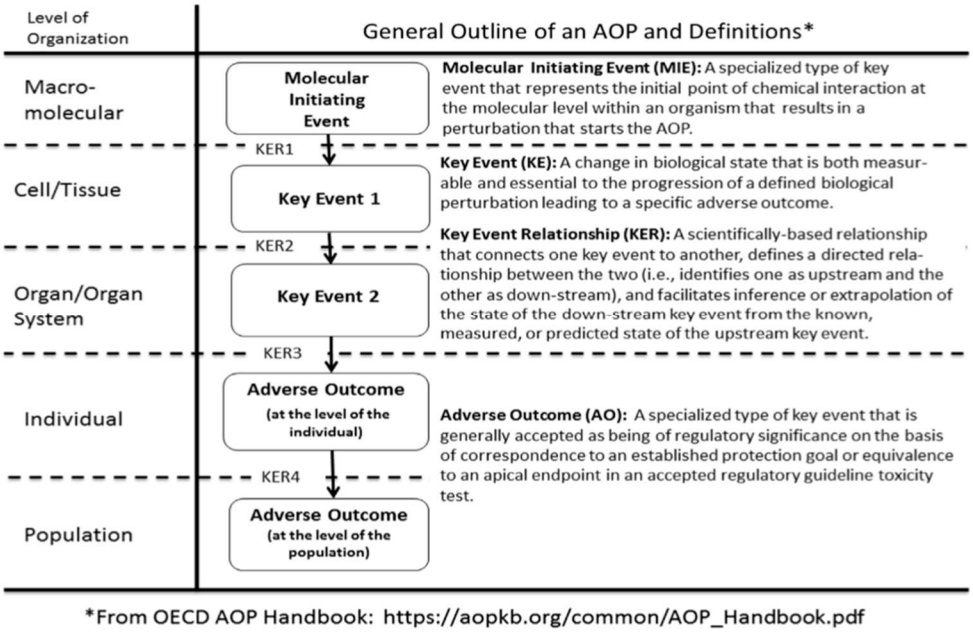
Introduction: There are large numbers of important commercial substances that require adequate risk assessment to protect human and environmental health. Traditional resource intensive standard *in vivo* toxicology cannot deliver this risk assessment and comply with ethical considerations on the use of animals. With the growing need to deliver robust hazard and risk assessment on more limited data sets, regulatory authorities in Canada, USA, European Union and the Asia Pacific region have issues various mandates and initiatives to develop novel and efficient strategies to assess hazard and risk. AOPs have the potential to help deliver cost effective ethical hazard and risk assessment by implementing scientific robust pathway- and mechanism-based approaches that can accommodate substances or groups of substances with varying amounts of toxicological information. However, to be useful, regulatory agencies need to be confident that the data supplied is adequate for human and environmental safety assessment, therefore further development, evaluation and standardisation of approaches are required.

The generation of mechanistic understanding has the potential to improve human and environmental risk assessment. The advancement of technology and mechanistic understanding of the molecular events (often generated by multiple approaches, e.g., *in vivo, in vitro* computation etc.) can be used to qualitatively and/or quantitatively predict potential toxicities in the absence of definitive data on the adverse effects. A major challenge is to integrate the data generated

from diverse sources at different levels of biological organisation in a manner that is transparent and informative enough for use in regulatory decision-making.

The AOP framework (Figure 3.9.4.10-1) outlines the mechanism of action (MOA) in a structured way and links the knowledge of the KEs (a change in a biological state that is measurable and essential for the progression of a defined biological perturbation) in a sequence that begins with a molecular initiating event (MIE; the initial point of chemical-biological interaction that sets an adverse outcome (AO) in motion) and proceeds through a series of higher order biological events, or KEs, that ultimately lead to an AO *in vivo* that is of interest for risk assessment. The KEs are linked to one another in series by Key Event Relationships (KERs).

Figure 3.9.4.10-1. (Reproduced from Becker et al., 2015). Framework for an AOP.



To ensure suitability of AOPs for specific application by the regulatory community, a weight of evidence (WoE) evaluation is incorporated in the AOP guidance handbook (OECD. 2013, 2014a) using the evolved Bradford-Hill (BH) WoE determinations in the application of MOA/species concordance analysis. The evolved BH considerations have been adopted for assessing the WoE of KEs, KERs and overall AOPs (OECD. 2014a). The guidance in the OECD AOP handbook has been informed and tested by example. The case examples discussed in this publication are considered essential for the continual evolution and refinement of the AOP process and lessons learned in the application of the guidance are highlighted in the publication. Additionally, a multi-criteria decision analysis (MCDA) model is introduced to illustrate an approach that may be useful for quantitatively weighting relevant BH considerations in AOP WoE analyses.

Application of evolved BH considerations for WoE in developing an AOP:

The challenges in conducting WoE evaluation for diverse AOPs and opportunities or improving the confidence in KEs, KERs and AOPs are discussed in the article. In a workshop titled "Advancing AOPs for Integrated Toxicology and Regulatory Applications" held in 2014, contributors of the OECD AOP Handbook (OECD 2014a) revised the evaluation to document levels of confidence by including categorical ratings of high (H) or strong, moderate (M) and low (L) or weak for KEs KERs and the over all AOP. The evaluation of KERs necessitates an understanding of the scientific support for (1) the Key Event$_{upstream}$, (2) the Key Event$_{downstream}$ and (3) the relationship between these events, summarised in Table 3.9.4.10-1 which includes rank ordered evolved BH considerations, with defining questions and guidance for assessing the relative level of confidence for biological plausibility, essentiality, and empirical evidence. In rank order the evolved BH considerations are (1) biological plausibility; (2) essentiality; and (3) empirical support (dose-response, temporality, consistency). Biological plausibility and empirical support is considered for each KE whereas essentiality of the KEs is considered in the context of the overall AOP. The OECD AOP handbook provides support for and is intended to promote

consistency of evaluation within and across AOPs and to explicitly capture the rationale of AOP developers for the levels of confidence assigned to KEs and KERs.

Table 3.9.4.10-1. (Reproduced from Becker et al., 2015). Guidance for assessing relative level of confidence in the overall AOP based on evolved Bradford-Hill weight of evidence considerations.

Biological Plausibility *Defining Question: Is there a mechanistic (i.e., structural or functional) relationship between Key Event$_{upstream}$ and Key Even$_{downstream}$ consistent with established biological knowledge?*		
High (Strong) Confidence: Extensive understanding of the Key Event Relationship based on extensive previous documentation and broad acceptance (e.g., mutation leading to tumors), i.e., an established mechanistic basis.	Moderate Confidence: The Key Event Relationship is plausible based on analogy to accepted biological relationships but scientific understanding is not completely established.	Low (Weak) Confidence: There is empirical support for a association between Key Events (see Empirical Evidence below), but the structural or functional relationship between them is not understood.
Essentiality[5] *Defining Question: Are downstream Key Events and/or the Adverse Outcome prevented if an upstream Key Event is blocked?*		
High (Strong) Confidence: Direct evidence from specifically designed experimental studies illustrating essentiality for at least one of the important Key Events (e.g., stop/reversibility/ recovery studies, antagonism, knockout models, etc.)	Moderate Confidence: Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a Key Event (e.g., augmentation of proliferative response in the Key Event$_{upstream}$ leading to an increase in Key Event$_{downstream}$ or in the Adverse Outcome).	Low (Weak) Confidence: No or contradictory experimental evidence of the essentiality of any of the Key Events.
Empirical Evidence[6] [7] *Defining Questions: Does the empirical evidence support that a change in Key Event$_{upstream}$ leads to an appropriate change in KeyEvent$_{downstream}$? Does KeyEvent$_{upstream}$ occur at lower doses and earlier time points than KeyEvent$_{downstream}$ and is the incidence of KeyEvent$_{upstream}$ greater than that for the KeyEvent$_{downstream}$? Are there inconsistencies in empirical support across taxa, species and stressors that don't align with an expected pattern for the hypothesized AOP? (Note: In many cases, evidence that contributes to quantitative understanding of a Key Event Relationship description will also provide empirical support for the relationship, and such relevant information should be considered as part of the overall weight of evidence evaluation of the concordance of empirical observations and consistency of the Key Event Relationship.)*		
High (Strong) Confidence: Multiple studies showing dependent change in both events following exposure to a wide range of specific stressors. Extensive evidence for temporal, dose-response and incidence concordance and no or few critical data gaps or conflicting data.	Moderate Confidence: Demonstrated dependent change in both events following exposure to a small number of specific stressors and some evidence inconsistent with an expected pattern that may be explained by factors such as experimental design, technical considerations, differences among laboratories, etc.	Low (Weak) Confidence: Limited or no studies reporting dependent change in both events following exposure to a specific stressor (i.e., endpoints never measured in the same study or not at all); and/or significant inconsistencies in empirical support across taxa and species that don't align with expected pattern for hypothesized AOP.

[1] Adapted from OECD AOP Handbook (OECD, 2014a,b,c), Annex 1. The BH considerations are rank ordered, as per Meek et al. (2014b) with respect for use in the overall weight of evidence determination of an AOP (i.e., biological plausibility > essentiality > empirical evidence). The following footnotes are verbatim from OECD AOP Handbook (OECD, 2014a,b,c), Annex 1, except as indicated with bracketed text.

[2] The guidance for "high", "moderate" and "low" draws on limited current experience. Additional delineation of the nature of relevant evidence in these broadly defined categories requires more experience with larger numbers of documented AOPs.

[3] "Direct evidence" implies specifically designed experiments to consider the relevant element. "Indirect evidence" normally relates to empirical support and is largely duplicative of Element 3 [empirical evidence].

[4] To the extent possible, each of the relevant Bradford Hill considerations is addressed for each of the KERs (biological plausibility and empirical support) and KEs (essentiality) and separate rationales provided.

[5] While the essentiality of each of the KEs is addressed separately, delineation of the degree of confidence is based on consideration of evidence for all of the KEs within the AOP and therefore, only one rationale is required.

[6] This is normally considered on the basis of tabular presentation of available data on temporal and dose-response aspects, in a template that documents the extent of support. See, for example, Meek and Klaunig (2010).

[7] Note that this relates to concordance of dose response, temporal and incidence relationships for KERs rather than the KEs; the defining question is not whether or not there is a dose response relationship for the KE but rather there is concordance with that for earlier and later KEs. This is normally demonstrated in studies with different types of stressors.

In this publication, the authors hope to contribute to further refinement of the AOP process and improve upon the guidance by reducing ambiguities and eliminating unnecessary duplicative evaluation of supporting data and increasing transparency in the AOP evaluation process. They do this by presenting six case studies from diverse AOPs and applying the evolved BH considerations to the AOPs. The six AOPs used to illustrate the WoE evaluation analysis are

- Aromatase inhibition leading to reproductive dysfunction in fish
- Aryl hydrocarbon receptor (AhR) activation leading to induction of cytochrome P450 (CYP) monooxygenases and oxidative stress.
- Juvenile hormone agonist-induction leading to increase in male offspring in the arthropod Cladocera.
- Binding of certain organophosphates to neuropathy target esterase (NTE) leading to delayed neuropathy.
- Agonist binding to oestrogen receptor a (ERa) leading to an increased risk of endometrial cancer.
- A chemical specific case example: induction of cytotoxicity and regenerative hyperplasia by oral hexavalent chromium (Cr(VI)) leading to duodenal tumours in mice – a chemical specific MoA analysis.

Aromatase inhibition leading to reproductive dysfunction in fish is evaluated in detail. To get a broader experience of using this framework to evaluate AOPs rather than focus on the specific AOPs, for the remaining 5 cases studies, the authors examined a subset of KEs and KERs to evaluate the WoE .

In this summary only the evaluation of the AOP considering the induction of cytotoxicity and regenerative hyperplasia by oral hexavalent chromium (Cr(VI)) leading to duodenal tumours in mice is summarised. This AOP has been shown to be relevant for captan and folpet and is discussed and summarised in detail elsewhere (Bhat. 2020). The reader is referred

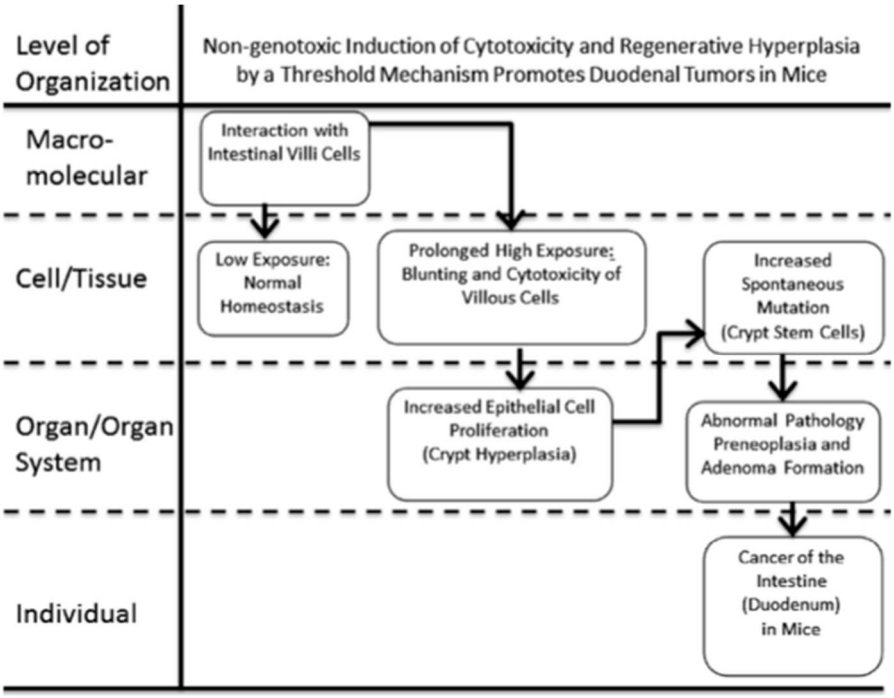
to this the original publication for more detailed use of the WoE approach using evolved BH considerations to document and present an AOP for use in regulatory decision making.

Case A5 (Becker et al. 2015): A chemical specific case example: Induction of cytotoxicity and regenerative hyperplasia by oral hexavalent chromium (Cr(VI)) leading to duodenal tumours in mice.

This AOP focuses on the tumour promotional MOA resulting from high-dose oral Cr(VI) exposure in mice. This AOP is a chemical-specific AOP and as knowledge of the MOA increases the breadth of application of a chemical specific AOP could expand to a broader chemical domain.

In the intestinal tract as well as other tissues, the multistep process of carcinogenesis is characterised by the canonical phases of initiation, promotion and progression. The initiation phase is generally considered to occur spontaneously or via exposure to chemicals. The promotion phase requires sustained proliferation often in the presence of decreased apoptosis in initiated cells, thereby conferring a selective advantage leading to clonal expansion and altered foci and ultimately tumours. Duodenal tumours in mice are believed to originate from mutations in stem cells that reside at the base of the crypts of Lieberkühn giving rise to proliferating progenitor or transit amplifying (TA) cells. TA cells occupy the length of the crypt and give rise to eight types of interstitial cells including enterocytes. Villus epithelium is rapidly turned over under normal conditions (approx. 3-5 days) and enterocytes move up from the base of the villus to the tip where they are sloughed off into the intestinal lumen. Exposure to sufficiently high concentrations of non-genotoxic substances can cause cytotoxicity to differentiated intestinal enterocytes resulting in increased loss of cells. This is compensated for by a burst of proliferation in the crypt to replenish the lost cells. However, sustained exposure to cytotoxic agents results in a chronic stimulation of proliferation and increases the opportunity for spontaneous mutation to occur. Clonal expansion is favoured in such a proliferating environment. This regenerative hyperplasia is a common mechanism of for the development of many tumour types. In the mouse duodenum, tumorigenesis can develop via a non-genotoxic mechanism of chronic cytotoxicity and regenerative hyperplasia and such mechanisms have been characterised as having a clear dose threshold leading the US EPA to reclassify captan. Studies clearly show that discontinuation of captan exposure leads to a reversal of captan mediated mouse duodenal toxicity (US EPA. 2004). Folpet, similar in structure to captan was also shown to act via a similar mechanism (Cohen. 2010, Gordon 2012). Following observation of mouse duodenal tumours in response to Cr(VI) exposure by the National toxicology programme (NTP) (NTP. 2008) extensive research to define the MOA and dose dependent sequence of events leading to mouse duodenal tumour formation has led to the development of the AOP for cytotoxicity and regenerative hyperplasia by oral Cr(VI) exposure in drinking water (Figure 3.9.4.10-2).

Figure 3.9.4.10-2. (Reproduced from Becker et al 2015). Depiction of the AOP of induction of cytotoxicity and regenerative hyperplasia by oral Cr(VI) leading to duodenal tumours in mice.



Biological plausibility (non-genotoxic induction of cytotoxicity and regenerative hyperplasia by a threshold mechanism promotes duodenal tumours in mice): The turnover on of normal villi epithelium is well understood as is hyperplasia in intestinal crypts in response to villous cytotoxicity. Additionally, prolonged cytotoxicity and associated with regenerative hyperplasia is a well-documented MOA for tumour development in a number of tissues for a large range of chemicals and has been described in toxicology textbooks since the mid 1980s. Therefore, the WoE for biological plausibility is high (strong).

Essentiality: Chronic cytotoxicity in enterocytes in small intestinal villi in mice is the MIE. Oral doses of Cr(VI) that overload the gastric capacity to reduced Cr(VI) to Cr(III) cause crypt hyperplasia eventually leading to duodenal tumorigenesis in mice. At does that do not exceed the gastric reducing capacity, duodenal villous cytotoxicity is not observed. Therefore, essentiality is focused on the KER, cytotoxicity leading to hyperplasia/proliferation. Destruction of villous enterocytes, caused by doses that exceed the cellular capacity to maintain homeostasis, causes a proliferative signal in the crypt resulting in hyperplasia. This is supported by data where exposing mice to Cr(VI) (180 mg/L) results in background levels of Cr in crypt cells, but >30-fold concentration of Cr in villous enterocytes.

The evidence for essentiality is moderate. In mice hyperplasia does not occur at doses below those that cause villous enterocyte cytotoxicity. Essentiality is supported by NTP studies in rats where specific villous blunting indicative of cytotoxicity did not occur in rats exposed to concentrations of Cr(VI) that caused cytotoxicity and tumorigenesis in mice. In rats there was no evidence of crypt hyperplasia and or intestinal tumours. The evidence is less than strong because no reversibility data existed for Cr(VI). However, reversibility has been demonstrated for captan showing that tumour progression is reversible upon cessation of dosing, indicating that there is a strong causal association (dose-response, temporality) and that tumour progression is secondary to cytotoxicity This has been accepted by the US EPA (US EPA, 2004).

Empirical support. At the time of publication, empirical support was deemed to be moderate to high (strong). Additional information, not available at the time of publication would have bolstered the WoE for KEs and KERs.

- **Empirical support of pre-MIE events:** Empirical support for the pre-MIE in this AOP include (1) reduction of Cr(VI) in the stomach and upper GI tract to Cr(III); (2) absorption on any Cr(VI) via sulphate and phosphate transporters small intestinal (SI) enterocytes. (Cr(VIII) is not readily bioavailable). Empirical evidence in the form of dose dependent increase in the Cr concentrations observed in intestinal enterocytes has been documented.

- **Empirical support for KER1(doses exceeding the gastric reducing capacity lead to cytotoxicity in intestinal villi):** At doses of Cr(VI) that exceed the gastric capacity to reduce Cr(VI) to Cr(III) induced cytotoxicity in villous epithelium in mice. This has been evaluated histopathologically as cytoplasmic vacuolization and blunting of the villi and a clear dose threshold exists. The empirical support for KE1 is high (strong).
- **Empirical support of KER2 (sustained cytotoxicity triggers crypt cell proliferation):** Hyperplasia occurs when stem cells in the intestinal crypts experience a prolonged stimulus to generate new epithelial cells. Hyperplasia has been observed and evaluated using histochemical and histopathological techniques. A clear dose and temporal response was observed. The empirical support for KER2 is high (strong).
- **Empirical support of KER3 (crypt hyperplasia leads to an increased probability of spontaneous mutations in cells within the crypt)**: The exact relationship between crypt hyperplasia and the occurrence of crypt cell mutations is not known nor does the methodology to measure this exact relationship exist. Therefore, the empirical evidence is indirect. The KE is postulated as sustained hyperplasia leading to increased probability of miss-repair of DNA leading to fixation of spontaneous mutations in daughter cells. There is no evidence of a direct mutagenic MOA following Cr(IV) exposure. The empirical support for this relationship is moderate.
- **Empirical support of KER4 (sustained increase in spontaneous mutations and sustained proliferative stimulus leads to formation of benign and malignant tumours)**: The KE is clonal expansion leading eventually to tumorigenesis. An increased spontaneous mutation coupled with chronic regenerative hyperplasia is well documented with clear dose and temporal dependance. The empirical support for this relationship is high (strong).

A detailed comparison of the MOA of captan induced and Cr(VI) induced mouse duodenal tumours, using the OECD AOP guidelines and evolved BH considerations is published by (Bhat et al. 2020) and demonstrates that captan and Cr(VI) share a common mechanism for the development of duodenal tumours in mice.

3.9.4.11 Thompson et al. (2017)

Reference:	Comparison of Toxicity and Recovery in the Duodenum of B6C3F1 Mice Following Treatment with Intestinal Carcinogens Captan, Folpet, and Hexavalent Chromium
Author(s), year:	Thompson, C. M., Wolf, J. C., McCoy, A., Suh, M., Proctor, D. M., Kirman, C. R., Haws, L. C., and Harris, M. A. (2017)
Report/Doc. number:	Toxicologic Pathology 45(8) 1091-1101
Guideline(s):	N/A published article.
GLP:	In-life portion of the study was conducted to GLP
Deviations	No applicable
Acceptability:	Reliable

Background

Duodenal tumours are rare in rodent toxicity studies. Chromium (Cr(VI), captan and folpet are a limited number of substances that have been shown to induce duodenal tumours in mice but not rats. These substances are not considered genotoxic *in vivo* and are considered to induce mouse duodenal and jejunum tumours via mechanism that involves chronic intestinal mucosal wounding and subsequent regenerative hyperplasia. The literature has identified common phenotypes that develop in mice upon exposure to Cr(VI), captan and folpet but a direct comparison of the toxicity induced by these mouse duodenal carcinogens was not available. The aim of this study was to perform a direct comparison on histological effects in mouse duodenum following exposure for 28-days to Cr(IV), captan and folpet. The reversibility of intestinal lesions was also studied following 28-day recovery a period.

Only data pertinent to captan exposure is summarised.

Material and Methods

Female B6C3F1 mice (n=20/group, with 10 animals per group used for recovery) approximately 9 weeks old and weighing between 16.4 and 23.1 g were acclimatised for 16 days prior to exposure to control diet (NTP-2000 irradiated meal diet) or the same diet containing captan (6000 or 16,000/12,000 ppm (see below)).

The study design is outlined below. The 6000 ppm captan dose was chosen as captan is structurally related to folpet, which was shown to induce intestinal hyperplasia in a 28-day mouse dosed at 6000 ppm) (Gordon et al. 2012). A top dose of 16,000 ppm captan was chosen to mirror the high dose used in an NCI (1997) cancer bioassay. However, this

was reduced to 12000 ppm from day 10 of the study as animals in the 16000 ppm captan group were of poor health by the end of the first week.

Captan (ppm)	Dosing phase (28 days) Animals (n)	Recovery phase (28 days) Animals (n)
0	20	10
6000	20	10
16000/12000	20	10
From day 10, the concentration of captan was reduced from 16000 to 12000 in the high dose group.		

Following 28 days exposure half of the animals in each group were sacrificed using CO_2 asphyxiation and the remainder were allowed to recover for a further 28-days before termination. The duodena were excised, for histopathological examination or immunohistochemistry analysis. Each duodenal segment was evaluated independently, and the findings reported using a scale to grade the severity: grade 1 = minimal, grade 2 = mild, grade 3 = moderate and grade 4 = severe.

To determine the level of regenerative hyperplasia, sections unstained by H&E were subjected to an antigen retrieval protocol followed by immunohistochemical staining for Ki67 using a rabbit anti-Ki67 monoclonal antibody (Thermo Scientific Fremont CA.) followed by detection using a goat anti-rabbit secondary antibody using standard techniques.

Findings

General observations: Two animals in the 16000 ppm captan group died during the first days of the experiment and a third was euthanized in moribund condition. Therefore, the captan dose was reduced to 12,000 ppm from day 10. No unscheduled deaths occurred for the remainder of the study. Four mice in the 12,000 captan dose group exhibited signs of dehydration and hunched posture until day 28 and two mice exposed to captan (6000 ppm) appeared thin. No clinical signs observed during the recovery period were considered treatment related. There was no statistical difference in body weight between the control and captan treated animals at the 28-day time point or following recovery.

Mean food intake between control and captan (6000 ppm) groups did not differ. The captan 12000 ppm group exhibited had sporadically reduced food intake compared to controls.

Histopathology observations: Treatment related observations at the end of the dosing phase (Table 3.9.4.11-1) tended to be more prominent in the proximal duodenum compared to the middle or distal segments. Crypt epithelial hyperplasia (minimal to mild), villous enterocyte hypertrophy (minimal to moderate), increased villous enterocytes (minimal to moderate), histiocytic cells in the villus lamina propria (minimal to mild), increased mononuclear cell infiltrates in the villi (minimal to mild) and rarefaction of the lamina propria (minimal to mild) was observed. With the exception of increased mononuclear cell infiltrates in duodenal villi of some control animals, all effects observed were treatment related. No severe grading (grade 4) was observed in any finding.

Following 28 days of recovery, the duodenal epithelium on the recovery phase control group tended to have greater proliferation compared to the dosing control group. Because of this, all animals in the recovery phase, regardless of group were scored relative to the appearance of those same diagnosis in the dosing control group and are summarised in Table 3.9.4.11-1. The prevalence and severity of observations in the treated mice in the recovery phase were generally diminished compared to those of treated mice in the dosing phase.

Table 3.9.4.11-1 Summary of histological data observed proximal duodenum.

	Exposure (28-day)			Recovery (28 day)		
	Control	6000 ppm	12000 ppm	Control	6000 ppm	12000 ppm
n – group size x - No. animals affected no. at Grade 1 2 3	n x grade 1 2 3	n x grade 1 2 3	n x grade 1 2 3	n x grade 1 2 3	n x grade 1 2 3	n x grade 1 2 3
Crypts, epithelium, hyperplasia	10 0 0 0 0	10 10 4 6 0	10 9 6 3 0	10 5 3 2 0	10 6 5 1 0	7 2 1 1 0
Villi, enterocytes, hypertrophy	10 0 0 0 0	10 10 0 1 9	10 10 0 1 9	10 6 2 3 1	10 3 3 0 0	7 5 5 0 0
Villi, enterocytes, increased	10 0 0 0 0	10 10 0 2 8	10 10 0 3 7	10 6 2 2 2	10 4 2 2 0	7 5 5 0 0
Villi, histiocytic cells	10 0 0 0 0	10 0 0 0 0	10 0 0 0 0	10 0 0 0 0	10 0 0 0 0	7 0 0 0 0
Villi, mononuclear cells, increased	10 3 3 0 0	10 2 2 0 0	10 4 4 0 0	10 2 1 1 0	10 4 0 4 0	7 0 0 0 0
Lamina propria, rarefaction	10 0 0 0 0	10 0 0 0 0	10 2 1 1 0			
No grade 4 findings were observed						

Evaluation of Ki67 by Immunohistochemistry showed that the staining for Ki67 was qualitatively similar in the duodena of treated and control mice. Ki67 staining occurred primarily in crypt epithelial cells where virtually all enterocytes were positively stained. There was a slight expansion of the crypt compartment in treated versus control animals.

Conclusions

Exposure to captan for 28-days resulted in a duodenal phenotype consistent with a mechanism of action of chronic mucosal toxicity followed by regenerative hyperplasia. Allowing animals to recovery for 28 days resulted in the predominant persistent changes (villous enterocyte hypertrophy and increased villous enterocytes) being comparable between control and treated mice. In all cases the severity of effect was less than that observed during the treatment phase, suggesting the effects are reversible. The persistence of histiocyes in villi and increased mononuclear cell infiltrates in the recovery phase were not considered surprising and these lingering inflammatory responses would resolve given adequate time. Histiocytic infiltration is often seen as a component of accumulation enteropathies (Gopinath et al. 1987) and may persist long after the original insult has subsided. These data, as well as the data generated on other substances in this report, support an adverse outcome pathway (AOP) for mouse small intestinal cancer that does not require carcinogens to be directly mutagenic.

3.9.4.12 Chappell et al. (2019)

Reference:	Comparison of Gene Expression Responses in the Small Intestine of Mice Following Exposure to 3 Carcinogens Using the S1500+ Gene Set Informs a Potential Common Adverse Outcome Pathway
Author(s), year:	Chappell, G. A., Rager, J. E., Wolf, J., Babic, M., LeBlanc, K. J., Ring, C. L., Harris, M. A. and Thompson, C. M.
Report/Doc. number:	Toxicol Pathol. 2019;47(7):851-64
Guideline(s):	N/A published article.
GLP:	In-life portion of the study was conducted to GLP and reported in Thompson et al. Toxicol Pathol 2017;45(8) 1091-1101
Deviations	No applicable
Acceptability:	Reliable

Background

Duodenal tumours are rare in rodents and humans. Oral exposure to hexavalent chromium (CR(VI)), captan and folpet were previously studied in parallel and shown to produce similar histopathological characteristics in female B6C3F1 mice. The study concluded that a common mechanism of action (MOA) for the development of duodenal tumours in mice exposed to Cr(VI), captan and folpet (Thompson et al. 2017). Tumours that develop by this mechanism developed following chronic mucosal toxicity followed by regenerative hyperplasia and did not require compounds to be directly mutagenic. The aim of this study was further elaborate on the proposed MOA using TempO-Seq technology and the S1500+ gene set to analyse transcriptomic response in the duodenal samples from Thompson et al. 2017 and anchor the transcriptomic responses to the histopathological findings reported in Thompson et al. 2017.

Due to the type of analysis performed it was not possible to completely isolate data specifically for captan exposure, however where appropriate only the data pertinent to captan exposure is discussed.

Material and Methods

Female B6C3F1 mice (n=20 / group, with 10 animals per group used for recovery) approximately 9 weeks old and weighing between 16.4 and 23.1 g were acclimatised for 16 days prior to exposure to control diet (NTP-2000 irradiated meal diet) or the same diet containing captan (6000 or 16,000/12,000 ppm (see below)).

The 6000 ppm captan dose was chosen as captan is structurally related to folpet, which was shown to induce intestinal hyperplasia in a 28-day mouse dosed at 6000 ppm) (Gordon et al. 2012). A top dose of 16,000 ppm captan was chosen to mirror the high dose used in an NCI (1997) cancer bioassay. However, this was reduced to 12000 ppm from day 10 of the study as animals in the 16000 ppm captan group were of poor health by the end of the first week.

The duodena were excised, flushed with phosphate buffered saline before being fixed with 10% neutral buffered formalin for histopathological examination or immunohistochemistry analysis. The duodenum was designated as proximal, middle and distal respectively and 3-4 mm long sections were collected at a distance of from the attached gastric pylorus and denoted: proximal 1 cm, middle 4 cm and distal 7 cm.

The following were analysed and compared

Transcriptomic analysis: Seven of the samples from each group were randomly selected for transcriptomic analysis. Subsequent transcriptomic analysis was carried out using TempO-Seq targeted RNA sequencing technology using the S1500+ gene list. (National Institute of Environmental Health Sciences) The sentinel genes cover all known canonical pathways from the Molecular Signature Database (version 4.0). The expression levels of 2754 mouse genes were measured using 3045 probes using the TempO-Seq platform.

Data processing: Data analysis was performed using multiple packages in R version 3.4 (cran.r-project.org/). Differentially expressed genes (DEGs) were identified from z-statistics generated using DESeq2 and compared against standard normal distribution curves adjusted for multiple testing using the Benjamin and Hochberg (BH) procedure. All differentially expressed probes (DEPs) were defined as having a false recovery rate of <10% based on BH-adjusted p values resulting in identification of unique DEGs in control versus exposed animals.

Comparison of global changes in gene expression was performed using the Hmisc package in R (version 1.6.20)..

Comparison of genes with differential expression across substances was performed with statistical analysis (Fisher exact test) between all gene lists testing the significance between 2 lists at a time using GeneOverlap R package (version 1.14.0).

Comparison of pathway level changes across substances, associated with transcriptional response profiles was performed with enrichment analysis using the Gene Set Enrichment Analysis (GSEA) method for statistical enrichment of pathways across ranked gene lists.

Findings

Histopathological finding. The histopathological findings for captan reported in Thompson et al 2017 are summarised below (Table 3.9.4.12-1).

Table 3.9.4.12-1: Histopathological findings

	Exposure (28-day)		
	Control	6000 ppm	12000 ppm
n – group size x - No. animals affected no. at Grade 1 2 3	n x grade 1 2 3	n x grade 1 2 3	n x grade 1 2 3
Crypts, epithelium, hyperplasia	10 0 0 0 0	10 10 4 6 0	10 9 6 3 0
Villi, enterocytes, hypertrophy	10 0 0 0 0	10 10 0 1 9	10 10 0 1 9
Villi, enterocytes, increased	10 0 0 0 0	10 10 0 2 8	10 10 0 3 7
Villi, histiocytic cells	10 0 0 0 0	10 0 0 0 0	10 0 0 0 0
Villi, mononuclear cells, increased	10 3 3 0 0	10 2 2 0 0	10 4 4 0 0
Lamina propria, rarefaction	10 0 0 0 0	10 0 0 0 0	10 2 1 1 0
Summary of the histological findings from (Thompson et al 20.17) in response to captan exposure for 28 days at 6000 and 12000 ppm. No grade 4 findings were observed			

Gene expression changes in the duodenum of mice associated with captan exposure. In response to captan exposure there were 307 probes (304) genes and 346 probes (339 genes) identified with significant differential expression as a result of exposure to captan via the feed in the 6000 ppm and 12000 ppm groups respectively (Table 3.9.4.13-2).

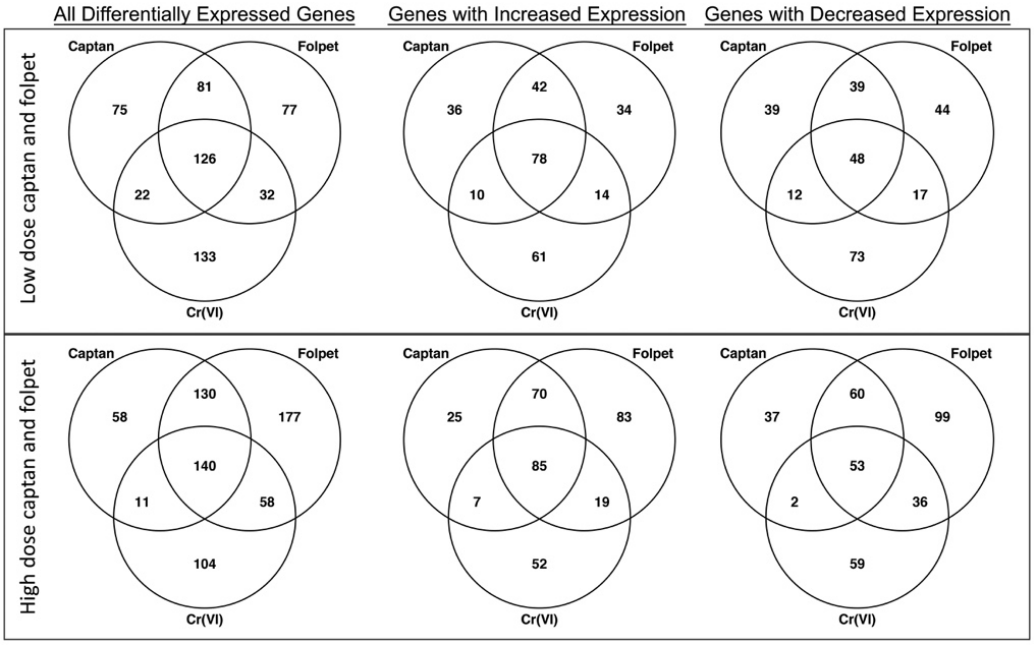
Table 3.9.4.12-2: Gene expression changes in the duodenum of mice

	Captan 6000 ppm	Captan 12000 ppm
All DEPs	307	346
Probes with increased expression	169	193
Probes with decreased expression	138	153
All DEGs	304	339
Probes with increased expression	166	187
Probes with decreased expression	138	152
Counts are out of 3045 probes representing 2754 genes in the mouse S1500+ gene list		

Comparative analysis between all transcriptional responses. Examining all genes measured, whether statistically regulated or not, at the probe level resulted in similar responses with respect to the magnitude of change between the agents tested (Cr(VI), captan and folpet when compared with control animals. Additionally, the total number of unique DEGs between any two groups was relatively low. Statistical comparisons between captan 12000 ppm and folpet 16000 ppm resulted in only 46 DEGs being different between the two groups.

Comparative analysis between genes with significant differential expression across substances: Examining genes that showed statistically significant changes in expression resulted in 126 genes overexpressed in common between Cr(VI) (180 ppm) and low dose captan and folpet and 140 genes differentially expressed in common between Cr(VI) (180 ppm) and high dose captan and folpet (Figure 3.9.4.13-1). The most significant number of intersecting genes was observed when comparing mice treated with the same substance at two doses. For captan, 6000 ppm versus captan 12000 ppm there were 207 and 270 overlapping genes respectively, with a 120 and 155 respectively showing increased expression and 87 and 133 respectively showing decreased expression.

Figure 3.9.4.12-1. Reproduced from Chappell et al (2017).



Comparison of the numbers of genes identified as differentially expressed by Cr(VI), captan, and folpet in the mouse intestine. Counts are out of a total of 2754 genes that were measured from the S1500+ gene set. The top panel shows a comparison between the Cr(VI) (180 ppm in drinking water) and the lower dose captan and folpet groups (6000 ppm in the feed). The bottom panel shows the comparison between the Cr(VI) (180 ppm in drinking water) and the higher dose captan and folpet groups (12 000 and 16 000 ppm, respectively, in feed). Note that all overlapping gene counts between all chemicals at all concentrations were significantly higher than those expected by chance (Fischer exact test P <0.001).

Comparative of exposure induced pathway alterations. An enrichment analysis across 1327 canonical pathways identified 143 upregulated pathways associated with Cr(IV) and/or captan and/or folpet at lower pesticide concentrations. At higher concentrations there was 145 upregulated pathways associated with Cr(VI) and/or captan and/or folpet. Of these, 21 pathways were commonly regulated between Cr(IV) and higher dose captan and folpet and 25 commonly regulated between Cr(VI) and lower dose captan and folpet. The overall transcriptomic changes were not significantly different between low and high dose exposures for captan or folpet, therefore comparative analysis was done with the higher-dose (cancer bioassay relevant) concentrations. The peroxisome proliferator activated receptor (PPAR) and hypoxia inducible factor 1 (HIF1) pathways were among the most significantly enriched gene sets. HIF1 relevant activator protein 1 (AP1) family pathway signalling was also prevalent. AP1 signalling is involved in cell growth and proliferation. In the high dose captan and folpet groups, 28 pathways were enriched independently of Cr(VI) and were related to metabolism, including xenobiotic metabolism e.g. biological oxidations, glutathione metabolism and specifically included multiple cytochrome P450 metabolism pathways, phase 2 conjugation pathways (Table 3.9.4.12-3).

Table 3.9.4.12-3. Reproduced from Chappell et all 2019) Upregulated pathways (p value <0.05) that were commonly associated with Cr(vi) (180 ppm via drinking water), captan (12000 ppm via feed), and/or folpet (16000 ppm via feed) exposures in the mouse intestine.

Table 3. Upregulated Pathways (*P* value <.05) That Were Commonly Associated With Cr(VI) (180 ppm via Drinking Water), Captan (12 000 ppm via feed), and/or Folpet (16 000 ppm via feed) Exposures in the Mouse Intestine.

		Cr(VI) (180 ppm)		Captan (12 000 ppm)		Folpet (16 000 ppm)	
Canonical Pathway Name	Pathway Database	*P* Value	% of Genes in Pathway with Increased Expression[a]	*P* Value	% of Genes in Pathway with Increased Expression[a]	*P* Value	% of Genes in Pathway with Increased Expression[a]
Metabolism of lipids and lipoproteins	REACTOME	1.73E-04	68%	3.64E-03	58%	1.27E-02	52%
Fatty acid triacylglycerol and ketone body metabolism	REACTOME	1.84E-04	75%	1.62E-02	71%	1.28E-02	60%
PPAR signaling pathway	KEGG	1.89E-04	73%	9.62E-03	67%	9.25E-03	67%
HIF1 TF pathway	PID	1.90E-04	60%	8.43E-04	63%	1.07E-02	55%
Glycolysis gluconeogenesis	KEGG	3.88E-04	78%	3.54E-04	74%	1.92E-04	74%
Membrane trafficking	REACTOME	5.68E-04	69%	1.76E-03	77%	2.86E-03	73%
PPARA activates gene expression	REACTOME	5.69E-04	76%	1.25E-02	76%	2.57E-02	67%
Peroxisome	KEGG	1.15E-03	82%	1.44E-03	76%	3.10E-03	65%
Fatty acid metabolism	KEGG	1.15E-03	71%	8.45E-03	82%	7.74E-04	59%
Glycolysis	REACTOME	1.18E-03	91%	1.84E-04	91%	9.95E-04	100%
Pentose phosphate pathway	KEGG	1.95E-03	90%	5.60E-04	100%	6.10E-04	100%
Glycolysis pathway	BIOCARTA	2.19E-03	80%	1.33E-03	100%	4.35E-03	100%
Peroxisomal lipid metabolism	REACTOME	2.35E-03	100%	2.49E-02	100%	3.42E-02	75%
Glucose metabolism	REACTOME	4.27E-03	65%	7.07E-04	78%	5.76E-04	65%
NUCLEARRS pathway	BIOCARTA	7.01E-03	80%	3.92E-03	80%	3.86E-03	70%
Fructose and mannose metabolism	KEGG	1.06E-02	67%	7.42E-04	75%	2.40E-03	83%
Gluconeogenesis	REACTOME	1.15E-02	69%	1.50E-02	69%	1.80E-03	69%
Pyruvate metabolism	KEGG	2.51E-02	56%	1.94E-02	78%	2.01E-03	78%
Tryptophan metabolism	KEGG	2.57E-02	61%	4.98E-03	67%	5.88E-04	61%
Pyruvate metabolism and citric acid TCA cycle	REACTOME	3.74E-02	50%	1.81E-02	80%	3.84E-02	90%
Lipoprotein metabolism	REACTOME	4.37E-02	76%	1.76E-04	76%	1.94E-04	76%
PPARA pathway	BIOCARTA	2.50E-03	85%	3.96E-02	77%	5.26E-01	77%
PTDINS pathway	BIOCARTA	4.00E-03	54%	5.62E-02	54%	3.56E-02	49%
Activation of the AP1 family of transcription factors	REACTOME	1.36E-02	82%	7.83E-02	82%	4.46E-02	82%
Valine leucine and isoleucine degradation	KEGG	1.59E-02	83%	4.98E-02	83%	2.61E-01	67%
mTOR 4 pathway	PID	1.68E-02	61%	1.61E-01	72%	2.31E-02	56%
Trans Golgi network vesicle budding	REACTOME	3.05E-02	63%	3.89E-01	63%	1.07E-02	63%
Glycerolipid metabolism	KEGG	3.65E-02	88%	1.12E-01	88%	1.32E-02	88%
Formation of ATP by chemiosmotic coupling	REACTOME	4.15E-02	89%	3.14E-01	56%	1.37E-02	56%
Lysosome vesicle biogenesis	REACTOME	4.18E-02	100%	5.51E-02	100%	3.90E-02	100%
Histidine metabolism	KEGG	4.63E-02	100%	5.62E-02	100%	7.05E-03	100%

Abbreviations: AP1, activator protein 1; BIOCARTA, BioCarta online maps of metabolic and signaling pathways; Cr(VI), hexavalent chromium; HIF1, hypoxia inducible factor 1; KEGG, Kyoto Encyclopedia of Genes and Genomes; PID, Pathway Interaction Database; PPAR, peroxisome proliferator activated receptor; REACTOME, Reactome database of reactions, pathways, and biological processes.

[a]Denominator represents the genes assayed that are in the pathway, not all genes reported to be in the pathway.

There were 138 commonly downregulated pathways identified with Cr(VI), captan (12000 ppm) or folpet (16000 ppm). Of these only 5 were modulated by all three substances (Table 3.9.4.12-4). In captan and folpet only, an additional 28 canonical pathways were down regulated and primarily involved transfer RNA and mRNA processing and many overlapped with those down regulated in the higher dose group.

Table 3.9.4.12-4. Reproduced from Chappell et all 2019) Down regulated pathways (p value <0.05) that were commonly associated with Cr(vi) (180 ppm via drinking water), captan (12 000 ppm via feed), and/or folpet (16 000 ppm via feed) exposures in the mouse intestine.

Table 4. Downregulated Pathways (*P* Value <.05) That Were Commonly Associated With Cr(VI) (180 ppm via Drinking Water), Captan (12 000 ppm via Feed), and/or Folpet (16 000 ppm via Feed) Exposures in the Mouse Intestine.

		Cr(VI) (180 ppm)		Captan (12000 ppm)		Folpet (16000 ppm)	
Canonical Pathway Name	Pathway Database	*P* Value	% of Genes in Pathway With Decreased Expression[a]	*P* Value	% of Genes in Pathway With Decreased Expression[a]	*P* Value	% of Genes in Pathway With Decreased Expression[a]
Systemic lupus erythematosus	KEGG	2.14E-04	83%	6.34E-03	58%	1.45E-02	69%
TCR (T cell Receptor) pathway	PID	8.52E-03	67%	7.48E-02	63%	2.64E-02	67%
Glycosphingolipid biosynthesis ganglio series	KEGG	2.21E-02	80%	1.50E-01	80%	4.48E-02	100%
Glycosphingolipid biosynthesis globo series	KEGG	2.68E-02	60%	1.58E-01	80%	4.36E-02	100%
Notch HLH (helix-loop-helix) transcription pathway	REACTOME	4.43E-02	50%	3.83E-01	100%	3.12E-03	50%

Abbreviations: Cr(VI), hexavalent chromium; KEGG, Kyoto Encyclopedia of Genes and Genomes; PID, Pathway Interaction Database; REACTOME, Reactome database of reactions, pathways, and biological processes.
[a]Denominator represents the genes assayed that are in the pathway, not all genes reported to be in the pathway.

Anchoring transcriptomic findings to histopathological data. Within in the duodenal tissues examined by histology (**Thompson et al. 2017**), captan and folpet were shown to cause crypt epithelial hyperplasia, villus enterocyte hypertrophy, increased villus enterocytes, and increased villus mononuclear cell infiltrate following 28 days of exposure. Pathways related to these phenotypes were enriched specifically for pathways involved in cell growth, proliferation and cell cycle signalling (e.g. HIF1, AP1, mTOR (mammalian target of rapamycin) and PPAR pathways) and were upregulated (Table 3.9.4.12-3).

Conclusions

The interpretation of rodent intestinal tumours with regard to human health risk is difficult as intestinal tumours are rare in 2 year cancer bioassays, and a limited number of substances are known to induce these in rodents. Of the known agents that induce rodent intestinal tumours, the hypothesis is that toxicity is caused by a common underlying mechanism of chronic mucosal wounding followed by regenerative hyperplasia. The substances known to cause intestinal tumours *in vivo* are not directly genotoxic. By examining the molecular events following exposure to Cr(VI), captan and folpet, and anchoring these in the histopathological phenotype, similar induction and significant overlap of gene and pathway modulation was observed. These data provide evidence that these structurally diverse rodent intestinal carcinogens cause toxicity through a common underlying process at the molecular and tissue levels. AP1 and HIF1 signalling are commonly involved in the response to all 3 carcinogens and are directly relevant to intestinal injury. Additionally, pathways related to cellular proliferation, growth, and signalling were phenotypically anchored to the histopathological changes observed, namely crypt epithelial hyperplasia, villus enterocyte hypertrophy, increased villus enterocytes and increased villus mononuclear cell infiltrate. These data provide evidence for the hypothesised MOA of chronic damage to the villi accompanied by regenerative hyperplasia and will provide a base from which to start to construct an adverse outcome pathway for rodent intestinal cancer.

3.9.4.13 Bhat et al. (2020)

Reference:	An adverse outcome pathway for small intestinal tumors in mice involving chronic cytotoxicity and regenerative hyperplasia: a case study with hexavalent chromium, captan, and folpet
Author(s), year:	Bhat, V. S., Cohen, S. M., Gordon, E. B., Wood, C. E., Cullen, J. M., Harris, M. A., Proctor, D. M. and Thompson, C. M.
Report/Doc. number:	Crit Rev Toxicol. 2020;50(8):685-706.
Guideline(s):	N/A published review article.
GLP:	N/A
Deviations	No applicable
Acceptability:	Reliable

Background

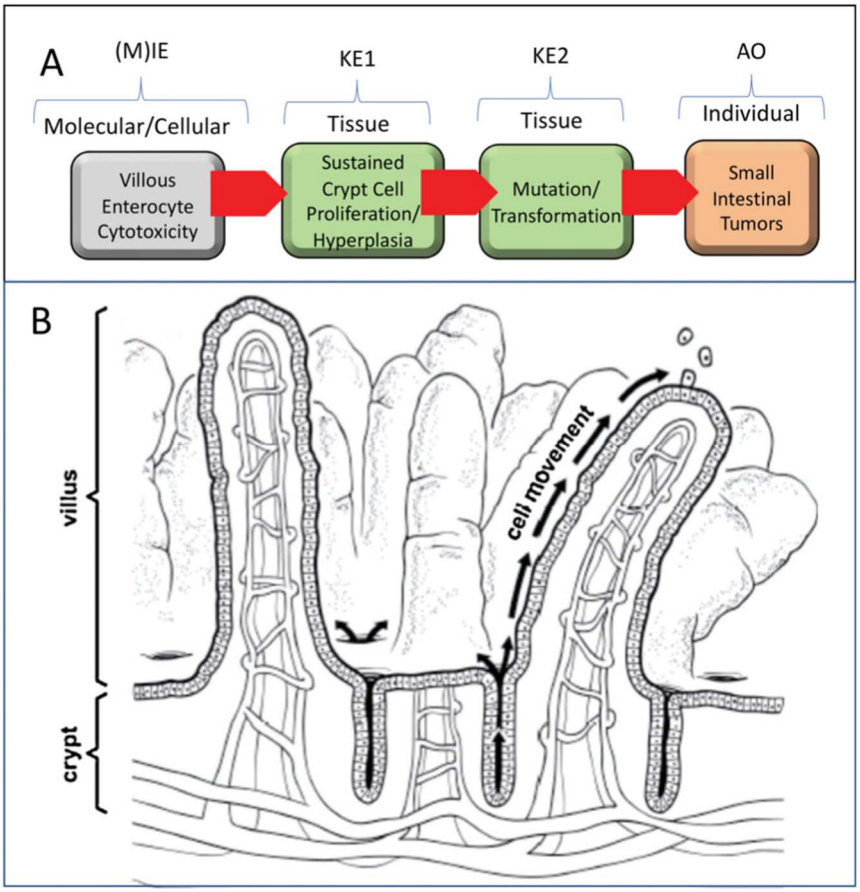
This publication is a review article discussing the development of an adverse outcome (AO) pathway (AOP) for substances that cause intestinal cancer in rodents. The review focus on hexavalent chromium (Cr(VI)), captan and folpet as case studies. This summary will summarise the discussion on the development of the AOP and will not focus specifically on captan. In addition, should the reader require additional information from the extensive reference list included in this publication, they are referred to the original publication. An extensive reference list has not been included in this summary.

Human health risk assessment has evolved to understanding the mechanistic basis for chemical-indued tumorigenesis thereby facilitating the development of a hypothesised mechanism of action (MOA) that can be used to inform quantitative approaches to deriving human and environmental safety criteria. Intestinal cancer is rare in rodent bioassays and historically lack a clear aetiology. In addition, the physiology and metabolic capacity of the small intestine (SI) is not well studied in relation to human health. As a result, the default regulatory position has been to assume that the MOA is mutagenicity and the shape of the dose-cancer relationship is low-dose linear for certain intestinal carcinogens. Following release in 2008 of the result of a National Toxicology Programme (NTP) study reporting Cr(VI) sites of carcinogenicity in rats (oral cavity) and mice (duodenal), a large body of work targeted at informing risk assessment have concluded that Cr(VI) caused duodenal tumours in mice via a non-mutagenic MOA and that non-linear risk assessment approaches are more appropriate. This approach has been adopted widely by risk assessment authorities resulting in a clear precedent for a cytotoxicity-mediated MOA for SI tumours. Captan and folpet are hypothesised to induce duodenal tumours in mice through a similar MOA of sustained damage to the SI villi resulting in chronic intestinal epithelial hyperplasia . As a result, US EPA Office of Pesticide Programmes has determined that captan is unlikely to be a human carcinogen at dose levels that do not cause irritation and regenerative hyperplasia in the SI. Studies comparing the SI effects of Cr(VI), captan and folpet (Thompson et al. 2017a+b and Chappell et al 2019), suggests that these compounds induce mouse intestinal tumours via a similar MOA thereby providing enough compressive data to support the development of a cytotoxicity-mediated AOP for mouse duodenal tumours. The aim of this review was to document the weight of evidence (WOE) and discuss the key events (KEs), the KE-relationships (KERs) leading to SI tumours and evaluate this WOE to support or refute the development of an AOP that can be used to support regulatory applications including hazard and risk assessment.

AOP development

The proposed AOP conforms to the Organisation for Economic Co-operation and Development (OECD) (**OECD 2018**) with the following modification to address (1) a relatively rare tumour location in rodent bioassays, (2) carcinogens that likely have non-specific initiating events (e.g. not receptor mediated), and (3) avoidance of necessary redundancy inherent to many frameworks. The AOP shown in Figure 3.9.4.13-1 (A) proposes that, upon entering the duodenum, cytotoxicity of the villous epithelium is the molecular initiating event (MIE). Over time, to compensate for the rate of enterocyte loss, the regenerative capacity of the gut epithelium declines, particularly at higher compound concentrations. The first KE is sustained regenerative crypt proliferation/hyperplasia. Due to the extensive repair capacity of the gut, this would likely require exposure times in the order of 6-12 months to create more opportunities for the second KE, spontaneous mutation/transformation, ultimately leading to SI tumours. When considering this AOP, it is important to note and understand characteristics of the SI. It is divided into three contiguous segments, duodenum (proximal SI), jejunum and ileum (distal SI). The SI is the primary site of nutrient absorption and large surface area created by the villi projections facilitate this (Figure 3.9.4.13-1 (B)). The villi are lined by mature differentiated enterocytes supplied by stem cells that reside at the base of the crypts or glands of Lieberkühn. The intestinal mucosa is amongst the most proliferative tissue in the body with cells moving from the villi crypts to the tip of the enterocytes (along the crypt villus axis) in approximately 2-3 days in rodent and 3-6 days in humans. A continuous supply of daughter cells emerging from the intestinal crypts move towards the villus where they become committed to non-dividing enterocytes and which are sloughed off the villi tips by passing intestinal content. The prevailing theory of intestinal cancer is that tumours arise from mutations that occur within the crypt stem cells. These cells remain at the base of the crypt and so have time to acquire multiple mutations leading to transformation. The crypts reside below the intestinal surface and are filled with mucous that is continuously secreted by goblet cells. Overall, the intestinal structure is similar in rodents and humans. The main difference is that human intestine contains structures called plicae, which are circular folds lined with villi that further increase the surface area of the SI. In rodents the villi are taller, resulting in increased surface area. In all three species (rat, mouse, human), the duodenal villi are taller than those in the jejunum and ileum. All three species contain a large intestine and colon that contain crypts but not villi.

Figure 3.9.4.13-1. Reproduced from Bhat et al 2020. Proposed AOP for cytotoxicity-mediated SI cancer in mice. (A) AOP diagram. (B) Structure of intestinal mucosa



See text for discussion regarding (M)IE. Future evolution of this AOP may better define the (M)IE associated with villous cytotoxicity. Villi are finger- or leaf-like projections into the lumen that are predominantly covered with mature, absorptive enterocytes, along with occasional mucus-secreting goblet cells. These cells live only for a few days, then die and slough into the lumen. The crypts, or glands of Lieberkühn, are tubular invaginations of the epithelium, lined largely with younger epithelial cells, which serve as a source of enterocytes to multiple villi. At the base of the crypts are stem cells, which divide continually and function as the source of all the epithelial cells in the crypts and on the villi.

Molecular initiating event; villus enterocyte cytotoxicity:

Overview: To date there is no clear evidence for a specific molecular initiating event or SI tumours. However, there is evidence for the general anatomical and cellular location of the initial point of interaction and key evidence supports reactivity of bioactivated compounds with cellular components leading to enterocyte injury and death. There is broad precedent for regulatory acceptance of cytotoxicity as an initial or early KE in non-mutagenic MOA. Guidelines from the US EPA and other health agencies internationally consider cytotoxicity and regenerative hyperplasia as a reference MOA relevant to a wide variety of chemical agents, target tissues and exposure routes, examples including captan and folpet which are regarded as carcinogens that act via a cytotoxic MOA. Others include thiamethoxam-induced mouse liver tumours, chloroform-induced kidney tumours in rats, acetochlor-induced nasal cavity tumours and saccharin-induced bladder tumours in rats. As a result, the authors have characterised villous enterocyte cytotoxicity as a (M)IE with molecular in brackets to denote that cytotoxicity is an initial or early KE in a non-mutagenic AOP/MOA which may not involve or require a specific molecular event. A common requirement for SI non-genotoxic carcinogens is the requirement to be transported to the SI lumen and inducing cytotoxicity in the duodenal epithelium. High concentrations of Cr(VI), that is not reduced to Cr(III) in the stomach can enter the duodenum where the higher pH limits Cr(VI) reduction. Cr(VI) in the SI lumen can react with protein thiols and/or DNA leading to cytotoxicity and depletion of cellular glutathione (GSH) levels. Captan or folpet are not readily metabolised in the acidic pH of the stomach. However, at the higher pH ot the intestinal lumen, they are readily hydrolysed by reaction with cellular thiols to form thiophosgene which causes cytotoxicity in the SI. Therefore, the SI is susceptible to damage by these structurally distinct agents. Transcriptomic analysis of duodenal tissue following 28-day exposure to Cr(VI), captan and folpet, at carcinogenic doses, resulted in clear activation of hypoxia inducible factor 1 (HIF-1) and activator protein 1 (AP1) signalling pathways (Chappell et al.

2019). Activation of these signalling pathways provide good evidence that upstream response leading to their activation was cytotoxicity. Pharmacological depletion of GSH results in shortened intestinal villi and indicates cytotoxicity which is prevented by GSH supplementation. Chemicals that deplete GSH in intestinal villi, very likely accelerate enterocyte sloughing, resulting in increased regenerative proliferation. Cr(VI), captan and folpet are all capable of interacting with GSH and other macromolecule thiols and potentially leading to decreased cellular GSH. However, data for effects on GSH are limited. Folpet decreases intestinal GSH 0.5-2 hours following exposure, but intestinal cellular GSH levels were higher following 6 hours exposure. Similarly, GSH levels and genes involved in GSH-synthesis following 13-week exposure to Cr(VI) are higher than control animals. These data support the idea that acute exposure to Cr(VI), folpet or captan decrease GSH levels, but induce compensatory GSH synthesis. It is not currently clear if changes to cellular GSH are an associative factor or specific MIE for these substances.

There is good evidence that duodenal villi are the initial target SI tumorigenesis in mice. Zynchrotron-based x-ray fluorescence (XRF) microscopy demonstrated that Cr(VI) (180 ppm) exposure in drinking water for 6 or 90 days resulted in Cr fluorescence concentrated in the tips of the villi and a lesser extent in the villi, but not in the crypts. In addition, tissue levels of Cr (mg/kg of tissue) after 90 days exposure where higher in the duodenum than either the stomach or the jejunum.

The intestinal lumen is a critical barrier to the gut with distinct compartmentalisation between the crypt and the villi. Under normal circumstances the invaginated crypts are not exposed to the contents of the intestinal lumen. This is supported by evidence showing that cytochrome P 450 (CYP)-1A1 induced by benzo[a]pyrene is induced in the intestinal villi following exposure by oral gavage but is induced in the intestinal crypts following exposure via intraperitoneal injection, thereby demonstrating that intestinal crypts should be considered as being part of the systemic compartment and not readily accessed from the lumen of the SI.

While Cr(VI) has been directly visualised in the villi but not the crypts by XRF, direct visualisation of captan and folpet in the duodenal villi has not been demonstrated. Higher duodenal captan concentrations occur in mice exposed compared to rats and this is considered by the US EPA to potentially explain the sensitivity of mice to these agents. There is indirect evidence for the presence of cytotoxic substances in the SI that can be determined histologically. Acute toxicity may be detected as necrosis shortly after high dose exposure. Mild enterocyte toxicity can be detected as increased crypt replication to compensate for greater enterocyte loss due to increased sloughing of enterocytes from the villi. When the cell loss rate increases, crypt hyperplasia occurs. If this is sustained and the proliferation rate is inadequate to replenish the loss of enterocytes, the villi become shorter/blunted and the enterocytes may become more cuboidal or squamous in shape in order to increase their surface area to adequately cover the basement membrane. These effects can also be observed if there is crypt toxicity. However, the XRF imaging of Cr(VI) exposed mice clearly shows healthy proliferating crypts, suggesting that crypt damage is not the cause of blunting observed following captan, folpet or Cr(VI) exposure.

Once inside cells, Cr(VI), captan or folpet do not undergo enzymatic metabolism. All three agents react with thiols (e.g., GSH). Captan and folpet hydrolyse to thiophosgene and tetrahydrophthalimide. Thiophosgene can readily react with thiols on cellular macromolecules including protein and DNA. The most likely route of toxicity of these agents is non-specific, protein binding, and alteration of the cellular redox balance. Importantly villous enterocytes are committed, non-proliferating cells that slough into the intestinal lumen with 24-48 h of reaching the villi. Therefore, the likely hood of intestinal tumours arising from DNA damage to differentiated enterocytes is not feasible and is not supported by the available data set for Cr(VI), captan or folpet.

Temporality: For this AOP the authors have separated the cytotoxicity MIE from sustained crypt cell proliferation (KE#1, below) by distinguishing exposures of ≤ 1 week from longer exposure studies. This is important as it distinguishes transient molecular interactions from sustained interactions. There is consistent evidence of villous enterocyte cytotoxicity as early as 1 to 7 days after exposure to Cr(VI), captan or folpet at tumorigenic concentrations. Extended exposure durations do not appreciably impact the magnitude of the cytotoxic response, likely due to the efficient regenerative repair capacity of the intestinal epithelium.

Key event #1; sustained crypt proliferation/hyperplasia

Sustained crypt cell proliferation/hyperplasia is distinguished from the MIE, by considering exposures longer than 1 week as evidence for sustained crypt hyperplasia. Sustained crypt hyperplasia is defined as clear evidence of an elongated crypt compartment and potentially altered villus structure. Crypt hyperplasia has been observed in exposure durations of 1-, 4-, 13- and 104-weeks following Cr(VI) exposure. Histopathological examination also revealed increased crypt length and increased crypt enterocyte numbers at 1 and 13 weeks demonstrating a dose and temporal progression with crypt hyperplasia occurring after 7-90 days of carcinogenic Cr(VI) exposure. Similar temporal crypt hyperplasia progression was observed following exposure to carcinogenic doses of captan and folpet. Crypt hyperplasia was evident following exposure to captan (3000 ppm) as early as 3 days but was not evident after 1 day.

Increased proliferation in mouse duodenal mucosa (measured by either bromodeoxyuridine (BrdU) incorporation, proliferating cell nuclear antigen (PCNA) or Ki67 mRNA levels) was also demonstrated following dietary captan exposure (6000 ppm) for various time frames between 28 and 91 days and for Cr(VI) exposure (≥ 60 ppm) between 7 and 90 days.

The OECD (2018) states that reversibility of a key event is evidence for it being essential and that it is necessary but not sufficient to drive the AO. It is difficult to prove reversibility of sustained hyperplasia without monitoring recovering animals for several months. This is uncommon in toxicity studies. However, captan exposure requires ≥ 6-months exposure to induce tumours and diffuse hyperplasia requires several months to resolve. In 6-, 12- and 18-month studies with exposure to capthan (6000 ppm), hyperplasia and tumour burden decreased to control levels following 6 months recovery. In recovery animals the SI exhibited muscular hypertrophy, submucosal fibrosis and ectasia of submucosal vessels. These lesions appeared to coincide with resolving foci of focal hyperplasia where the epithelial repair exceeded the repair of mesenchymal tissue. These studies provide evidence that the regenerative hyperplasia observed is not as a result of genetic damage leading to dysregulated cell growth, otherwise recovery after dosing would not be observed. In dietary captan and folpet (6000 ppm) exposure studies, as well as Cr(VI) drinking water exposure studies, for 4-weeks with 2-week recovery, ~50% reduction in crypt hyperplasia was observed. Histological evidence did not support a DNA-reactive mechanism for the observed hyperplasia and subsequent decrease following removal of the compounds.

Cr(VI), captan and folpet do not induce SI tumours in rats. Rats did exhibit signs of crypt hyperplasia and villous damage, but to a much lesser extent than mice. In rats Cr(VI) dose selected for the 2-year bioassay was 180 ppm based on reduction in bodyweight, reduced water consumption and increased glandular stomach ulceration at higher concentrations in shorter-term assays. In mice the top concentration was chosen based on reduction in bodyweight and water consumption. It is established that Cr(VI) is reduced in the acid pH of the stomach. In rats, the transit time from the stomach to the duodenum is 4-times slower than in mice possibly facilitating greater reduction of Cr(VI) to Cr(III) in the rat stomach and greater potential for Cr(VI) to irritate the rat stomach compared to mice. Had rats been able to tolerate greater Cr(VI) concentrations, greater intestinal damage would likely have occurred. Transcriptomic analysis indicate that rat and mouse duodenal responses to Cr(VI) are comparable.

In summary, there is consistent data (direct cellular and histopathological observations) for crypt cell regenerative hyperplasia occurring in mice as early as 1-7 days following exposure to Cr(VI), captan or folpet at tumorigenic dose levels. Crypt hyperplasia must be sufficiently maintained across dose and time to result in tumorigenesis. Reversibility studies suggest that exposure must be maintained for up to 6-months and recovery can take as long as 6 months following cessation of dosing. Cr(VI) induces a milder phenotype in rats that does not result in SI tumours. Thus, the AOP is specific to SI tumours in mice as the AO has not been observed in rats exposed to Cr(VI), captan or folpet.

Key event #2; mutation/transformation

There is a greater probability of spontaneous mutation with each round of cell division. In normal SI the cell proliferation is limited to the crypt compartment with stem/progenitor cells residing at the base of the crypt. These cells divide asymmetrically, giving rise to daughter cells that move through the transit-amplifying region of the crypt and into the villi within 2-3 days in rodents and 3-6 days in humans. It is possible that some cells in a hyperplastic SI may not be fully differentiated by the time they reach the reach the villus and may divide. However, due to rapid turnover of the intestinal epithelium, exacerbated by toxicity and migratory pressure from the crypt, the most likely outcome is rapid cell death and/or sloughing into the intestinal lumen. It is possible that genetically engineered mice harbouring activating mutations in Wnt/β-catenin and NF-kB signalling develop crypt-like foci on villi. However, no such foci have been observed in the small intestine of animals exposed to Cr(VI), captan or folpet. The prevailing view on the theory of intestinal cancer is that mutations occur and accumulate in the stem/progenitor cells in the crypt. This is evident from studies where controlled deletion of adenomatous polyposis coli (Apc) gene in crypt stem cells results in rapid transformation and adenoma formation. However, targeted deletion of APC in amplifying daughter cells results in stalled microadenoma on the intestinal villi.

The AOP or MOA framework, states that genotoxic studies should be conducted in tissues that receive high doses of carcinogen, at the carcinogenic site and in highly proliferating tissue. The SI fulfils all of these criteria for Cr(VI) captan and folpet. No mutagenic signal has been described for the any of these chemicals in the SI or in any *in vivo* genotoxicity assay. These results should be considered strong evidence for the lack of a genotoxic MIE and supports the proposed AOP, requiring regenerative hyperplasia in response to non-specific cytotoxicity. All three molecules, however, are positive in a number of *in vitro* genotoxicity assays. In order to interact with DNA the chemicals must be available to DNA. The XFR mapping of Cr fluorescence in intact duodenum indicates Cr-DNA binding could only occur in differentiated enterocytes. In addition, positive *in vitro* genotoxicity result is followed up by *in vivo* tests and negative results in these studies are interpreted as the chemicals being safe for humans and are classified accordingly.

In summary, there is strong evidence that early genotoxicity events do not occur in the SI exposed to Cr(VI), captan or folpet at doses that induce carcinogenicity, and the results are consistently negative genotoxicity results should be supportive of spontaneous mutation resulting from chronic regenerative hyperplasia.

Adverse outcome; Small intestine tumours in mice:

Collectively, seven carcinogenicity studies exist for Cr(VI) (n=1), captan and folpet (n = 3 each) in mice alone. Small intestine tumours are observed after lifetime exposure of B6C3F1 or CD-1 mice only. Intestinal tumours are not seen in rats regardless of strain tested (Fisher 344, Sprague Dawley, Wistar, CD or Osborne-Mendel). These data are consistent with small intestinal tumours occurring only in mice but not rats exposed for life-time to comparable mouse tumorigenic doses. The AO is concurrent with or preceded by each of the AOP KEs in a dose dependent and/or temporal manner. There is no evidence of the SI tumorigenesis occurring in mice as exposure levels that are not accompanied by the KEs in the AOP.

Stressors:

The OECD 2018 define stressors as chemical or non-chemical factors that might trigger an AOP. The AOP summarised here shares features with celiac disease, which is unrelated to Cr(VI), captan or folpet. Celiac disease arises from gluten intolerance and manifests in the proximal SI with a grading severity based on intraepithelial lymphocytes, degrees of crypt hyperplasia from none, to mild and hyperplasia with villi blunting, to increased epithelial lymphocytes and hyperplasia with normal crypt depth but flattened mucosa. These phenotypes can also be induced by pharmacological agents, e.g., non-steroidal anti-inflammatories, cytopathic viruses (coronavirus) and rotavirus. These agents all produce phenotypes comparable to those described by Cr(VI), captan and folpet. In contrast, parvovirus infection results in radiomimetic injury, so called because it resembles effects seen following radiation where rapidly dividing cells are killed more readily than others resulting in villous blunting from death of the crypt epithelium. Physical damage from coarse digestate is also reported to produce a phenotype of villus blunting and increased proliferation of intestinal epithelium comparable to that described for Cr(VI), captan and folpet. These studies support the hypothesis that damage to the intestinal epithelium by mechanisms distinct to Cr(VI), captan or folpet result in a phenotype comparable to that induced by Cr(VI), captan or folpet.

Additionally, transcriptional analysis of duodenal tissue from mice exposed to Cr(VI), captan or folpet result in similar upregulation of pathways consistent with cellular metabolism, stress, inflammation/immune cell response and cell proliferation (including HIF-1 and AP1 signalling) that have been linked to intestinal injury and carcinogenesis.

Alternative modes of action

Two published articles propose a mutagenic MOA for SI tumours following Cr(VI) exposure (McCarroll et al. 2010, Zhitkovich 2011). This work was published prior to the extensive research that led to the development of this AOP. Much of the evidence was generated in non-target tissue using *in vitro* techniques. Evidence for a mutagenic MOA resulted from a dose dependent increase in the size of comet tails in leukocytes after 1 day of Cr(VI) exposure at gavage doses as low as 0.6 mg/kg/day. These investigators did not differentiate potential effects in the crypt versus the villi and the location of DNA damage was not considered. It was also concluded that the primary data gap supporting a mutagenic MOA to be the lack of historical evidence of cytotoxicity in either the 90-day or 2-year rat and mouse NTP bioassays. Neither researchers integrated the intestinal histopathology and biology described in this summary.

Oxidative stress has also been proposed as a MOA as Cr(VI) can react with GSH and ascorbate. There is some transcriptomic evidence of induction of GSH synthetic genes, no oxidative DNA damage has been reported in duodenal tissue following Cr(VI) exposure at carcinogenic dose levels for up to 9 months. Oxidative stress has been shown to result in increased mutation frequency from potassium bromate ($KBrO_3$) exposure. $KBrO_3$ causes SI tumours in mice with increased DNA G to T transversions in a threshold manner consistent with an observed threshold in mutation frequencies. An OECD guideline *gpt* transgenic rodent assay with Cr(VI) would have been expected to detect an increased mutation frequency if oxidative stress were the MIE.

No agents have been shown to cause SI tumours in mice or humans via a mutagenic MOA. Genotoxic carcinogens, e.g., x-ray irradiation, benzo(a)pyrene, *n*-methyl-*N*-nitrosourea and dimethylhydrazine, have all been shown to cause SI tumours after oral exposure. However, these occur in the presence of multiple other local and systemic tumours which were observed at higher rates or at lower doses. Studies with these agents provide evidence that tumorigenesis as a result of exposure arise via a mutagenic MOA and affect a wider variety of tissues. Cr(VI), captan, and folpet affect the proximal SI in mice only with no evidence of a mutagenic MOA.

No alternative MOA has been postulated for captan or folpet although DNA reactivity of these substances is discussed in the literature.

Overall assessment of the AOP

The OECD 2018 AOP guidance addresses the weight of evidence for the AOP as a basis to consider appropriate regulatory application (priority setting, testing strategies or risk assessment). The assessment of the confidence in an AOP and associated KEs is determined by essentiality of the KEs, biological plausibility of the KERs and empirical support for the KERs. A summary of the evidence supporting essentiality is provided in Table 4 of Bhat et al 2020 and it is considered (STRONG) for the (M()IE (villous enterocyte cytotoxicity), STRONG for KE1 (sustained crypt cell proliferation/hyperplasia) and STRONG for KE2 (mutation/transformation). Support for biological plausibility of the KER is summarised in Table 5 in Bhat et al 2020 and provides STRONG evidence of biological plausibility for the (M)IE => KE1(villous enterocyte cytotoxicity leads to sustained crypt cell proliferation/ hyperplasia), STRONG for KE1 => KE2 (sustained crypt cell proliferation/ hyperplasia leads to mutation and transformation) and STRONG for KE2=>AO (mutations and transformation lead to small intestinal tumours in mice). The empirical support for KERs is STRONG for (M)IE => KE1(villous enterocyte cytotoxicity leads to sustained crypt cell proliferation/ hyperplasia), STRONG for KE1 => KE2 (sustained crypt cell proliferation/ hyperplasia leads to mutation and transformation) and STRONG for KE2=>AO (mutations and transformation lead to small intestinal tumours in mice).

A cytotoxicity-mediated regenerative hyperplasia-based MOA for Cr(VI) captan and folpet has been acceptied by the US EPA in 2004 and in 2012, Health Canada 2016 and FSCH in 2017 and 2019.

Biological domain of applicability

The OECD 2019 guidance defines the biological domain of applicability for an AOP based on the groups of organisms for which the measurements represented by the KEs and KERs are operative. For Cr(VI) captan and folpet there are no obvious sex differences in susceptibility. Susceptibility based on life-stage cannot be examined with the current data set. While there is no evidence of the AO in rats, evidence for milder KEs in rats exist and data suggest that entire AOP might be relevant to rats under higher exposure conditions.

With respect to humans the MOA/AOP for Cr(VI) or folpet have been previously reviewed and updated again in Bhat et al. 2020) using the World Health Organisation International Programme on Chemical Safety (WHO/IPCS) human relevance framework which address (1) Is the WOE sufficient to establish the MOA in animals? (2) Can human relevance of the MOA be reasonably excluded on the basis of fundamental, qualitative differences in KEs between experimental animals and humans? (3) Can human relevance of the MOA be reasonably excluded on the basis of quantitative differences in either kinetic or dynamic factors between experimental animals and humans and (4) Conclusion: Statement of confidence, analysis, and implications. These criteria are briefly discussed below.

1). The WOE is sufficient to establish the AOP/WOE in mice.

2). The human relevance cannot be reasonably excluded in humans on the basis of fundamental qualitative interspecies differences. However, based on the Cr(VI), captan and folpet exposure levels in mice (Cr(VI): 30-180 ppm in drinking water, captan: 6000 – 16000 ppm in feed, or folpet: 1000 – 12000 ppm in the feed) were required to be sustained for long periods of time (minimal 6-12 months). The human equivalent dose (HED) for Cr(VI) would 0.02 mg/kg/day, equivalent to 0.54 ppm of Cr(VI) in drinking water daily for a lifetime. This equates to 180-fold higher than the concentration of Cr(VI) found in US drinking water (3 pbb). The HED estimate only includes induction of hyperplasia which is not sufficient to cause SI cancer.

3). There are quantitative differences in the kinetic factors between rodents and humans and non-linearity in pharmacokinetic reduction of Cr(VI) to Cr(III). Physiologically based pharmacokinetic models (PBPK) predict greater Cr(VI) to Cr(III) reduction in human versus mice. Similar models do not exist for captan and folpet however, aggregate exposure of humans to captan and/or folpet through food and drinking water are at least three orders of magnitude lower than exposures causing SI tumours in mice and thus not a concern for SI carcinogenesis in humans.

4). To conclude, in the context of the WHO/ICPC human relevance framework, the WOE is sufficient to establish a MOA in animals with qualitatively plausible KEs in humans. However, the KEs become quantitatively implausible in humans when accounting for species differences in toxicokinetics as background human exposure levels. Confidence in the AOP is high and the implication suggest the AOP/MOA is unlikely to be quantitively relevant for humans. The AOP may be relevant to rats if sufficient dose and time were achieved as the KEs, but not the AO, have been identified in rats exposed to Cr(VI).

To conclude, while the AOP is qualitatively plausible in humans (and rats), the doses required to induce SI tumours in humans are not quantitatively plausible. The AO is only observed in mice and has not been observed or defined for any other known chemical stressor. Interspecies toxicokinetic differences in gastric transit time, environmental exposure levels in relation to carcinogenic exposures further suggest the AOP is unlikely to be quantitively plausible for humans.

Essentiality of key events

The OECD 2019 guidance on the essentiality of KEs states that the KE can only be assessed relative to manipulation of a given KE, (e.g., experimentally blocking or exacerbating the event) on the downstream sequence of KEs. This is difficult to perform for this MOA. However, studies inhibiting Cr(VI) uptake in the SI have not been performed, but the NTP have conducted a 2-year bioassay with Cr(III) and no tumours were observed. Long term studies with captan (≥ 6-months) have demonstrated the essentiality of sustained crypt hyperplasia in tumour formation, and by extension, more opportunities for spontaneous mutation and transformation ultimately leading to tumours. The lack of induced mutation frequency in the duodenum following carcinogenic doses of Cr(VI) in drinking water could also be interpreted as evidence that crypt hyperplasia must be sustained for prolonged durations in order for mutations to occur. This is not an unreasonable conclusion as *gpt* mutation in the duodenum of transgenic C57BL/6 mice exposed to tumorigenic doses of Cr(VI) for 28 or 90 showed no increased in the mutation frequency. In addition, transcriptomic analysis and phenotypic anchoring studies using captan or folpet supported a cytotoxicity mediated MOA. The presence of Cr in villus tips without aberrant foci indicative of transformation after 13 weeks exposure and the reversibility of the early KEs in mice exposed to Cr(VI), captan and folpet are all direct evidence of essentiality of the KEs.

Evidence assessment and quantitative considerations for key event relationships

Biological plausibility of each of the KEs is the most influential consideration in assessing the WOE and degree of confidence in an AOP for potential regulatory application and a quantitative weighting scheme is used to assess confidence in the AOP based on the rank ordering of the Bradford Hill considerations (OECD 2018). The assessment can be viewed in Table 7 (Bhat et al. 2020). Using the quantitative scoring and weighting approach the AOP and its KEs were found to be highly supported by the WOE (95% confidence in the AOP and KEs) when assessed toward the evolved Bradford Hill considerations of biological plausibility, essentiality and empirical support as described in the OECD 2018 guidance on AOP development. The primary considerations that lowered the confidence score were the relatively few evaluations directly measuring the IE and the lack of structural analogy of chemical stressors that operate via this AOP. No other structurally related chemicals to captan and folpet share a common major metabolite.

Conclusions:

This AOP proposes that duodenal tumours in mice occur as a result of chronic villous enterocyte cytotoxicity and accompanying regenerative hyperplasia. The supporting KEs have been measured at the molecular, cellular and tissue level, within concentrations and time frame of the observed AO using Cr(VI), captan and folpet as reference chemical stressors. The biological plausibility, essentiality and empirical support for the IE and KEs was judged to be strong and provide high confidence according to the OECD 2018 guidelines. Evidence for other potential MOAs was weak, obtained in vitro in non-target tissue after high or non-physiological routes of exposure. Genotoxicity using exposure conditions consistent with the measured KEs and AO were negative. Based on the WOE for this AOP and is associated KEs, it can be used for regulatory applications including hazard identification and risk assessment.

Minimal selected references (please see Bhat et al. 2020 for a full reference list supporting this summary).

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3.10 Reproductive toxicity

3.10.1 Animal data

3.10.1.1 Study 1 (1982)

Reference:	Three generation reproduction study in rats
Author(s), year:	Anonymous (1982b)
Report/Doc. number:	International Research and Development Corporation, unpublished report No. 153-096 (Company file: R-6340/TMN-0785)
Guideline(s):	In-house method
GLP:	Partially
Deviations to OECD 416 (2001):	No 1:1 mating, pairs without progeny were not evaluated to determine the cause of infertility, oestrus cycle and sperm parameters were not evaluated, data on sexual maturation were not evaluated, no organ weights were determined, no histopathology was performed.
Acceptability:	Yes

Material and Methods

In a three generation study, captan (purity 89%, batch number SX-944, obtained from Ortho Chevron Chemical Company, Richmond, California) was administered in the diet to COBS CD strain rats (from Charles River Breeding Laboratories, Inc., Massachusetts) at dosage levels of 0, 25, 100, 250 and 500 mg/kg bw/day (15 males and 30 females/dose group). The parental animals were dosed for approximately 100 days after which each F_0 male was cohabited with two F_0 females within the same treatment group. F_0 rats were mated twice to produce two litters, F_{1a} and F_{1b}. On lactation day 4, litters were randomly reduced to 10 pups per litter, of equal sex ratio if possible. Pups from the F_{1b} litter (15 males and 30 females) were randomly selected from each dose group to produce F_1 parental animals. F_1 parental animals were administered test substance as described for the F_0 parents and mated three times to produce F_{2a}, F_{2b} and F_{2c} litters. Offspring from the F_1 generation´s third mating (F_{2c}) were delivered by caesarean section on gestation day 19. Foetuses were examined for abnormalities. Pups from the F_{2b} litters were selected to comprise the F_2 generation parental animals and mated to produce the F_{3a} and F_{3b} litters. In each case, apart from the F_{2c} offspring, two-thirds of the litters remaining at weaning (after selection for parental generations if applicable) were sacrificed and necropsied. Animals not selected for necropsy were sacrificed and discarded.

Parameters evaluated were:

- parental and pup behaviour, appearance and mortality (twice daily),
- parental body weight (weekly), litter weight (on lactation days 1, 4, 7, 14), individual pup weight by sex (on lactation day 21 = weaning),
- parental food consumption (weekly),
- reproductive parameters (male and female fertility, gestation length, litter size, sex ratio of litter, growth, viability and survival of pups).

Pups sacrificed and necropsied were examined for gross pathological abnormalities. In the teratology phase (F_{2c} mating, caesarean section on gestation day 19) the number and location of viable and non-viable foetuses, early/late resorptions, number of total implantations and corpora lutea were recorded and foetuses were examined for abnormalities.
Diet was analysed for the content and homogeneity of test substance.

Findings

General observations:
No treatment-related effects were seen in the general behaviour or appearance of the treated parental rats or pups. Parental survival of the F_0 generation was 100%. In the F_1 generation, deaths occurred at 100 mg/kg bw/d (one female), at 250 mg/kg bw/d (one male) and at 500 mg/kg bw/d (two females). In the F_2 generation, one parental female died in each of the control and the 250 mg/kg bw/d treatment group. Survival of parental rats was therefore considered unrelated to treatment.

Parental body weights:
Throughout all generations, a dose-dependent decrease in male and female body weights in 100, 250 and 500 mg/kg bw/day treatment groups was observed. This was statistically significant in the 250 and 500 mg/kg bw/day treatment groups at all times in both sexes and occasionally significant at 100 mg/kg bw/day (for males at study week 34 of F_0 and F_1 generations, and for females at study weeks 14 (F_0), 34, 47 and 66 (F_1) and 79 (F_2)). However, in females the decrease in body weight at 100 mg/kg bw/day remained always less than 10% compared to controls.
At 25 mg/kg bw/d mean female body weights in each generation were comparable to the control. Although a slight reduction in the 25 mg/kg bw/day parental weight of males was evident during the F_0 and F_2 generations, there was no statistical significance. F_2 generation male body weight at sexual maturity was similar to the control.

Table 3.10.1.1-1 Three generation reproduction study in the rat: Parental group mean body weights

	Dose group (mg/kg bw/day)				
	0	**25**	**100**	**250**	**500**
Males					
F_0, week 0	126	129	128	127	128
F_0, week 14	530	503	487	**460****	**438****
F_0, week 34	653	617	603*	**566****	**534****
F_1, week 34	187	186	**158****	**152****	**118****
F_1, week 47	515	513	480	**433****	**370****
F_1, week 66	614	627	577	**517****	**448****
F_2, week 67	216	198	203	**176****	**151****
F_2, week 79	486	469	451	**406****	**385****
F_2, week 98	614	586	566	**500****	**469****
Females					
F_0, week 0	108	111	108	108	108
F_0, week 14	273	270	258*	248**	**238****
F_0, week 34	337	332	323	311**	**297****
F_1, week 34	148	140	135**	**123****	**98****
F_1, week 47	289	280	267**	**242****	**219****
F_1, week 66	346	337	322**	**297****	**269****
F_2, week 67	159	157	154	**139****	**124****
F_2, week 79	282	274	266*	**241****	**221****
F_2, week 98	344	330	330	**305****	**283****

Significantly different from the control, * ($p < 0.05$), ** ($p < 0.01$).
Bold: Body weight decrease ≥ 10% compared to controls

Parental food consumption:
Slight to moderate decreases in parental food consumption (g food/rat/day) were observed in males and females of all treatment groups in all three generations (Table 3.10.1.1-2). Exceptions were slight increases over the control in 25 mg/kg bw/day F_1 males and 100 mg/kg bw/day F_2 females and similar food consumption in F_2 females at 25 mg/kg bw/day compared to the control.

Table 3.10.1.1-2 Three generation reproduction study in the rat: Parental food consumption

Dose (mg/kg bw/day)	Average mean food consumption (g/rat/day)	
	males (% difference from control)	females (% difference from control)
F_0		
0	27.9	25.3
25	26.4 (-5.4)	24.0 (-5.1)
100	26.4 (-5.4)	23.7 (-6.3)
250	23.8 (-14.7)	21.6 (-14.6)
500	22.6 (-19.0)	19.3 (-23.7)
F_1		
0	28.4	26.3
25	29.0 (+2.1)	25.3 (-3.8)
100	27.1 (-4.6)	25.7 (-2.3)
250	24.5 (-13.7)	23.1 (-12.2)
500	22.2 (-21.8)	21.1 (-19.8)
F_2		
0	28.2	26.6
25	27.2 (-3.5)	26.6 (+0.0)
100	27.1 (-3.9)	27.8 (+4.5)
250	24.6 (-12.8)	25.6 (-3.8)
500	23.2 (-17.7)	21.5 (-19.2)

Pup body weights:
In general, dose-related reductions in pup body weights were seen in all litters throughout lactation at 100, 250 and 500 mg/kg/day. These reductions were statistically significant in all six matings (F_{1a}, F_{1b}, F_{2a}, F_{2b}, F_{3a} and F_{3b}) in the 250 and 500 mg/kg/day dose groups throughout lactation to weaning, and at most body weight intervals in the 100 mg/kg/day dose group. At lactation day 21, male and female mean pup body weights were significantly lower than the controls in all six matings. Mean pup body weights were slightly lower than the controls in the 25 mg/kg/day dose group with occasional statistical significance. However, even if significant, the decreases in pup body weight remained always less than 10% at the dose level of 25 mg/kg bw/day. Also reductions in bodyweight gain remained always less than 10% at low dose. At lactation day 21, mean pup body weights were slightly lower at 25 mg/kg bw/day than the controls in all six matings with statistical significance noted for F_{1b} litters only. A summary of pup body weights and body weight gain is given in Table 3.10.1.1-3.

Table 3.10.1.1-3 Three generation reproduction study in the rat: Pup body weight (g) and body weight gain (g)[a] during lactation

Observation	0 mg/kg bw/day	25 mg/kg bw/day	100 mg/kg bw/day	250 mg/kg bw/day	500 mg/kg bw/day
Day 1					
F1a Litter	7.3	6.8*	6.7*	6.4*	6.0**
F1b Litter	7.2	6.9	6.6*	6.6*	6.0**
F2a Litter	7.0	6.7	6.7	6.1**	5.8**
F2b Litter	7.1	6.6*	6.6**	6.4**	6.4**
F3a Litter	6.9	6.8	6.7	6.4**	5.9**
F3b Litter	6.9	6.8	6.6	6.4*	6.0**
Day 4 Before reduction					
F1a Litter (body weight gain)	10.6 (+3.3)	10.0 (+3.2)	9.3** (+2.6)	8.9** (+2.5)	7.9** (+1.9)
F1b Litter (body weight gain)	10.4 (+3.2)	10.1 (+3.0)	9.7 (+3.1)	9.0** (+3.3)	7.6** (+1.6)
F2a Litter (body weight gain)	10.3 (+3.3)	10.0 (+3.3)	9.5* (+2.8)	8.3** (+2.2)	7.6** (+1.8)
F2b Litter (body weight gain)	10.5 (+3.4)	9.8 (+3.2)	9.3** (+2.7)	9.2** (+2.8)	8.7** (+2.3)
F3a Litter	10.3	10.1	9.8	8.7**	7.8**

(body weight gain)	(+3.4)	(+3.3)	(+3.1)	(+2.3)	(+1.9)
F3b Litter (body weight gain)	10.3 (+3.4)	10.1 (+3.3)	9.5 (+2.9)	8.7** (+2.3)	7.8** (+1.8)
Day 4 After Reduction					
F1a Litter (body weight gain)	10.6 (+3.3)	10.0 (+3.2)	9.3** (+2.6)	8.9** (+2.5)	8.1** (+2.1)
F1b Litter (body weight gain)	10.4 (+3.2)	10.0 (+3.1)	9.7 (+3.1)	9.0** (+2.4)	7.6** (+1.6)
F2a Litter (body weight gain)	10.3 (+3.3)	10.0 (+3.3)	9.4* (+2.7)	8.3** (+2.2)	7.6** (+1.8)
F2b Litter (body weight gain)	10.5 (+3.4)	9.7* (+3.1)	9.2** (+2.6)	9.2** (+2.8)	8.6** (+2.2)
F3a Litter (body weight gain)	10.3 (+3.4)	10.1 (+3.3)	9.8 (+3.1)	8.7** (+2.3)	7.7** (+1.8)
F3b Litter (body weight gain)	10.2 (+3.3)	10.0 (+3.2)	9.5 (+2.9)	8.6** (+2.2)	7.8** (+1.8)
Day 7					
F1a Litter (body weight gain)	15.6 (+8.3)	14.6 (+7.8)	13.4** (+6.7)	12.4** (+6.0)	10.4** (+4.4)
F1b Litter (body weight gain)	16.4 (+9.2)	15.3* (+8.4)	14.8* (+8.2)	13.1** (+6.5)	10.2** (+4.2)
F2a Litter (body weight gain)	15.7 (+8.7)	15.3 (+8.6)	14.3* (+7.6)	11.6** (+5.5)	9.9** (+4.1)
F2b Litter (body weight gain)	16.4 (+9.3)	15.6 (+9.0)	14.1** (+7.5)	13.2** (+6.8)	12.0** (+5.6)
F3a Litter (body weight gain)	15.6 (+8.7)	15.8 (+9.0)	14.6 (+7.9)	12.9** (+6.5)	11.1** (+5.2)
F3b Litter (body weight gain)	16.7 (+9.8)	15.8 (+9.0)	14.9** (+8.3)	13.2** (+6.8)	10.7** (+4.7)
Day 14					
F1a Litter (body weight gain)	27.7 (+20.4)	26.9 (+20.1)	25.4* (+18.7)	22.0** (+15.6)	18.0** (+12.0)
F1b Litter (body weight gain)	31.8 (+24.6)	30.2* (+23.3)	28.3** (+21.7)	24.7** (+18.1)	19.1** (+13.1)
F2a Litter (body weight gain)	28.9 (+21.9)	27.4 (+20.7)	26.1* (+19.4)	21.0** (+14.9)	17.4** (+11.6)
F2b Litter (body weight gain)	32.2 (+25.1)	30.3* (+23.7)	27.2** (+20.6)	23.5** (+17.1)	22.1** (+15.7)
F3a Litter (body weight gain)	29.8 (+22.9)	29.8 (+23.0)	28.2 (+21.5)	24.0** (+17.6)	19.0** (+13.1)
F3b Litter (body weight gain)	32.5 (+25.6)	30.6 (+23.8)	29.5** (+22.9)	15.6** (+9.2)	20.0** (+14.0)
Day 21 Male					
F1a Litter	42.0	41.1	38.2**	34.7**	27.8**
F1b Litter	49.9	49.0*	46.2**	29.2**	30.8**
F2a Litter	47.6	47.3	41.2**	33.3**	26.6**
F2b Litter	50.4	48.6	43.96**	38.0**	34.6**
F3a Litter	47.1	45.9	43.3*	38.9**	31.5**
F3b Litter	51.9	50.5	48.0**	41.3**	31.4**
Day 21 Females					
F1a Litter	40.9	39.7	36.9**	33.6**	26.7**
F1b Litter	48.0	47.1*	44.8**	38.0**	28.8**
F2a Litter	44.7	44.2	38.9**	32.3**	26.6**
F2b Litter	48.1	45.9	41.7**	36.4**	32.8**
F3a Litter	44.9	44.3	42.1*	37.3**	30.5**
F3b Litter	50.4	47.7	46.0**	39.8**	30.7**

[a] Body weight gain from Day 1 pup body weight

Statistically significant difference from control group (* p<0.05; ** p<0.01)

Foetal body weights:
No treatment-related differences were evident in the mean fetal body weights of the F_{2c} foetuses in the 25, 100 and 250 mg/kg bw/d dose groups when compared to the controls. The mean foetal body weights in the 500 mg/kg bw/day dose group were significantly lower than the control (Table 3.10.1.1-4).

Reproductive parameters:
No consistent treatment-related effects on male and female fertility or length of gestation were observed. A slight decrease in male and female fertility was noted in all captan treated groups at the F_{1a} mating compared to controls, with statistical significance in the 250 mg/kg bw/day males and in the 500 mg/kg bw/day females. However, since no continuing trend of fertility reduction occurred as compared to the controls during subsequent matings, the decreases were not considered treatment-related (Table 3.10.1.1-5).

Two females at 500 mg/kg bw/day showed high incidences of peri-natal pup losses at the F_{1b} mating, but as these were isolated incidences in this generation and were not seen in other matings or other generations, no association with treatment is made.
Pup survival during lactation was unaffected in the 25 and 100 mg/kg bw/day dose groups. At 250 mg/kg bw/day a reduction in survival was noted through lactation day 4 of the F_{1a}, F_{2a} and F_{3a} litters, reaching statistical significance for the F_{2a} and F_{3a} litters. Consistent treatment-related reductions in pup survival were observed at lactation day 4 at 500 mg/kg bw/day in the litters of all three generations (Table 3.10.1.1-6).

Litter size was reduced in F_{1a} and F_{1b} litters in all treatment groups (reaching statistical significance for the F_{1b} litter at 500 mg/kg bw/d) compared to the controls. In the F_{2a} and F_{2b} litters, the litter sizes were reduced at 250 and 500 mg/kg bw/day compared to the controls.

Teratology (F_{2c} caesarean section observations):
No treatment-related or statistically significant differences were noted in male and female fertility, mean numbers of nonviable foetuses, early and late resorptions and post-implantation loss per dam or foetal sex distribution in any of the captan treated groups compared to the control. A very slight reduction in the mean number of viable foetuses per dam and a corresponding slight decrease in total implantations was determined at 250 and 500 mg/kg bw/day, as well as a slight decrease in the number of corpora lutea per dam at 500 mg/kg bw/day, however no statistical singnificance was determined (Table 3.10.1.1-7).
There were no biologically meaningful or statistically significant differences in the number of foetuses or litters with malformations in the captan treated groups compared to the control.

Table 3.10.1.1-5 Three generation reproduction study in the rat: Fertility indices

Females	**Females Pregnant / Total Females Mated (Fertility Index in %)**					
Dose group (mg/kg bw/day)	**Litter F_{1a}**	**Litter F_{1b}**	**Litter F_{2a}**	**Litter F_{2b}**	**Litter F_{3a}**	**Litter F_{3b}**
0	26/30 (87)	24/30 (80)	27/30 (90)	29/30 (97)	28/30 (93)	25/29 (86)
25	22/30 (73)	22/30 (73)	28/30 (93)	30/30 (100)	27/30 (90)	26/30 (87)
100	21/30 (70)	27/30 (90)	28/30 (93)	27/29 (93)	30/30 (100)	30/30 (100)
250	19/30 (63)	22/30 (73)	30/30 (100)	28/30 (93)	30/30 (100)	30/30 (100)
500	17/30* (57)	25/30 (83)	28/30 (93)	27/30 (90)	25/30 (83)	28/30 (93)
Males	**Males Fertile / Total Males Mated (Fertility Index in %)**					
Dose group (mg/kg bw/day)	**Litter F_{1a}**	**Litter F_{1b}**	**Litter F_{2a}**	**Litter F_{2b}**	**Litter F_{3a}**	**Litter F_{3b}**
0	15/15	15/15	15/15	15/15	15/15	15/15

	(100)	(100)	(100)	(100)	(100)	(100)
25	12/15 (80)	12/15 (80)	14/15 (93)	15/15 (100)	15/15 (100)	15/15 (100)
100	13/15 (87)	15/15 (100)	15/15 (100)	15/15 (100)	15/15 (100)	15/15 (100)
250	10/15* (67)	13/15 (87)	15/15 (100)	14/15 (93)	15/15 (100)	15/15 (100)
500	11/15 (73)	14/15 (93)	14/15 (93)	15/15 (100)	13/15 (87)	15/15 (100)

Significantly different from the control, * ($p < 0.05$).

Table 3.10.1.1-6 Three generation reproduction study in rats: Survival of pups

Dose (mg/kg/day)	**Survival Index (%)**				
	Pups surviving/live pups at birth		**Pups surviving/no. retained on day 4**		
	day 1	**day 4**	**day 7**	**day 14**	**day 21**
F_{1a}					
0	99	99	100	100	100
25	100	99	100	99	99
100	98	98	100	100	100
250	92	92	100	99	99
500	96	93*	100	100	99
F_{1b}					
0	100	99	100	100	100
25	100	100	100	100	100
100	96	95	100	100	99
250	99	98	100	100	100
500	97*	93	99	94	93
F_{2a}					
0	100	99	100	100	99
25	100	99	100	96	96
100	99	98	100	100	100
250	96*	95*	100	100	100
500	87**	85**	100	92	92
F_{2b}					
0	99	99	100	100	100
25	100	97	100	100	100
100	100	98	99	99	99
250	100	100	100	100	100
500	97	96	98*	98	98
F_{3a}					
0	100	98	100	100	98
25	100	99	100	100	100
100	99	98	100	100	99
250	99	97*	99	99	98
500	96*	89**	99	98	98
F_{3b}					
0	100	99	100	100	100
25	99	98	100	100	100
100	99	98	100	100	99
250	99	97	100	99	99
500	98	93*	100	100	100

Significantly different from the control, * ($p < 0.05$), ** ($p < 0.01$).

Table 3.10.1.1-7 Three generation reproduction study in rats: Maternal and foetal observations at Caesarean section (F_{2c})

	Dose group (mg/kg/day)				
	0	**25**	**100**	**250**	**500**
Total no. of females mated	30	30	29[a]	30	29[a]
Total no. of gravid females	21	23	23	26	23
No. of females dying prior to caesarean section (gravid)	0	0	0	0	1
Number of females that delivered	2	7	4	3	5
No. of females examined at Cesarean section	30	30	29	30	28
Nongravid	9	7	6	4	6
Gravid	21	23	23	26	22
Dams with resorptions only	0	0	0	2	0
Dams with viable fetuses	21	23	23	24	22
Viable foetuses/dam	12.4	12.1	11.9	11.3	11.7
Postimplantation loss/dam	1.3	1.6	1.5	1.5	1.2
Total implantations/dam	13.7	13.7	13.4	12.8	12.9
Corpora lutea/dam	15.9	16.1	15.4	15.2	14.0
Foetal sex distribution	M: 125 F: 135	M: 114 F: 127	M: 114 F: 137	M: 146 F: 125	M: 129 F: 116
Mean foetal body weight (g)	2.1	2.2	2.1	2.0	1.9*

* Significantly different from the control ($p < 0.05$).
[a] One female died prior to mating
[b] Includes females that delivered and/or died

Gross Pathology:
There were no treatment-related abnormalities in any of the pups examined after weaning or in any of the parental rats that died during the study.

Diet analysis indicated that captan levels ranged from 90 to 100% of the nominal levels.

Conclusions

In the three-generation study, there was a dose-related and statistically significant reduction in parental body weight at 100 mg/kg bw/day and above, with marked reductions at 500 mg/kg bw/day. There were no significant effects on fertility, length of gestation or litter size at birth. Pup survival was reduced at 250 and 500 g/kg bw/day and there was also a constant and dose-related reduction in pup weight, which was consistent at 250 and 500 mg/kg bw/day, frequent at 100 mg/kg bw/day and occasionally statistically significant at 25 mg/kg bw/day. A reduction in foetal body weight was also seen in an F_2 litter (removed by caesarean section for teratological examination) at the highest dose. The incidence of gross abnormalities was not increased by captan administration. There were no indications of any cumulative effects of treatment in successive generations.

In the DAR of captan (2005), it was concluded from the study that the NOEL for parental toxicity was 25 mg/kg bw/day based on occasionally statistically significant parental body weight reductions observed at 100 mg/kg bw/day. Since these reductions were always less than 10% in females compared to controls, here a NOAEL for maternal toxicity of 100 mg/kg bw/day is proposed based on significant reductions in body weight greater than 10% and decreased food consumption greater than 10% observed at 250 mg/kg bw/day and above. The paternal NOAEL (as well as the overall parental NOAEL) remains at 25 mg/kg bw/day based on occasionally statistically significant parental body weight reductions > 10% observed at 100 mg/kg bw/day in males.
The NOAEL for fertility was 500 mg/kg bw/day, and the NOAEL for pup survival was 100 mg/kg bw/day, based on reduced survival observed at 250 and 500 mg/kg bw/day.
In the DAR of captan (2005), it was concluded that it was not possible to establish a NOEL for pup toxicity, based on the reduction in body weight at the lowest dose tested (25 mg/kg bw/day). Since occasionally significant pup weight reductions at 25 mg/kg bw/day were always less than 10% compared to control pups and furthermore not observable in F_3 pups, a NOAEL for pup toxicity of 25 mg/kg bw/day is proposed based on significant reductions in pup body weight greater 10% observed at 100 mg/kg bw/day and above. This conclusion is also in line with the results of the one generation reproduction study in rats by Study 2 (1982c), section 3.10.1.2.

There was no evidence of teratogenicity at any dose.

3.10.1.2 Study 2 (1982)

Reference:	One generation reproduction study in rats with captan
Author(s), year:	Anonymous (1982c)
Report/Doc. number:	International Research and Development Corporation, unpublished report No. 153-190 (Company file: R-6339/TMN-0784)
Guideline(s):	In-house method
GLP:	Yes
Deviations to OECD 415 (1983):	The study met the essential criteria of the test guideline.
Acceptability:	Yes

Material and Methods

As there was no evidence of any cumulative effects of treatment in successive generations in an earlier study (Study 1, 1982b), only one generation was tested in this study. Captan technical (purity not specified, batch number SX-1086; on the basis of the evidence available it is considered reliable to conclude that the purity was close to 89%) was administered in the diet to COBS CD strain rats (from Charles River Breeding Laboratories, Michigan) at dosage levels of 0, 6, 12.5 and 25 mg/kg bw/day (15 males and 30 females/dose group). The parental animals were dosed for 102 days after which each F_0 male was cohabited with two F_0 females within the same treatment group. On lactation day 4, litters were reduced to 10 pups per litter, of equal sex ratio if possible. One day after weaning (weaning = lactation day 21), pups and dams were sacrificed and necropsied. Parental males were sacrificed and necropsied after the last female inseminated by them was sacrificed. Females that did not deliver were sacrificed and examined for confirmation of pregnancy status after the last female parent and litter had been necropsied.

Parameters evaluated were:

- parental and pup behaviour, appearance and mortality (twice daily),
- parental body weight (weekly), litter weight (lactation days 1, 4, 7, 14), individual pup weight by sex (on lactation day 21),
- parental food consumption (weekly),
- reproductive parameters (male and female fertility, gestation length, litter size, sex ratio of litter, growth, viability and survival of pups).

Test diet was analysed for active ingredient content and homogeneity.

Findings

General observations:
Parental appearance and behaviour, body weight and food consumption, were not affected by treatment. Offspring appeared normal with no differences from the control evident.

Reproductive parameters:
No treatment-related effects on male and female fertility or length of gestation were observed. Litter size, pup viability at birth, pup survival through lactation and pup weight at birth were unaffected by treatment. Mean pup body weight in the 25 mg/kg bw/day dose group was slightly reduced, although not statistically significantly, compared to the control on lactation days 4, 7 and 14. Mean body weights for males and females in this dose group were comparable to the control at weaning. A summary of mean body weights of pups is given in Table 3.10.1.2-1.

Table 3.10.1.2-1 One generation reproduction study in rats: Pup body weight during lactation

Dose (mg/kg bw/d)	Mean (± sd) weight of pups on lactation day						
	1	4		7	14	21	
		before reduction	after reduction			male	female
0	7.4 ± 0.84	11.3 ± 1.59	11.3 ± 1.58	17.3 ± 1.96	30.6 ± 3.29	43.0 ± 5.92	41.8 ± 5.62
6	7.5 ± 0.89	11.4 ± 1.09	11.4 ± 1.10	17.6 ± 1.50	30.9 ± 3.36	43.8 ± 4.28	42.9 ± 4.04
12.5	7.3 ± 0.84	10.9 ± 1.34	10.9 ± 1.30	16.9 ± 1.85	30.3 ± 2.79	43.2 ± 5.25	42.6 ± 4.64
25	7.3 ± 0.73	10.7 ± 1.14	10.7 ± 1.16	16.2 ± 1.42	28.9 ± 2.26	42.5 ± 3.92	41.6 ± 3.75

Gross pathology:
No treatment-related gross pathological findings were observed in parental rats.
At necropsy of the F_1 pups, hydronephrosis was observed in two pups in the 12.5 mg/kg bw/d dose group, but was not attributed to the treatment with captan according to the study author. No treatment-related gross pathological findings were observed in pups.

Levels of captan in the diet: The mean concentration of captan in diet ranged from 95 to 103% of nominal levels. Results indicated that the test article was distributed homogeneously in the diet with concentrations ranging from 79 to 120% of nominal. Stability data for seven days indicated that captan was stable in the diet.

Conclusions

In a one generation study, no parental toxicity was evident. Pup weights at 25 mg/kg bw/day were marginally and not statistically significantly lower than those in the control group. In the DAR of captan (2005), it was concluded from this two studies that the NOEL for pup toxicity was 12.5 mg/kg bw/day.
Since in the three generation study by (Study 1, 1982b) pup weight reductions at the dose level of 25 mg/kg bw/day were, even if occasionally significant, less than 10% compared to control pups (and not observed at all in F_3 pups), the NOAEL for pup toxicity is considered to be 25 mg/kg bw/day. This conclusion is supported by the results of this study.

3.10.1.3 Study 3 (1987)

Reference:	Captan teratology study in the rat
Author(s), year:	Anonymous (1987a)
Report/Doc. number:	Life Science Research Israel Ltd., unpublished report No. MAK/097/CAP (Company file: R-4364)
Guideline(s):	USEPA Guideline 83.3; OECD 415 (1981)
GLP:	Yes
Deviations to OECD 414 (2001):	The study met the essential criteria of the test guideline. Dosing occurred solely during the period of organogenesis.
Acceptability:	Yes

Material and Methods

In a developmental toxicity study, pregnant CD strain rats (Sprague-Dawley origin) were administered captan (purity 91%, batch number BN 331552) in a vehicle of 0.5% carboxymethylcellulose (containing 0.05% acetic acid) at dose levels of 0, 18, 90 and 450 mg/kg bw/day (22 dams/dose) on days 6 to 15 of gestation. Dams were sacrificed on day 20 of gestation.
Parameters evaluated in dams were clinical signs (daily), body weight (on gestation days 0, 3, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17 and 20), food consumption (twice weekly) and gross pathology. Litter parameters evaluated were number of corpora lutea, weight of gravid uterus, distribution of live and dead foetuses and resorption sites, placental weights, foetal weights and sexes. Foetuses were examined for external, visceral and skeletal anomalies and the thoracic and abdominal contents were examined.

Findings

General observations:
All animals survived to scheduled terminal sacrifice on gestation day 20.

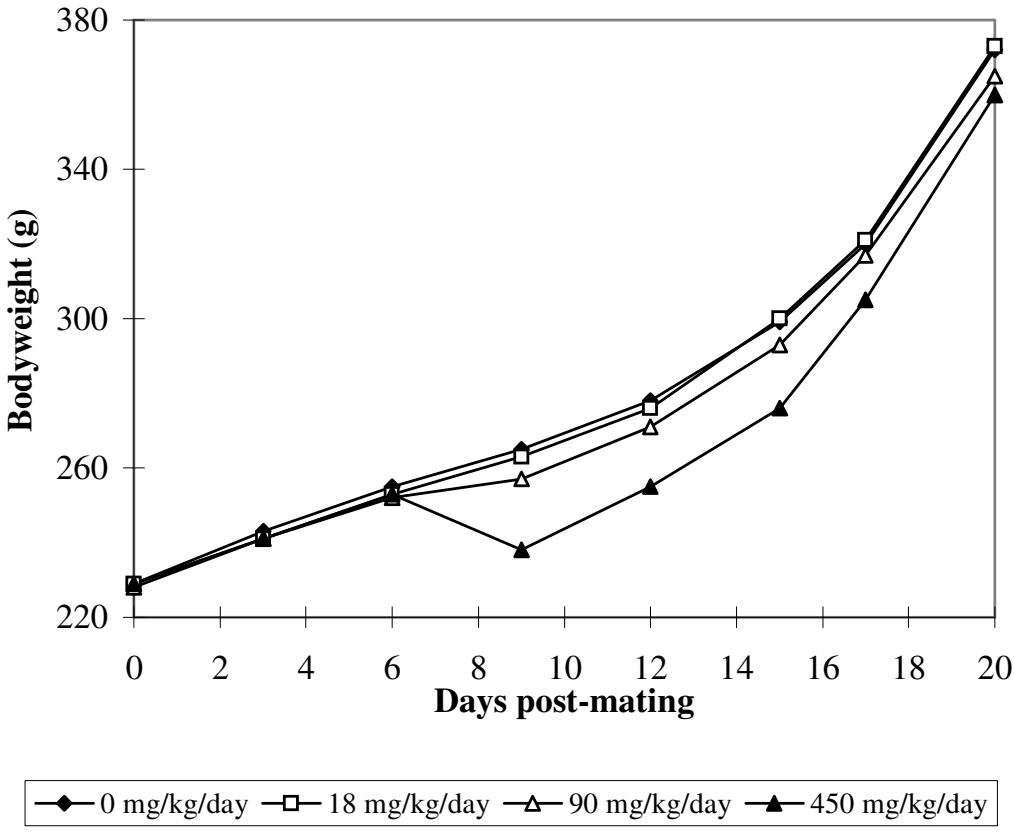
A higher incidence of hair loss ($p < 0.05$) and lack of grooming were observed in 450 mg/kg bw/day dams. Food consumption was significantly reduced in 450 mg/kg bw/day dams during the entire dosing period and on days 7 to 9 in the 90 mg/kg bw/day dams. A significant compensatory increase in food consumption in the 450 mg/kg bw/day dams was observed post-dosing (Table 3.10.1.4-1).
Maternal body weight was significantly reduced over the dosing period in the 450 mg/kg bw/day dams. By day 20 no treatment-related differences from the control were apparent in any dose group. The 90 mg/kg bw/day dams showed a very slight reduction in body weight (less than 4%) compared to the control and this was statistically significant on days 7 and 8 (Figure 3.10.1.4-1).

Table 3.10.1.4-1 Developmental toxicity study in the rat: group mean (± SD) food consumption, body weights and body weight gain of dams

Gestation day	Dose group (mg/kg bw/day)			
	0	18	90	450
	Food consumption (g/animal/day)			
1 - 3	26.0 ± 2.6	25.1 ± 2.1	25.3 ± 2.2	25.3 ± 2.2
4 - 6	25.0 ± 3.6	24.5 ± 2.0	24.3 ± 2.5	25.2 ± 2.4
7 - 9	25.1 ± 3.3	24.4 ± 3.0	20.7 ± 2.6***	13.4 ± 3.6***
10 - 13	25.9 ± 2.7	25.4 ± 2.6	24.7 ± 2.1	21.4 ± 2.8***
14 - 16	26.7 ± 2.9	26.3 ± 2.6	25.7 ± 2.3	22.2 ± 3.9***
17 - 20	27.7 ± 2.7	28.6 ± 2.7	28.4 ± 2.7	32.4 ± 3.8***
	Body weight (g)			
0	229.2 ± 9.8	228.7 ± 9.4	228.4 ± 10.8	229.0 ± 7.7
6	254.6 ± 12.1	252.9 ± 12.9	252.0 ± 14.7	253.1 ± 9.4
7	257.5 ± 13.2	255.7 ± 12.6	248.5 ± 14.5*	245.6 ± 11.2**
8	261.9 ± 13.4	258.8 ± 13.9	251.6 ±13.6*	239.3 ± 11.6***
15	299.3 ± 18.8	299.8 ± 16.2	292.5 ± 16.4	276.1 ± 15.1***
20	372.0 ± 23.6	373.3 ± 22.6	364.7 ± 25.5	359.7 ± 19.7
	Body weight gain (g)			
0-20	142.8 ± 18.0	144.6 ± 19.1	136.3 ± 19.1	130.7 ± 16.8*
6-20	117.3 ± 14.1	120.5 ± 13.7	112.7 ± 16.6	106.1 ± 15.9

* significantly different from the control, $p < 0.05$
** significantly different from the control, $p < 0.01$
*** significantly different from the control, $p < 0.001$

Figure 3.10.1.4-1 Developmental toxicity study in the rat: body weight of dams (mean)



Pregnancy and litter data:
Foetal viability as measured by post-implantation loss, was not adversely affected by treatment. Although pre-implantation loss was elevated in the 90 mg/kg/day treatment group, the incidence in the 450 mg/kg/day group was not affected and therefore this difference is considered to be incidental. Mean foetal weight was significantly reduced in the 450 mg/kg bw/day dose group (mean foetal weights of 3.49 g and 3.27 g in the control and 450 mg/kg bw/day dose groups, respectively), whereas at 90 mg/kg bw/day foetuses were slightly larger than control foetuses.

Foetal assessment:
No treatment-related foetal malformations were observed in any treated group. However, the incidence of small foetuses (< 3.0 g) was significantly increased in the 450 mg/kg bw/day dose group. Observations of free-hand foetal sections did not reveal any treatment-related major abnormalities. A number of minor skeletal variants were observed in the 450 mg/kg bw/day dose group and these were apparently treatment-related. The number of foetuses with a 14th (lumbar) rib unilaterally or bilaterally was slightly elevated in this dose group as was the incidence of foetuses with one or two vertebral hemicentra incompletely fused (when tested on a litter basis). The incidence of reduced ossification of the pubic bone was more frequent in the 450 mg/kg bw/day dose group. An increased incidence of rudimentary 13th ribs in the 18 mg/kg bw/day dose group foetuses was not considered to be treatment-related. A summary of the significant skeletal variations is presented in Table 3.10.1.4-2.

Table 3.10.1.4-2 Developmental toxicity study in the rat: incidence of skeletal variations

Incidence of foetal skeletal variations	Dose group (mg/kg bw/day)			
	0	18	90	450
Number of foetuses affected				
Number foetuses examined	169	163	157	164
14th lumbar rib (unilaterally)	2 (1.18)	0 (0.00)	1 (0.64)	8 (4.88)*
14th lumbar rib (bilaterally)	0 (0.00)	0 (0.00)	1 (0.64)	5 (3.05)*
Reduced ossification of pubic bone	1 (0.59)	2 (1.23)	3 (1.91)	8 (4.88)*
Number of litters affected				
Number litters examined	22	22	22	22
one or two vertebral hemicentra incompletely fused	8 (9.38)	13 (15.73)	11 (10.33)	16 (13.28)*

* Significantly different from the control ($p < 0.05$).
For foetuses number in parenthesis = percentage. For litters number in parenthesis = % mean which is sum of individual litter proportions divided by number of litters in group.

Conclusions

A teratology study was carried out in the rat. Food consumption was reduced in the 450 mg/kg bw/day dose group compared to the control and maternal body weight was reduced in this dose group over the entire dosing period. A reduction in food consumption and maternal body weight was also noted in 90 mg/kg bw/day dams during the first two days of treatment. Foetotoxicity, as shown by reduced foetal body weight, was observed at 450 mg/kg bw/day. The incidence of small foetuses and skeletal defects classified as variants was also increased at this dose level. The observed axial skeletal abnormalities could be related to the gastrointestinal maternotoxicity and, as a consequence, to an embryonic nutrient imbalance. There was no indication of test substance related teratogenicity under the conditions of the study.

In the DAR of captan (2005), the NOAEL for maternal toxicity was concluded to be 18 mg/kg bw/day based on the treatment-related effects on food consumption and body weight at 90 mg/kg bw/day. Althouh statistically significant ($p < 0.05$), the reductions in maternal body weight observed on gestation days 7 and 8 at 90 mg/kg bw/day were very small, less than 4% compared to the control. The significant reduction in food consumption in 90 mg/kg bw/day dams on days 7 to 9 appears to have had little effect on body weight. Therefore, a NOAEL for maternal toxicity of 90 mg/kg bw/day is considered to be more appropriate.

The NOAEL for foetal toxicity was 90 mg/kg bw/day based on the reduced foetal weight and slight increases in the incidence of certain minor skeletal variations at 450 mg/kg bw/day.

3.10.1.4 Study 4 (1991)

Reference:	Captan: Teratogenicity study in the rabbit
Author(s), year:	Anonymous (1991)
Report/Doc. number:	ICI Central Toxicology Laboratory, UK, unpublished report No. CTL/P/3039 (Company file: R-6148/TMN-0791).
Guideline(s):	USEPA Guideline 83-3
GLP:	Yes
Deviations to OECD 414 (2001):	The study met the essential criteria of the test guideline. However, overall the pregnancy rate was low (55-65%) in all dose groups including the control group. Dosing occurred solely during the period of organogenesis.
Acceptability:	Yes

Material and Methods

In a developmental toxicity study, captan (purity 91.2%, batch number WRC 11240-37-1) was administered by oral gavage to pregnant New Zealand White rabbits at dose levels of 0, 10, 30 and 100 mg/kg bw/day in a vehicle of corn oil on days 7 to 19 of gestation (20 dams/dose). The day of artificial insemination was designated Day 1 of gestation.
Parameters evaluated in dams were clinical signs and behaviour (daily), body weight (on gestation days 1, 4, 7 to 19, 22, 26 and 30), and food consumption (on gestation days 4, 7, 10, 13, 16, 19, 22, 26 and 30).

On day 30, all surviving females were sacrificed, examined macroscopically and the uteri examined for intact gravid uterus weight, number of corpora lutea, live foetuses and intra uterine deaths.
Foetuses were weighed, sexed and examined for external, visceral and skeletal abnormalities.
Dosing solutions were analysed for captan content and homogeneity.

Findings

Analysis of dosing formulations: Achieved concentrations of captan in dosing preparations were ± 4% of nominal levels. Homogeneity and stability of captan in solution were satisfactory.

General observations:
Treated dams had an increased incidence of few or no faeces compared to the control. Increased incidences of diarrhoea in all captan treated groups and mucus in the faeces in 100 mg/kg bw/day dams were observed. One 100 mg/kg bw/day female was killed intercurrently following signs of abortion.
There was a dose-related reduction in maternal body weight gain in 30 and 100 mg/kg bw/day dams, which was most marked during gestation days 7 to 10. Compensatory body weight gain was evident during the post-dosing period in the 100 mg/kg bw/day dams (Table 3.10.1.5-1). A dose-related reduction in food consumption was also seen at 30 and 100 mg/kg bw/day, followed by a compensatory increase in food consumption by 100 mg/kg bw/day dams, particularly on days 26 to 30 (Table 3.10.1.5-2). In the low dose group, there were no effects on maternal body weight gain or food consumption.

Table 3.10.1.5-1 Developmental toxicity study in the rabbit: Body weight changes of dams

Period (days)	Body weight gain (g) at dose (mg/kg bw/day)			
	0	10	30	100
1 – 7 (pre-dosing)	145.2	137.5	172.9	159.6
7 – 19 (dosing)	238.2	205.5	57.2*	-159.3**
7 - 10	15.6	40.2	-68.0*	-142.5**
10 - 13	85.9	52.5	68.8	11.2**
13 - 16	145.9	122.8	59.3*	23.0**
16 - 19	-9.3	-9.8	-2.9	-51.0
19 – 30 (post-dosing)	249.4	288.8	300.3	502.3**
19 - 22	32.1	53.1	51.4	150.4**
22 - 26	125.6	141.9	146.7	176.9
26 - 30	91.7	93.8	102.2	175.0**
1 – 30 (overall)	632.7	631.8	530.4	502.6

Significantly different from the control, * ($p < 0.05$), ** ($p < 0.01$).

Table 3.10.1.5-2 Developmental toxicity study in the rabbit: Food consumption by dams

Period (days)	Food consumption (g/day) at dose (mg/kg bw/day)			
	0	10	30	100
1 – 7 (pre-dosing)	205.3	209.7	208.9	188.5
7 – 19 (dosing)	163.7	158.8	126.7*	56.8**
7 - 10	156.9	170.6	108.7**	45.7**
10 - 13	183.3	186.4	156.4	77.3**
13 - 16	162.1	176.4	125.0	90.9*
16 - 19	152.3	151.7	116.8	42.5**
19 – 30 (post-dosing)	197.8	200.5	178.6	204.7
19 - 22	196.9	213.8	167.4	157.2
22 - 26	212.6	222.6	254.5	224.3
26 – 30	183.7	184.5	164.9	251.6**

Significantly different from the control, * ($p < 0.05$), ** ($p < 0.01$).

Pregnancy and litter data:
There was no treatment-related effect on pregnancy rate, which was 65, 65, 55 and 65% for the 0, 10, 30 and 100 mg/kg bw/day dose groups, respectively.

The incidence of pre-implantation loss in 100 mg/kg bw/day dams was reduced (not significantly) compared to controls. Post-implantation loss at 100 mg/kg bw/day was significantly increased compared to the control and reflected the increased incidences of early and late intra-uterine deaths. Mean total implantation loss was not affected by captan treatment and as a result there was no adverse effect on the mean number of live foetuses compared to the controls.
Mean foetal weight in the 100 mg/kg bw/day dose group was significantly lower than the control, with a consequent reduction (not statistically significant) in mean gravid uterine weight. A summary of litter data is given in Table 3.10.1.5-3.

Table 3.10.1.5-3 Developmental toxicity study in the rabbit: Litter data

Litter data parameter	**Dose (mg/kg bw/day)**			
	0	**10**	**30**	**100**
Corpora lutea (mean)	10.54	10.23	9.80	10.42
Pre-implantation loss (transformed mean)	0.490	0.428	0.369	0.291
Post-implantation loss (transformed mean)	0.311	0.337	0.325	0.485*
Implantations (mean no.)	8.46	8.23	8.50	9.50
Intra-uterine deaths (mean % early)	3.1	4.7	0.0	10.7
Intra-uterine deaths (mean % late)	3.5	4.2	9.5	11.9
Live foetuses (mean no.)	7.85	7.38	7.50	7.17
Mean gravid uterine weight (g)	514.8	481.2	472.7	440.3
Mean litter weight (g)	339.2	319.9	310.3	271.4
Mean foetal weight (g)	45.64	44.90	43.30	37.99**

Significantly different from the control, * ($p < 0.05$), ** ($p < 0.01$).

Foetal assessment (major defects):
The incidence of foetuses with major defects was significantly increased in the 100 mg/kg bw/day dose group compared to the control. Eight foetuses from five litters had at least one major abnormality compared to one foetus in the control group. Four of the eight foetuses affected in the 100 mg/kg bw/day dose group were from one litter. There was no compound-related effect on the incidence of defects involving the cardiac blood vessels but there was an increased incidence of foetuses with major defects of the head in both the 30 and 100 mg/kg bw/day groups. In the latter group, two of the three affected foetuses were litter mates. Of the remaining types of major defects all but one occurred in the 100 mg/kg bw/day group. The possibility of the major defects being compound-induced cannot be dismissed though the small numbers involved and type of specific minor defects and the fact that several foetuses were litter mates, provides no strong evidence for a compound relationship.

Foetal assessment (minor defects):
The incidence of minor external/visceral defects was increased in the 30 and 100 mg/kg bw/day dose groups compared to the control and was mainly due to an increase in the incidence of cysts on the liver. The overall incidence of minor skeletal defects was unaffected by treatment, although the incidence of unossified 6th and 7th lumbar transverse processes and asymmetric alignment of the pelvic girdle was increased at 100 mg/kg bw/day.

Foetal assessment (variants):
There were no external or visceral variants. Each foetus had at least one skeletal variant. Treatment-related effects were seen on the incidence of the following variants in the 100 mg/kg bw/day dose group: odontoid partially ossified (increase); normal length extra 13th ribs (increase); 27 pre-sacral vertebrae (increase): asymmetric development of 1st and 2nd sacral vertebrae (increase); 4th, 5th, 6th and 7th lumbar transverse processes partially ossified; 2nd lumbar transverse processes partially ossified; 3rd lumbar transverse processes fully ossified. The effects on ossification of the lumbar transverse processes reflect reduced ossification which may be associated with reduced foetal weight. The majority of these variants were also seen in the 30 mg/kg bw/day dose group. The overall incidence of foetuses with extra 13th rib was increased in 30 and 100 mg/kg bw/day dose groups compared to the control (60.8, 52.1, 77.3 and 77.6% foetuses affected at 0, 10, 30 and 100 mg/kg bw/day, respectively).

Foetal assessment (manus and pes assessment):
An increase in manus scores, reflecting the decrease in ossification was observed in the 100 mg/kg bw/day dose group (mean score 2.56, $p < 0.01$) compared to the control (mean score 2.24). The mean pes score was not affected by treatment and was 2.0 for all dose groups.

A summary of the incidence of defects and variants is given in Table 3.10.1.4-4. The type and incidence of major defects is summarised in Table 3.10.1.5-5.

Table 3.10.1.5-4 Developmental toxicity study in the rabbit: Summary of foetal defects and variants

Incidence of foetal defects and variants	**Dose (mg/kg bw/day)**			
	0	**10**	**30**	**100**
Number of litters examined	13	13	10	12
External and visceral:				
Number of foetuses examined	102	96	75	86
Number of foetuses with major defects	1	0	2	6*
Number of foetuses with minor defects only	1	3	6*	8**
Skeletal defects:				
Number of foetuses examined	102	96	75	85
Number of foetuses with major defects	0	0	1	4*
Number of foetuses with minor defects only	37	41	25	36
Variants:				
Number of foetuses examined	102	96	75	85
Number of foetuses showing variants	102	96	75	85

Significantly different from the control, * ($p < 0.05$), ** ($p < 0.01$).

Table 3.10.1.5-5 Developmental toxicity study in the rabbit: Summary of type and incidence of major defects.

Type and incidence of major defects	**Dose (mg/kg bw/day)**			
	0	**10**	**30**	**100**
Gross torso malformations	-	-	-	1 (61A)
Encephalocoele, open eyes, gross malformations of the skull	-	-	-	1 (70K)
Microphthalmia	-	-	1 (51A)	-
Mid-brain ventricles extremely dilated, cebocephaly	-	-	-	1 (72D)
Mandibles fused, lower jaw shortened	-	-	1 (49F)	-
Maxillae fused	-	-	-	2 (72D, 72G)
Aorta extremely enlarged	1 (100)	-	1 (49F)	1 (63B)
Pulmonary artery extremely enlarged	-	-	1 (49F)	-
Pulmonary artery extremely reduced	-	-	-	1 (63B)
Subclavian artery absent	-	-	1 (49F)	-
11th rib and thoracic arch absent	-	-	-	1 (72E)
Omphalocoele	-	-	-	1 (72B)
Forepaw extremely flexed	-	-	1 (51A)	2 (64B, 70K)
Pollex absent - bilateral	-	-	-	1 (70K)
Number of foetuses with major defects in the head region	0	0	2	3
Number of foetuses with major defects involving the cardiac blood vessels	1	0	1	1
Number of foetuses with other major defects	0	0	1	5
Number of foetuses with at least one major defect	1	0	2	8

Foetus identity in parenthesis (Dam + foetus letter).

Gross pathology dams:
There was no evidence of treatment-related effects on the incidence of macroscopic findings.

Conclusions

In a teratology study in rabbits, maternal toxicity was observed at 30 and 100 mg/kg bw/day as manifested by a dose-related reduction in body weight gain and food consumption during dosing compared with the controls. Maternal body weight loss was observed at 100 mg/kg bw/day during the dosing period.
Embryotoxicity, seen as an increase in post-implantation loss, was observed at 100 mg/kg bw/day. Mean foetal body weight was decreased in the highest dose group. An increased incidence of a variety of skeletal variants was also observed at this dose and, to a lesser extent, at 30 mg/kg bw/day. An increased incidence of major abnormalities was observed in the 100 mg/kg bw/day dose group. Minor visceral anomalies were observed at 30 and 100 mg/kg bw/day. Although increases in major defects at these dose levels provided no consistent evidence of a treatment-relationship due to the diverse nature of the abnormalities seen, an association with the administration of captan cannot be dismissed.
The overall NOAEL (maternal and foetal) was 10 mg/kg bw/day.

3.10.1.5 Study 5 (1987)

Reference:	Captan: Teratology study in the rabbit
Author(s), year:	Anonymous (1987b)
Report/Doc. number:	Life Science Research Israel Ltd., unpublished report No. MAK/099/CAP (Company file: R-4429/TMN-0792)
Guideline(s):	USEPA Guideline 83-3
GLP:	Yes
Deviations from study protocol and deviations from OECD 414 (2001):	An incidence of mis-dosing occurred (9 animals - 1, 4 and 4 from low, intermediate and high dose group, respectively – received three times their nominal dose. The mis-dosed animals were removed from the study and replaced, and 4 additional control animals were added). At least 5 cases of mortality not related to treatment with captan occurred (including 2 cases of lung dosing and one pasteurella infection). Dosing occurred solely during the period of organogenesis. Low number of dams per dose group.
Acceptability:	Supplementary data due to deviations mentioned above

Material and Methods

In a developmental toxicity study, captan (purity 91%, batch number BN 331552) was administered by oral gavage to pregnant New Zealand White rabbits at dose levels of 0, 10, 40 and 160 mg/kg bw/day (group sizes of 18, 14, 15 and 16 dams, respectively) in a vehicle of 0.5% carboxymethylcellulose (containing 0.05% acetic acid) on days 7 to 19 of gestation inclusive (day of mating = day 0 of gestation). Parameters evaluated in dams were clinical signs (daily), body weight (days 0, 3, 7 to 19, 22, 25 and 29 of gestation), food consumption (twice weekly). On day 29 of gestation, females were sacrificed and examined macroscopically. The number of corpora lutea in each ovary, the weight of gravid uteri, the distribution of live/dead foetuses and resorption sites, placental weights, foetal weights and lengths and anomalies/abnormalities of foetuses were assessed.

Findings

General observations:
A total of 7 animals died or were sacrificed before term (1, 2 and 4 animals from the low, intermediate and high dose group, respectively): The low dose animal was sacrificed after abortion on day 27 and was shown to have been suffering from bronchopleuropneumonia (not reatment-related). One intermediate dose animal suffered from Pasteurella infection and was sacrificed after abortion on day 23, whereas the second intermediate dose animal was found dead after six days dosing and showed congested lungs and a urinary bladder distended with bloody urine (both not treatment-related). Two high dose animals were found dead during the dosing period and showed evidence of lung dosing at the post-mortem examination. Another 160 mg/kg bw/day dam was sacrificed while aborting on day 26. Foetuses in the left uterine horn were autolytic and those in the right horn were dead. The relation of these foetal deaths to treatment was unclear. A second 160 mg/kg bw/day dam was sacrificed after aborting on day 18. Abortuses were autolysed and the antecedent foetal death was considered to be treatment-related.
Reduced faecal output was observed in 160 mg/kg bw/day dose group animals, presumably related to reduced food intake in these animals.
Food consumption was dramatically reduced in the 160 mg/kg bw/day dams over the entire dosing period. A compensatory increase in food consumption was observed on gestation days 24 to 26 (Table 3.10.1.6-1). In the 40 mg/kg bw/day dams a moderate reduction in food consumption was observed on gestation days 15 to 19. The significance of a decrease in food consumption in 10 mg/kg bw/day dams on gestation days 27 to 29 was not clear, but was probably unrelated to treatment.

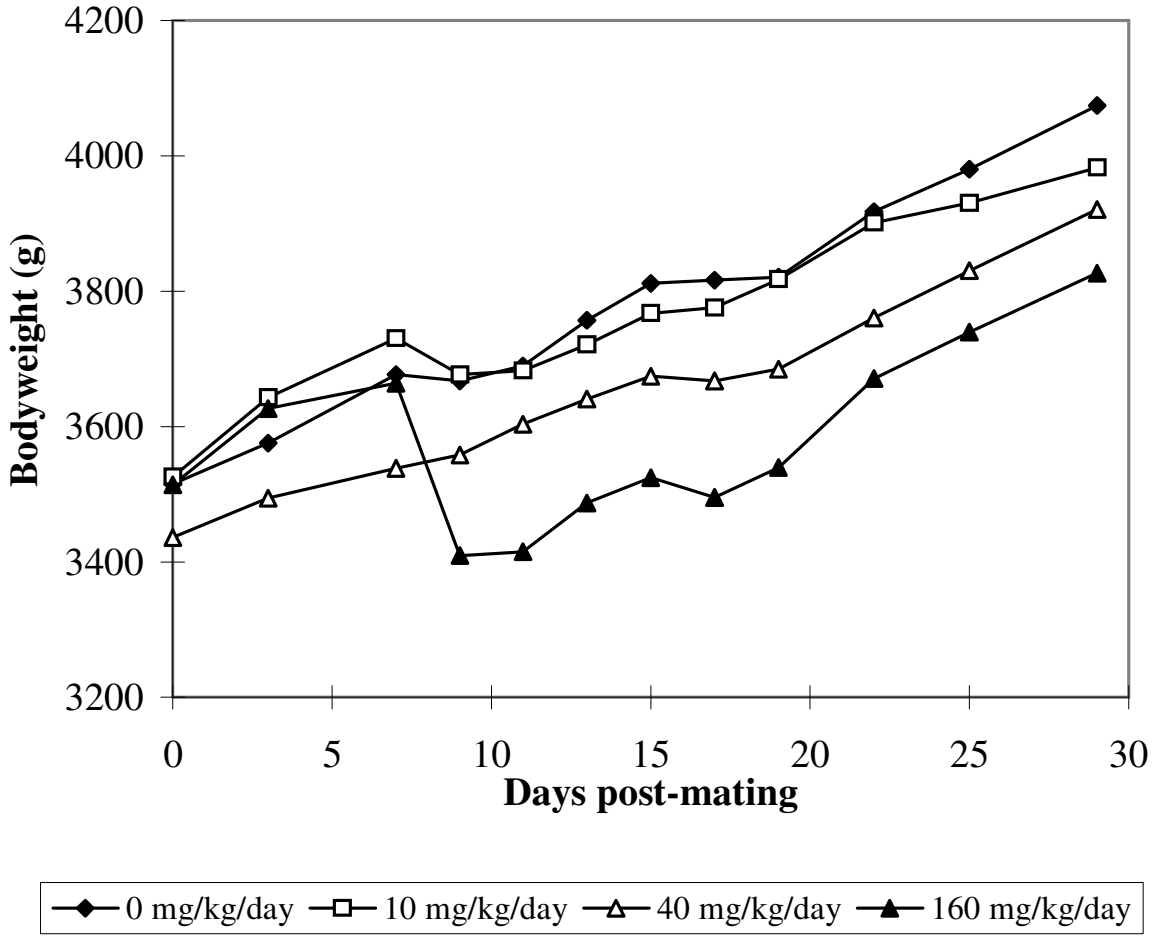
Body weight was decreased at 160 mg/kg bw/day compared to the controls over the entire dosing period and did not recover substantially in the post-dosing period (Figure 3.10.1.6-1).

Table 3.10.1.6-1 Developmental toxicity study in the rabbit: Mean food consumption by dams (g/animal/day) and mean maternal body weight (g/animal)

Gestation day	**Dose group (mg/kg bw/day)**			
	0	**10**	**40**	**160**
	Mean food consumption (g/animal/day)			
1 - 4	193	215	210	216
5 - 7	238	228	210	229
8 - 10	210	203	196	35***
11 - 14	204	198	184	58***
15 - 19	223	212	186**	104***
20 - 23	202	199	199	212
24 - 26	168	143	183	216***
27 - 29	179	133***	177	192
	Mean body weight (g)			
0	3515	3526	3436	3514
9	3668	3677	3558	3409**
10	3683	3665	3587	3382***
15	3812	3768	3674	3525***
19	3821	3818	3685	3540***
22	3918	3901	3761*	3671**
29	4074	3982	3921	3827**

Significantly different from the control, * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), students t-test.

Figure 3.10.1.6-1 Developmental toxicity study in the rabbit: Body weight of dams (mean)



Pregnancy and litter data:
Gravid uterine weight, in line with the reduced maternal body weight, was reduced at 160 mg/kg bw/day, but also at 40 mg/kg bw/day. Intrauterine foetal death (post-implantation loss) was significantly increased in the 160 mg/kg bw/day dose group compared to the control with one case of total resorption (Table 3.10.1.6-2). Administration of captan had no consistent effect on the incidence of pre-implantation loss. Foetal weight and crown rump length were unaffected by treatment.

Table 3.10.1.6-2 Developmental toxicity study in the rabbit: Summary of litter parameters (group mean ± SD)

Litter data parameter	Dose group (mg/kg bw/day)				
	0	10	40	160	
				includes case of total litter resorption	excludes case of total litter resorption
Resorptions					
early	0.4 ± 0.8	0.4 ± 0.5	0.3 ± 0.6	1.8 ± 2.8	1.0 ± 1.2
late	0.2 ± 0.5	0.2 ± 0.6	0.2 ± 0.4	0.3 ± 0.7	0.4 ± 0.7
total	0.6 ± 1.0	0.6 ± 0.9	0.4 ± 0.9	2.1 ± 2.8*	1.4 ± 1.4
Implantation loss					
pre (%)	10.0 ± 2.4	7.3 ± 2.3**	21.0 ± 2.1***	10.0 ± 2.9	11.0 ± 2.9
post(%)	5.8 ± 2.0	6.9 ± 1.8	5.7 ± 2.1	19.6 ± 11.2***	13.2 ± 2.8***
Gravid uterus					
Weight (g)	591.6 ± 107.9	596.3 ± 87.5	503.0 ± 90.5	542.1 ± 136.8	-

Significantly different from the control, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (students t-test).

Foetal assessment:
There were no remarkable malformations observed at necropsy and no apparent increases in the number of minor foetal variations. The principle findings at skeletal evaluation were an increase in the number of foetuses in the 160 mg/kg bw/day dose group with a 13th lumbar rib (57.1% and 79% in the control and 160 mg/kg bw/day dose groups, respectively) and a shift in the articulation of the ilium from the first to the second sacral vertebra in the 160 mg/kg bw/day dose group (Table 3.10.1.6-3).
Reduced or incomplete ossification of the hyoid bone was significantly increased in the 10 and 160 mg/kg bw/day dose groups, but not in the 40 mg/kg bw/day dose group. The absence of this finding in the intermediate dose group indicates that, at least in the 10 mg/kg bw/day dose group, this finding is not treatment-related.

Table 3.10.1.6-3 Developmental toxicity study in the rabbit: Summary of skeletal variations

Incidence of foetal skeletal variations	Dose group (mg/kg bw/day)			
	0	10	40	160
Number of foetuses affected (%)				
Number foetuses examined	156	127	100	100
Reduced or incomplete ossification of hyoid bone	14 (9.0)	26 (20.5)**	12 (12.0)	24 (24.0)**
13th lumber rib present bilaterally	89 (57.1)	73 (57.5)	68 (68.0)	79 (79.0)***
Ilium articulating with 1st or 1st and 2nd sacral vertebra (bilaterally)	69 (44.2)	56 (44.1)	36 (36.0)	23 (23.0)***
Ilium articulating with 1st or 1st and 2nd sacral vertebra (unilaterally)	10 (6.4)	6 (4.7)	5 (5.0)	1 (1.0)*
Number of litters affected				
Number litters examined	16	13	12	11
Reduced or incomplete ossification of hyoid bone	7 (8.9)	7 (20.5)	4 (12.5)	10 (22.5)***
13th lumber rib present bilaterally	16 (57.6)	12 (59.0)	12 (66.2)	11 (79.7)**
Ilium articulating with 1st or 1st and 2nd sacral vertebra (bilaterally)	16 (44.9)	12 (43.9)	11 (36.9)	10 (23.9)*
Ilium articulating with 1st or 1st and 2nd sacral vertebra (unilaterally)	8 (6.8)	5 (5.4)	5 (5.8)	1 (1.3)*

Significantly different from the control, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Conclusions

In a developmental toxicity study in rabbits, maternal toxicity was observed at 160 mg/kg bw/day as shown by a decrease in body weight and body weight gain. Food consumption in this group was also decreased during dosing, followed by an

increase post- dosing compared to the control. In the 40 mg/kg bw/day dose group food consumption was slightly decreased during the last part of the dosing period.
Embryo-and foetotoxicity was observed at 160 mg/kg bw/day. Increased post-implantation loss, including one total resorption, and an increase in foetal defects classified as skeletal variants were observed in the 160 mg/kg bw/day dose group. The observed axial skeletal abnormalities could be related to the gastro-intestinal maternotoxicity and, as a consequence, to an embryonic nutrient imbalance. One dam also aborted at this dose level following death of all foetuses. There was no increase in the number of major malformations in treated groups compared to the control.
In the original DAR of captan (2005), the NOAEL for maternal toxicity was set at 10 mg/kg bw/day based on reduced food consumption and body weight in dams administered captan at 40 mg/kg bw/day. Although statistically significant ($p < 0.05$), the transient reduction in maternal body weight on gestation day 22 was very small (only 4%) compared to the control. The significant reduction in food consumption in 40 mg/kg bw/day dams at days 15 to 19 appears to have had only little effect on maternal body weight. Therefore, a NOAEL for maternal toxicity of 40 mg/kg bw/day is considered to be more appropriate
The NOAEL for foetal toxicity was 40 mg/kg bw/day based on foetal death (post-implantation loss), including one case of total resorption and one case of abortion and an increase in the proportion of foetuses with certain skeletal variations in the 160 mg/kg bw/day dose group.

3.10.1.6 Study 6 (1981)

Reference:	Effect of technical captan on pregnancy of the New Zealand White rabbit
Author(s), year:	Anonymous (1981)
Report/Doc. number:	Huntingdon Research Centre, unpublished report No. CHR/15/8114 (Company file: TMN-0787)
Guideline(s):	In-house method
GLP:	Yes
Deviations:	Not applicable. Animals were obtained from three different sources, Chesire Rabbit Farms (Cheshire), Ranch Rabbits (Sussex) and Buxted Rabbit Co. Ltd. (Sussex). Low number of dams per dose group. Large differences between initial group mean body weights among dose groups.
Acceptability:	Supplementary data only due to deviations mentioned above

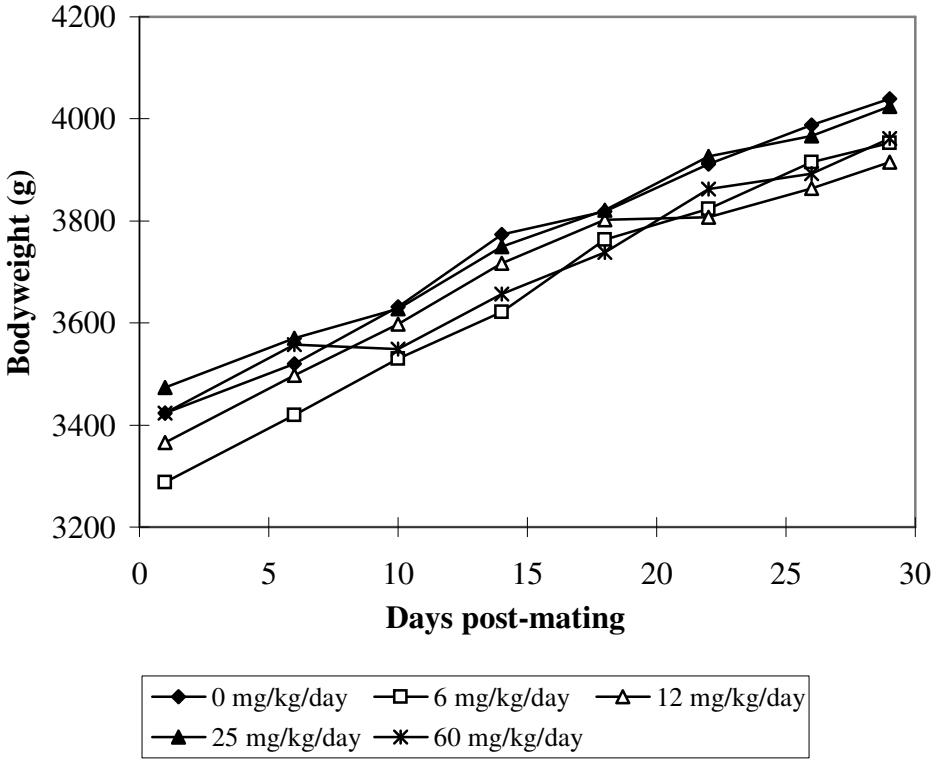
Material and Methods

In a developmental toxicity study, captan (purity 89%, batch number SX-1086), in a vehicle of 0.5% sodium carboxymethylcellulose, was administered by oral gavage to pregnant New Zealand white rabbits on days 6 to 28 of gestation at dose levels of 0, 6, 12, 25 and 60 mg/kg bw/day (15 dams/dose). Dams were sacrificed on day 29 of gestation. Parameters evaluated in dams were clinical signs (daily) and body weight (on days 1, 6, 10, 14, 18, 22, 26 and 29 of gestation). Litter parameters evaluated were number of corpora lutea, gravid uterine weight, number and distribution of live young, embryonic/foetal deaths, individual foetal weight, foetal abnormalities (classified as major malformations, minor anomalies and variants) and crown rump length.

Findings

General observations:
Occasional deaths occurred in all groups (2, 1, 2, 2 and 1 at 0, 6, 12, 25 and 60 mg/kg bw/day, respectively) and were considered unrelated to treatment with captan. Among animals surviving to termination an increased incidence of non-specific signs such as anorexia, reduced faecal output and water intake were observed in the 60 mg/kg bw/day dose group and possibly in the 25 mg/kg bw/day dose group during the first part of the dosing period (days 6 to 18).
Body weight gain was reduced compared to controls from the initiation of dosing until termination in all dose groups except at 6 mg/kg bw/day (Figure 3.10.1.7-1). These differences were considered to be due to initial weight loss during the first four days of dosing (60 mg/kg bw/day) and retarded weight gain throughout the entire dosing period (25 mg/kg bw/day) or from day 18 (12 mg/kg bw/day). There were no treatment-related gross macroscopic findings in dams at autopsy.

Figure 3.10.1.7-1 Developmental toxicity study in the rabbit: Body weight of dams (mean)



Pregnancy and litter data:
At 60 mg/kg bw/day there was a higher incidence of non-pregnancy (Table 3.10.1.7-1). However, from the results of a preliminary study (Huntingdon Research Centre Report No. CHR/14/80550) and in the absence of increased pre-implantation loss in the current study, this was considered to be coincidental by the study author.

Table 3.10.1.7-1 Developmental toxicity study in the rabbit: Adult performance (number of animals) and mean maternal body weight and body weight gain (g/animal)

	Dose group (mg/kg bw/day)				
	0	**6**	**12**	**25**	**60**
Mated	15	15	15	15	15
Dead or killed	2	1	2	2	1
Non-pregnant	1	0	0	1	6
Abortion or total resorption	0	1	2	3	0
Live fetuses at sacrifice	12	13	11	9	8
Gestation day	**Mean body weight (g)[a]**				
1	3422.9	3241.2	3316.4	3560.6	3422.5
6	3519.6	3373.1	3439.5	3681.7	3558.1
10	3630.8	3486.2	3546.8	3753.3	3549.4
14	3772.9	3585.0	3653.6	3851.7	3657.5
18	3817.9	3726.9	3746.4	3911.7	3738.1
22	3912.1	3790.0	3780.5	4001.1	3862.5
26	3987.5	3881.5	3840.0	4066.7	3892.5
29	4040.4	3908.2	3885.9	4118.9	3960.6
Gestation day	**Mean body weight gain (g/animal)**				
6-29 (dosing)	520.8	535.1	446.4	437.2	402.5

[a] Includes all animals with live young at termination (excluding animals showing abortion or total resorption).

There was no treatment-related effect on litter size and post-implantation loss. A lower number of implantations per dam at the lowest dose level was considered to be incidental. Non-significant reductions in the gravid uterine weight, litter weight, mean foetal weight and crown rump length were observed in the 60 mg/kg bw/day dose group compared to the control (Table 3.10.1.7-2). These differences suggest slight foetotoxicity at the highest dose, but were considered to be indirectly related to treatment and as a result of the effects on maternal body weight gain.

Table 3.10.1.7-2 Developmental toxicity study in the rabbit: Group mean litter weights and measurements

Dose group (mg/kg bw/day	Uterine weight (g)	Litter weight (g)	Foetal weight (g)	Crown rump length (mm)
0	549	360.7	43.3	97.9
6	449	321.0	48.0	100.4
12	516	364.4	41.7	95.9
25	521	368.5	43.3	97.8
60	475	333.1	39.9	94.4

Foetal assessment:
There was no significant effect of treatment on the incidence of major malformations, minor anomalies (visceral and skeletal) or skeletal variants.

Conclusions

In this developmental toxicity study in the rabbit, maternal body weight loss occurred at 60 mg/kg bw/day during initial dosing and was associated with non-specific clinical signs (anorexia, reduced faecal output and water intake). Maternal body weight gain reduction during dosing (greater 10%) was observed at 12 mg/kg bw/day and above. Foetal and litter weights as well as crown-rump length were reduced at 60 mg/kg bw/day, however not significantly, indicating slight foetotoxicity. There was no significant increase in the incidence of abnormalities at any dose level compared to the control. The NOAEL for maternal toxicity was 6 mg/kg bw/day based on decreased body weight gain at 12 mg/kg bw/day and above. The NOAEL for foetal toxicity was 25 mg/kg bw/day based on effects on uterine, litter and foetal weight and crown rump length at 60 mg/kg bw/day.

3.10.1.7 Study 7 (2006)

Reference:	Prenatal toxicity study in the rabbit by oral gavage administration
Author(s), year:	Anonymous (2006a)
Report/Doc. number:	MAK 862/052833, Company file: R-18199
Guideline(s):	OECD Test guideline 414 (1981), EU guidelines B.31, OPPTS guideline No. 870.3700, No. 12-Nousan-8147
GLP:	Yes
Deviations:	The weight of the gravid uterus was not recorded for females 35, 43, 57 and 61 due to an error. The vehicle was corrected to 0.5% w/v Tween 80 with 0.7% w/v carboxymethylcellulose. These deviations are considered not to affect the scientific validity of the study.
Acceptability:	Yes

Executive Summary

In a developmental toxicity study, Captan (95.1% purity) in 0.5% w/v Tween 80 with 0.7% w/v carboxy-methyl¬cellulose prepared in water was administered to three groups of 25 female New Zealand White rabbits by gavage at dose levels of 10, 20, or 45 mg/kg bw/day from days 6 to 28 after mating.
There were no treatment-related effects on mortality. Reduced body weight gain and food consumption were detected in a dose-related manner. These effects were associated with general clinical signs of thin build, little water drunk and few/pale/dark faeces, most noticeable at 45 mg/kg/day. Microscopic examination of sections of the duodenum from animals treated at 45 mg/kg/day revealed a higher incidence of minimal/slight eosinophils infiltrating the duodenum compared with controls.
Furthermore, increase in the mean number of early resorptions per litter and consequently post implantation loss were also noted. There was a low incidence of foetuses/litters with absent kidney and ureter (4 foetuses; 2 litters), the aetiology of which was uncertain. The low incidence and distribution of other major abnormalities did not suggest a relationship with treatment. Slightly higher numbers of foetuses had 12/13 or 13/13 ribs with associated 20 thoracolumbar vertebrae (a common variant in the rabbit) and offset alignment of the pelvic girdle compared with controls. Mean foetal and litter weights were lower than in controls. A slight delay in development associated with the lower mean foetal weights was indicated by higher incidences of foetuses/litters with incompletely ossified/unossified epiphyses, astragalus, pubes and metacarpals/phalanges and atelectatic lungs. Prenatal development was not affected by treatment at 20 or 10 mg/kg/day.
The developmental NOAEL is 20 mg/kg bw/day, based on evidence of a slight developmental delay at 45 mg/kg/day.
For the pregnant females, absolute body weight was unaffected at 10 or 20 mg/kg/day, and at 10 mg/kg/day bodyweight gain was only significantly affected from 11 days after start of treatment. This was subsequent to a fall in food intake although this recovered from Day 21 despite continued treatment. The maternal no-observed-effect-level (NOEL) would therefore appear to be less than 10 mg/kg/day.

Material and Methods

A. MATERIALS

1. Test Material: Captan (Merpan Technical)

Description	Off-white powder
Batch/Lot no:	601 385 40
Purity	95.1%
Production date	13.01.2005
Expiry date	November 2006
Compatibility	Not compatible with alkaline material.
Storage conditions	Ambient temperature (approx. 21°C) in dry conditions, protected from light

2. Vehicle and/or positive control

Vehicle: 0.5% w/v Tween 80 with 0.7% w/v carboxymethylcellulose in water (homogeneity and stability have been confirmed)

3. Test system:

Species/ strain:	Rabbit / New Zealand White
Age:	21 – 29 weeks (on day 0 of gestation)
Body weight:	2.99 – 4.09 kg (on day 0 of gestation)
Sex:	Females

Source: Charles River Laboratories, Domaine de Oncins, L'Arbresle, Cedex, France
Number of animals: 108 females in groups 1-4 (25 females per group)
Adaptation period: 18 days
Housing: Housed individually in LCP cages (high impact plastic and perforated counter-sunk floor panels); environmental enrichment with stainless steel key ring attached in front of each cage and untreated Aspen chew block
Food: Standard rabbit breeding diet, SAFE (Scientific Animal Food and Engineering) 110-10 from SAFE, Route de Saint Bris, 89 290 Augy, France; restricted to 150 g/animal/day during acclimatization up to one week prior to the onset of mating and 200 g/animal/day thereafter. No antibiotic or other chemotherapeutic or prophylactic agent was added. Once daily each rabbit was given a handful of autoclaved hay to promote gastric motility.
Water: Tap water ad libitum via polycarbonate bottles fitted with sipper tubes

4. Environmental conditions:
Temperature: 15 – 23°C
Humidity: 40 – 70%
Photoperiod: 14 hours light/10 hours dark
Group assignment: Females were allocated to group and cage position in sequence of mating on Day 0 of gestation. The animals were given unique new identity numbers, using an ear tag. Females mating on any day were evenly distributed amongst the groups. The study consisted of one control and three treated groups of rabbits.

B. STUDY DESIGN
1. In-life dates: 10. February – 2. August 2005 at Huntingdon Life Sciences Ltd., Eye, Suffolk, England

2. Mating
A colony of stock males used was maintained specifically for the purpose of mating. These healthy males were considered to be not part of the study and were returned to stock after mating. On each day of mating, sufficient numbers of healthy females, showing evidence of vaginal oestrus, were paired on a one male to one female ratio. Pairs were observed to naturally mate and were then left together in the cage for at least a further 30 minutes. Individual mating records were kept, and any reason for premature separation was documented. Mated females were then removed and 25 IU of luteinising hormone was administered intravenously to promote successful ovulation. The day of mating was designated Day 0 of gestation.

3. Administration of test article
Three groups of 25 female rabbits received Captan via gavage at doses of 10, 20, or 45 mg/kg bw/day from gestation day (GD) 6 until GD 28 at a volume of 5 mL/kg based on most recent body weight. A similarly constituted control group received the vehicle, 0.5% w/v Tween 80 with 0.7% w/v carboxy-methyl-cellulose prepared in water for formulation, at the same volume-dosage throughout the same period. All animals were dosed in sequence of cage-number within each group, once each day at approximately the same time. Females 59 (20 mg/kg/day group) and 86 (45 mg/kg/day) were only dosed from Day 6 to Day 23 for reasons of animal welfare.

Table 3.10.1.8-1 Study design - Animal assignment

Group	No. of females	Animal number	Treatment	Dosage [mg/kg bw/day]	Concentration [mg/mL]	Volume dosage [mg/kg]
1	25	1 – 25	Control	0	0	5
2	25	26 – 50	Captan	10	2	5
3	25	51 – 75	Captan	20	4	5
4	25	76 – 100	Captan	45	9	5

4. Statistical analysis
Where appropriate, group mean values, each with standard deviation (SD), were calculated from individual data. Mean data was restricted to data derived from females/litters with live young at GD 29.

For binary (e.g. absence/ presence) categorical data (= non-parametrical values), the proportion of animals (or litters/ foetuses) was analysed using Fisher´s Exact test for each treated group versus control.
For continuous data (parametrical values), Bartlett´s test was applied to test the homogeneity of variance between groups. Dependent on the outcome, different post-tests were performed: A frequency analysis was applied if 75% of data (across all groups) were the same value followed by a Mantel test for a trend in proportions and also pairwise Fishers's Exact tests for each dose group against the control. In case of non-significance at the 1% level, a parametric analysis was applied followed by a Williams test (monotone trend) or a Dunnett's test (non-monotone). In case of a significant Barlett's test at the 1% level, a logarithmic or square-root transformation was tried and followed by a Shirley´s tests (monotonic trend) or a Steel´s tests (not monotonic). Significant differences between control and treated groups were expressed at the 5% ($p < 0.05$) or 1% ($p < 0.01$) level.

C. METHODS

1. Observations

All animals were observed for viability and clinical signs including behavioural changes, reaction to treatment, or illness at least twice daily throughout the study.

2. Mortality and abortion

Debilitated animals were observed carefully and killed to prevent unnecessary or prolonged suffering. A complete necropsy was performed for the animal killed for humane reasons as described in the Necropsy section.

3. Body weights and food consumption

Body weights of each rabbit were recorded weekly during acclimatisation, on day of mating (GD 0) and thereafter daily until necropsy.
The weight of food supplied to each animal, that remaining and an estimate of any spilled was recorded daily from GD 1.

4. Termination

The animals were sacrificed one day before calculated parturition, i.e. on GD 29 by a lethal intravenous injection sodium pentobarbitone. Foetuses were killed by subcutaneous injection of sodium pentobarbitone. In case of pregnancy loss, the animals were sacrificed on the same day that abortion was detected.

5. Necropsy

After ventral midline incision the neck and associated tissues and the thoracic, abdominal and pelvic cavities and their viscera were exposed and examined in situ. External and cut surfaces of the organs and tissues were examined as appropriate. The caecum, colon, duodenum (including the sphincter of Oddi), ileum (including Peyer's patches), jejunum, rectum and stomach and any abnormal tissues were retained in 10% Neutral Buffered Formalin.
Tissue sections of duodenum (including the sphincter of Oddi) were dehydrated, embedded in paraffin wax, sectioned at approximately four to five micron thickness and stained with haematoxylin and eosin.

Regarding reproductive assessment, the gravid uterus (incl. ovaries) was weighed prior to dissection, and for each animal, the number of corpora lutea in each ovary as well as the number of implantation sites, the number and distribution of resorption sites (classified as early or late) and live and dead foetuses were recorded for each uterine horn. For apparently non-pregnant animals, and for apparently empty horns, the number of uterine implantation sites was checked after staining with ammonium sulphide (modification of the Salewski staining technique). For animals exhibiting pregnancy loss, expelled uterine contents were identified and examined, as appropriate.

All foetuses and placentae were dissected from the uterus and weighed individually. Foetuses were individually identified within the litter, each foetus and placenta was externally examined and any abnormalities were recorded. The neck and thoracic and abdominal cavities of all foetuses from each litter were dissected, the contents examined for visceral abnormalities and sex recorded. Following examination, one half of the foetuses in each litter were decapitated and their heads initially stored in Bouin's fluid for evaluation of visceral anomalies. The remaining intact foetuses and torsos were eviscerated and fixed in industrial methylated spirits prior to processing and staining with Alizarin Red for skeletal assessment.
For all animals the duodenum including the sphincter of Oddi was examined.

Findings

1. Survival

No treatment-related mortalities occurred.

One female in the 20 mg/kg/day group (Group 3 Female Number 73) was killed for humane reasons on Day 27 after mating due to a wet abrasion and ulceration on the cervical region and a swollen muzzle. An open wound with pale, purulent material surrounded by dark necrotic tissue (approx. 25 mm) on the ventral neck region was observed at necropsy. There were also a few encrustations around the muzzle. The animal was found to be pregnant but there was only one live foetus in the uterus with the other implantations being late resorptions.

One animal which had received Captan at 45 mg/kg/day (Group 4 Female Number 99) was found to have had a total litter resorption.

2. Body weight and food consumption

Bodyweight was not statistically different from control at 10 or 20 mg/kg/day, the differences being less than 4%, and was only significantly different from the control at 45 mg/kg/day on GD 19 and 21. Bodyweight change (GD 6 to GD 29) was affected by treatment at all dosages of Captan; reflecting a dose dependency.

Food consumption was reduced by treatment at all dosages of Captan; a dose response was evident. The effects, however, appeared to be transient in nature as all dose groups appeared to recover several days before cessation of treatment.

Table 3.10.1.8-2 Maternal body weight / body weight gain per group (± SD)

Body weight/ gain [mean kg]	**Group 1 Control (n = 24)**	**Group 2 [10 mg/kg bw/day] (n = 24)**	**Group 3 [20 mg/kg bw/day] (n = 23)**	**Group 4 [45 mg/kg bw/day] (n = 23)**
GD 0	3.53 ± 0.23	3.51 ± 0.26	3.54 ± 0.20	3.61 ± 0.24
GD 6	3.62 ± 0.23	3.60 ± 0.25	3.63 ± 0.21	3.73 ± 0.26
GD 29	3.88 ± 0.28	3.75 ± 0.29	3.76 ± 0.20	3.78 ± 0.26
Pre-treatment: GD 0 to GD 6	0.09 ± 0.05	0.09 ± 0.06	0.09± 0.06	0.12 ± 0.08
Treatment: GD 6 to GD 29	0.26 ± 0.15	**0.15 ± 0.16[a]**	**0.13 ± 0.17[a]**	**0.06 ± 0.19[b]**

n: number of dams

[a] - $p<0.05$; [b] - $p<0.01$

3. Clinical signs

There was an increased incidence of thin build recorded amongst animals receiving Captan at 10, 20 or 45 mg/kg/day when compared with controls. There was also an increased incidence of little water drunk and few, pale or dark faeces amongst these animals. In both cases the incidences being noticeably higher at 45 mg/kg/day. A small number of animals receiving Captan at 45 mg/kg/day were observed to be underactive (3/25) or, to have red staining on the cage under tray (2/25). Other clinical signs observed were those commonly seen at these laboratories in this type of caging, and they show no relationship to treatment. There was no effect of treatment on the weight of the gravid uterus.

Table 3.10.1.8-3 Most relevant clinical signs per group – females

Observations	Group 1 Control	Group 2 10 mg/kg bw/day	Group 3 20 mg/kg bw/day	Group 4 45 mg/kg bw/day
Build deformity				
Thin	0/22	5/25	5/24	14/25
Behaviour				
Little water drunk	5/22	10/25	14/24	20/25
Excreta				
Few faeces	1/22	10/25	16/24	25/25
Pale faeces	0/22	5/25	6/24	20/25
Dark faeces	1/22	4/25	4/24	7/25
Pellets reduced size	1/22	5/25	3/24	7/25
Staining (yellow/brown/grey)				
Paws/tail/body surface/muzzle	22/22	22/25	17/24	18/25
Cervical region/perigenital region	4/22	3/25	0/24	0/25

4. Macroscopic Examination

Macroscopic observations at necropsy confirmed the presence of the increased incidence of thin build among animals treated with Captan, being more noticeable at 45 mg/kg/day.

There were no other findings that showed a conclusive relationship to treatment with Captan.

5. Histopathologic Examination

An increased incidence of eosinophils infiltrating the duodenum at the minimal and slight level was seen in rabbits at 45 mg/kg/day compared with controls. One case of focal erosion was also seen at the same dosage.

6. Reproduction data

One female in each group was found to be not pregnant. Captan treatment did not affect the mean numbers of corpora lutea and implantations, or the extent of pre-implantation losses.

Table 3.10.1.8-4 Summary of reproduction data – values per group

Observation	Dose [mg/kg bw/day]			
	Group 1 Control	**Group 2 10 mg/kg**	**Group 3 20 mg/kg**	**Group 4 45 mg/kg**
Number of females assigned (Mated)	25	25	25	25
Number of females pregnant (Pregnancy rate %)	24 (96)	24 (96)	24 (96)	24 (96)
Number of females aborted	0	0	1	1
Corpora lutea/ group (± SD)	10.0 ± 1.8	10.9 ± 2.4	10.5 ± 1.3	10.8 ± 1.4
Implantations (± SD)	8.7 ± 2.4	9.3 ± 2.1	8.8 ± 2.6	9.4 ± 1.8
Total resorptions/ group	0.5	0.5	0.9	**1.6[b]**
Early resorptions/ group	0.4	0.3	0.7	**1.0[a]**
Late resorptions/ group	0.1	0.3	0.3	0.5
Mean foetal weight [g] ± SD Males Females	41.2 ± 5.5 41.1 ± 5.4 40.7 ± 4.9	38.2 ± 4.3 39.0 ± 4.3 37.4 ± 5.0	39.9 ± 6.7 40.9 ± 6.6 38.0 ± 6.0	**35.5 ± 5.2[b]** **36.0 ± 5.5[b]** **35.1 ± 5.8[b]**
Sex-ratio (% male)/ group	50.9	48.2	51.5	53.5
Total number of foetuses (alive) / group (± SD)	8.2 ± 2.2	8.8 ± 1.8	7.9 ± 2.5	7.8 ± 1.8
Male foetuses (alive)/ group (± SD)	4.8 ± 1.8	3.8 ± 2.0	5.0 ± 2.4	3.8 ± 1.6
Female foetuses (alive)/ group (± SD)	4.2 ± 1.8	4.2 ± 2.1	3.4 ± 1.6	4.9 ± 2.2
Pre-implant. loss (%) / group	12.9	13.3	16.4	12.9
Post-implant. loss (%) / group	4.9	5.0	9.3	**15.3[b]**

SD: Standard deviation
[a] $p < 0.05$ from the control; [b] $p < 0.01$ from the control

At 45 mg/kg/day, there was a statistically significant increase in the mean number of early resorptions per litter and consequently mean post-implantation loss compared with controls; 6/23 litters contained 2 or more early resorptions compared with 3/24 controls; 11/23 litters contained no resorptions compared with 19/24 Controls. The mean live litter size was marginally lower than in controls; the sex ratio was considered to be unaffected by treatment. One female examined on GD 29 was found to have lost its litter in utero, with all implantations early resorptions. A relationship to treatment could not be discounted, but cannot be confirmed as it was an isolated event.
Treatment at 20 or 10 mg/kg/day was considered not to have affected embryo-foetal survival. At 20 mg/kg/day, the mean number of early resorptions per litter and the mean percentage of post-implantation loss were slightly higher than in controls. However, no effect of treatment was inferred because the differences largely reflected a single litter containing 5 early resorptions and did not attain statistical significance, and the number of litters containing 2 or more early resorptions was the same as in the control group.

7. Examination of the foetuses

At 45 mg/kg/day, mean foetal and litter weight were statistically significantly lower than in controls.
Foetal weights were considered unaffected by treatment at 20 or 10 mg/kg/day. At 45 mg/kg/day, four foetuses (two litters) had the finding absent kidney and ureter, which was not found in the control group in this study. Although of uncertain aetiology a relationship to treatment could not be unequivocally discounted. The low incidence and distribution of other major abnormalities did not suggest a relationship with treatment.
There was a slight increase in the incidence of foetuses with the variant 12/13 or 13/13 ribs with associated 20 thoracolumbar vertebrae and offset alignment pelvic girdle, compared to a relatively low concurrent control. However, for 12/13 or 13/13 ribs the litter incidence was similar in all groups. The incidences of foetuses/litters with incompletely/unossified epiphyses, astragalus, pubes, metacarpals/phalanges and atelectatic lungs were higher than concurrent controls, indicating a slight developmental delay associated with the decrease in mean foetal weight seen in this group.

There were no effects of treatment on foetal morphology at 20 or 10 mg/kg/day.

Table 3.10.1.8-5 Foetal Examinations – major abnormalities

Observation	Dose [mg/kg bw/day]			
	Group 1 Control	**Group 2 10 mg/kg**	**Group 3 20 mg/kg**	**Group 4 45 mg/kg**
Number of foetuses [litters] examined	197 (24)	211 (24)	182 (23)	180 (23)
Number of foetuses [litters] affected	1 (1)	2 (1)	2(2)	9 (5)
Mean % litter affected	4.2	4.2	8.7	21.7
Acrania: multiple cervical, thoracic and lumbar vertebral/rib abnormalities, brachyury: amelia: markedly misshapen scapula, long bones and clavicle: unfused tibia and fibula: transposition of dilated pulmonary trunk and narrow ascending aorta: incomplete aortic arch: muscular ventricular septal defect: gastroschisis	-	-	-	1 (1)
Fused frontal/parietal	1 (1)	-	-	-
Hydrocephaly: domed cranium	-	-	-	1 (1)
Hydrocephaly with confluent lateral ventricles: medially displaced nares: absent bilateral upper major incisors: thin nasal septum: shortened muzzle	-	1 (1)	-	-
Fused upper major incisors	-	1 (1)	-	-
Dilated ascending aorta/aortic arch, narrow pulmonary trunk, marked: caudally displaced ductus arteriosus: muscular ventricular septal defect	-	-	1 (1)	-
Umbilical hernia	-	-	-	2 (1)
Absent kidney and ureter	-	-	1 (1)	4 (2)
Lumbar scoliosis	-	-	-	1 (1)

Table 3.10.1.8-6 Foetal Examinations – minor skeletal variations

Observation	Dose [mg/kg bw/day]			
	Group 1 Control	**Group 2 10 mg/kg**	**Group 3 20 mg/kg**	**Group 4 45 mg/kg**
Number of foetuses [litters] examined	196 (24)	209 (24)	180 (23)	171 (23)
Number of foetuses intact [litter]	99 (24)	107 (24)	91 (23)	86 (23)
Number of foetuses affected [litter]	20 (14)	11 (6)	17 (10)	14 (11)
Mean % litter affected	58.3	25	43.5	47.8

Table 3.10.1.8-7 Foetal Examinations – minor visceral variations

Observation	Dose [mg/kg bw/day]			
	Group 1 Control	Group 2 10 mg/kg	Group 3 20 mg/kg	Group 4 45 mg/kg
Number of foetuses [litters] examined	196 (24)	209 (24)	180 (23)	171 (23)
Number heads examined at detailed visceral	97 (23)	102 (24)	89 (23)	85 (23)
Number of foetuses affected [litter]	19 (11)	11 (7)	10 (7)	22 (9)
Mean % litter affected	45.8	29.2	30.4	39.1

Conclusions

It is concluded that the no-observed-adverse-effect-level (NOAEL) of Captan for prenatal development is 20 mg/kg/day, based on evidence of a slight developmental delay and increased fetal and litter incidence in major abnormalities at 45 mg/kg/day.
A statistically significant increase in early resorptions and post-implantation loss was observed at 45 mg/kg bw/day. For the pregnant females absolute bodyweight was unaffected at 10 or 20 mg/kg/day and at 10 mg/kg/day bodyweight gain was only significantly affected from 11 days after start of treatment. This was subsequent to a fall in food intake although a recovery was noted from Day 21 despite continued treatment. The maternal NOAEL would therefore appear to be less than 10 mg/kg/day.

3.10.2 Human data

No relevant studies

3.10.3 Other data (e.g. studies on mechanism of action)

3.10.3.1 Position paper (2018)

Reference:	Captan: A Review of the Potential to Induce Developmental Toxicity
Author(s), year:	Anonymous (2018)
Report/Doc. number:	Position paper, Project No. 000101576
Guideline(s):	Not applicable
GLP:	Not applicable
Deviations:	Not applicable
Acceptability:	Yes

Introduction

This review has been undertaken to thoroughly investigate the potential of Captan to induce developmental effects in mammalian species with respect to potential classification.
Captan´s fungicidal mode of action is chemical reactivity towards the fungal organism/spores. This mode of action also drives the mammalian toxicity profile (local irritative effects at point of first contact). It is important to note that the reactivity of Captan is driven by the reactive group, trichloromethylsulfanyl-moeity, and that the reactivity is immediate and not prolonged (half-life in blood is 0.97 seconds). It is thus highly unlikely that the active substance would reach systemic circulation at all via field exposure scenarios (dermal, inhalation).

Rat studies with captan

Captan did not adversely affect the development of rat offspring in two reproduction studies.
In a prenatal developmental toxicity study, Captan did not induce fetal malformation in the rat at the highest dose level administered, 450 mg/kg bw day, despite the marked maternal toxicity resulting in reduced fetal body weight and an increase in a small number of skeletal variations. The developmental NOAEL was higher than the maternal NOAEL.

Reference to the evaluation of the developmental toxicity of Captan in the rat is also found in the published literature and is summarised in Appendix 3 of this position paper. There is no indication from the published literature that Captan induces fetal malformation in the rat.

Rabbit studies with captan

Four studies of the prenatal developmental toxicity of Captan in the rabbit have been conducted. With respect to maternal toxicity, the studies (Study 5 1987b & Study 4 1991), where treatment was for the period of major organogenesis, the NOAEL was 10 mg/kg bw/day. The studies of Study 7 (2006a) & Study 6 (1981) where the period of treatment was longer, the maternal NOAEL was < 10 mg/kg bw/day. The latter studies also indicated that the NOAEL for developmental effects was 20/25 mg/kg bw/day. Therefore, Captan induced effects on the fetus, only in the presence of maternal toxicity which was marked in the rabbit at 45 mg/kg bw/day (Study 7, 2006a).

Table 3.10.3.1-1 Summary of no observed adverse effect levels (NOAELs) in rabbit developmental studies

Reference	Dose levels of Captan (mg/kg bw/day)	Treatment period (gestation days)	Maternal NOAEL (mg/kg bw/day)	Developmental NOAEL (mg/kg bw/day)
Study 7, 2006a	10, 20, 45	6-28	<10	20
Study 6, 1981	6, 12, 25, 60	6-28	6	25
Study 4, 1991	10, 30, 100	7-19	10	10
Study 5, 1987b	10, 40, 160	7-19	10	40

The developmental effects were generally similar across the studies with an increase in post-implantation loss and a decrease in fetal weight being secondary to the maternal toxicity induced. Also, a reduction in ossification for several areas of the skeleton was considered secondary to the reduction in fetal body weight. An increased incidence of extra ribs sometimes associated with an additional vertebrae and misalignment of the pelvic girdle was also observed; this is a common finding in rabbit developmental toxicity studies considered not to be adverse.
Fetal malformations were seen in all studies and were sometimes of highest incidence at the highest doses of Captan tested. Nevertheless, the malformations were varied and not consistent in type to indicate any association with Captan. The malformations observed were considered to be spontaneous in origin and part of the spectrum of change occurring in the NZW rabbit.
Reference to the evaluation of the developmental toxicity of Captan in the rabbit is also found in the published literature and is summarised in Appendix 3 of this position paper. There is no indication from the published literature that Captan induces fetal malformation in the rabbit.

Rabbit – evaluation of four comparable studies with structurally similar chemicals

In reviewing the potential of Captan to induce developmental effects in the rabbit, it is relevant to consider not only the metabolite tetrahydrophthalimide (THPI), but also the structurally similar chemical folpet and its metabolite phthalimide. In 2005, four rabbit prenatal developmental toxicity studies were commissioned in the same facility, using the New Zealand White rabbit from an accredited closed colony (Charles River Laboratories, Domaine de Oncins, BP 0109, F69592 L'Arbresle, France) and conducted to the same study design (essentially OECD TG 414, 2001). All studies were reported by the same Study Director. This series of studies provides a directly comparable set of data without the inherent variation between different laboratories and methodologies, different rabbit colonies and year of study conduct etc.

Table 3.10.3.1-2 Summary of the comparable rabbit developmental toxicity studies

Reference	Study 7 (2006a)	Study 8 (2006b)	Study 11 (Folpet CLH) (2006c)	Study 15 (Folpet CLH) (Study (2006d)
Test substance	Captan	THPI	Folpet	Phthalimide
Study dates 'in life'	February/April 2005	May/July 2005	March/April 2005	May/June 2005
Dose levels [a]	10, 20, 45 mg/kg/day	5, 10, 22.5 mg/kg/day	10, 30, 60 mg/kg/day	5, 15, 30 mg/kg/day
Maternal NOAEL	< 10 mg/kg/day	22.5 mg/kg/day	< 10 mg/kg/day	30 mg/kg/day
Developmental NOAEL	20 mg/kg/day	22.5mg/kg/day	10 mg/kg/day	30 mg/kg/day

[a] The dose levels of the metabolites were selected on the basis of molar equivalence with the dose levels of the parent chemical.

The particular strength of these four studies relates to the understanding of fetal malformation in the rabbit. Rabbit colonies have varying types and incidences of spontaneous malformation which occur more frequently in comparison with the rat. This makes interpretation of study data more difficult. To aid interpretation, it is necessary not only to refer to historical control data but more importantly, it is necessary to look for signs of commonality and reproducibility in the types of malformation observed in order to distinguish between those that are of spontaneous aetiology and those that are chemically induced.

The type and incidence of malformation observed in the four studies (Table 3.10.3.1-3) clearly demonstrates the spectrum of spontaneously occurring abnormality. There are no consistent findings to indicate chemically-induced malformation despite the sometimes higher incidence in the higher dose groups. The complexity of some of the multiple malformations also serves to illustrate the limitations of historical control data which frequently do not include a similar finding.

The results of the four comparable studies, clearly demonstrate that neither Captan and its metabolite THPI nor the structurally similar chemical folpet and its metabolite phthalimide, are developmental toxicants in the rabbit.

Table 3.10.3.1-3 Summary of fetal malformations from the rabbit developmental toxicity studies of Captan and THPI (fetal incidence)

Malformation	Dose level of Captan (mg/kg bw/day)				Dose level of THPI (mg/kg bw/day)			
	0	10	20	45	0	5	10	22.5
Number of fetuses with malformations	1/197	2/211	2/182	9/180	0/149	1/224	4/191	0/215
Number of litters with malformations	1/24	1/24	2/23	5/23	0/18	1/24	3/21	0/24
Acrania: multiple cervical, thoracic and lumbar vertebral/rib abnormalities: distorted/misshapen ribcage: thoracic kyphosis: lumbar lordosis/scoliosis: spina bifida and interruption of vertebral column: brachyury: amelia: markedly misshapen scapula, long bones and clavicle: unfused tibia and fibula: transposition of dilated pulmonary trunk and narrow ascending aorta: incomplete aortic arch: muscular ventricular septal defect: gastroschisis				1/180				
Fused frontal/parietal	1/197							
Hydrocephaly: domed cranium				1/180				
Hydrocephaly with confluent lateral ventricles: medially displaced nares: absent bilateral upper major incisors: thin nasal septum: shortened muzzle		1/211						
Fused upper major incisors		1/211						
Dilated ascending aorta/aortic arch, narrow pulmonary trunk, marked: caudally displaced ductus arteriosus: muscular ventricular septal defect			1/182					
Umbilical hernia				2/180				
Absent kidney and ureter			1/182	4/180			1/191	
Lumbar scoliosis				1/180			1/191	
Absent paraflocculus: lumbar to caudal spina bifida: fused 2nd to 3rd lumbar vertebral centra resulting in kyphosis: malrotated hindlimb						1/224		
Absent paraflocculus: lumbar to caudal spina bifida occulta: brachyury: gastroschisis: imperforate anus: malrotated and hyperflexion left hindlimb							1/191	
Encephalocoele: disorganised/misshapen cranium							1/191	

Table 3.10.3.1-4 Summary of fetal malformations from the rabbit developmental toxicity studies of Folpet and Phthalimide (fetal incidence)

Malformation	Dose level of Folpet (mg/kg bw/day)				Dose level of Phthalimide (mg/kg bw/day)			
	0	10	30	60	0	5	15	30
Number of fetuses with malformations	3/227	3/177	1/184	11/191	1/199	2/178	2/192	3/187
Number of litters with malformations	2/25	1/22	1/22	5/22	1/21	2/20	2/22	3/25
Partially fused nasals				1/191				
Fused nasals to frontals: misshapen/disorganised nasals/frontals: absent nasofrontal suture: large ossified area extending from frontal/frontal suture to nasal region: misshapen zygomatic arch: short/thickened cornua of hyoid: cleft palate				1/191				
Fused olfactory lobes: confluent lateral ventricles	1/227							
Small/misshapen/oval lens with lenticular irregularities/opaque areas				8/191				
Narrow ascending aorta: muscular ventricular septal defect			1/184					
Multiple thoracic vertebral irregularities		2/177						
Absent kidney and ureter	2/227	1/177						1/187
Lumbar/sacral scoliosis, Lumbar scoliosis		2/177					1/192	
Sacral/caudal spina bifida, Lumbar/sacral spina bifida				1/191			1/192	
Single central naris: absent upper incisors: single nasal cavity: narrow nasal septum, misshapen nasal turbinates and conchae: absent olfactory lobes, fused frontal lobes: hydrocephaly								1/187
Distorted ribcage: termination of vertebral column lumbar region: lumbar spina bifida and scoliosis: abnormal orientation of pelvis: fused/misshapen pubis and ischium: unilateral amelia: one central rudimentary hindpaw consisting of calcaneum, metatarsal, phalanx, 2nd phalanx & claw: narrow pulmonary trunk, marked: gastroschisis: absent kidney, ureter, adrenal & ovary: small and misshapen kidney: fleshy tail								1/187
Bent humerus, scapula: dilated ascending aorta/aortic arch: diaphragmatic hernia						1/178		
Umbilical hernia					1/199			
Hindlimb syndactyly: hindpaw brachydactyly: hindpaw hyperextension						1/178		

Other mammalian species

Published studies in the literature have been reviewed and the detail is included in Appendix 3 of this position paper.
No potential of Captan to induce developmental effects in the mouse was indicated by the oral, inhalation and subcutaneous routes of exposure.
No evidence of teratogenicity due to Captan in two species or primate was indicated in a study with some limitations.
A study of Captan in hamsters concluded that teratogenicity could not be ascribed to any of the chemicals tested. The study had major limitations and was considered not to provide any reliable data to conclude on the effects in the hamster.
The published literature included a number of studies which evaluated the metabolite of Captan, THPI, and the structurally related chemical folpet and its metabolite, phthalimide. There was no evidence from any of the studies to indicate that Captan, THPI, folpet and phthalimide were developmental toxicants in rats or rabbits.

Conclusion

This review has been undertaken to thoroughly investigate the potential of Captan to induce developmental effects in mammalian species with respect to potential classification. The results are summarised as follows:

- The half-life of Captan in human blood at 37°C is 0.97 seconds. This very short half-life indicates that in humans and mammalian prenatal developmental toxicity studies, the developing offspring in utero will not be exposed to Captan. The principal metabolite, tetrahydrophthalimide (THPI) may reach the developing offspring following maternal administration of Captan.
- A series of directly comparable rabbit developmental toxicity studies, conducted in 2005, provide clear evidence that neither Captan and its metabolite THPI, nor the structurally related chemical folpet and its metabolite, phthalimide induce malformation in the rabbit.
- Review of other rabbit developmental toxicity studies, including those in the published literature, confirm that neither Captan nor THPI induce malformation in the rabbit.
- Review of rat developmental and reproduction toxicity studies, including those in the published literature, confirm that neither Captan nor THPI induce malformation in the rat.
- Review of the data available in the published literature confirm that neither Captan nor THPI induce malformation in the mouse or primate.
- Review of developmental toxicity studies including those in the published literature confirm that the structurally similar chemical folpet, and its metabolite phthalimde, do not induce malformation in several species including the rat and rabbit.

On the basis of the available data it is evident that Captan is not a developmental toxicant in several mammalian species including the rat and rabbit. Classification of Captan for reproductive toxicity is not warranted.

3.10.3.2 Study 8 (2006)

Reference:	Tetrahydrophthalimide prenatal toxicity study in the rabbit by oral gavage administration
Author(s), year:	Anonymous (2006b)
Report/Doc. number:	Huntingdon Life Sciences Ltd.,UK, No: MAK864/053232 R-18202
Guideline(s):	OECD 414 (1981)
GLP:	Yes
Deviations:	No
Acceptability:	Yes

Summary

The influence of Tetrahydrophthalimide, on embryo-fetal survival and development in New Zealand White rabbits was assessed following oral administration during the organogenesis and fetal phases of gestation. Three groups of 25 female rabbits received Tetrahydrophthalimide by gavage at dosages of 5, 10 or 22.5 mg/kg/day from Days 6 to 28 after mating. A similarly constituted Control group received the vehicle, 0.5% w/v Tween 80 with 0.7% w/v carboxymethylcellulose prepared in water for formulation, at the same volume-dosage throughout the same period. Animals were killed on Day 29 after mating for reproductive assessment and fetal examination.

Clinical signs, bodyweight and food consumption were monitored. Adult females were examined macroscopically at necropsy on Day 29 after mating, samples of duodenum were processed and subsequently examined microscopically. All fetuses were examined macroscopically at necropsy and subsequently by detailed internal visceral examination of the head or skeletal examination.

There were no treatment-related deaths and no signs were attributed to treatment. Bodyweight, gravid uterine weight and food consumption were considered not to be unaffected by treatment.

Macroscopic examination did not reveal any treatment-related observations and microscopic examination of sections of the duodenum from animals in the Control and 22.5 mg/kg/day groups did not reveal any treatment-related findings.

Treatment did not adversely affect pregnancy outcome.

Litter parameters as assessed by the numbers of corpora lutea, implantations, resorptions, live young, sex ratio and the extent of pre- and post-implantation loss were considered unaffected.

Fetal and placental weights were considered to be unaffected by treatment with Tetrahydrophthalimide.

Fetal pathology examinations did not reveal any findings that were considered to be treatment-related.

Based on the results of this study it is concluded that the no-observed-adverse-effect-level (NOAEL) for both maternal toxicity and prenatal toxicity is at least 22.5 mg/kg/day Tetrahydrophthalimide.

MATERIAL AND METHODS

MATERIALS

1. Test Material:	Tetrahydrophthalimide
Lot/Batch #:	S17363
Purity:	98.4%
Stability:	Expiry date 31.03.2009
Storage conditions	At room temperature, protected from light
2. Control Materials:	
Vehicle	0.5% w/v Tween 80 with 0.7% w/v carboxy-methylcellulose prepared in water for formulation
3. Test System	
Species:	Rabbit
Strain	New Zealand White rabbits
Number/Sex	25 females per group
Age	18 to 26 weeks
Weight	2.78 – 5.17 kg
Supplier	Charles River Laboratories, F69592 L'Arbresle, France
Acclimatisation period	2 weeks
Diet	Standard rabbit breeding diet, SAFE (Scientific Animal Food and Engineering) 110-10 from SAFE, Route de Saint Bris, 89 290 Augy, France
4. Test Conditions	
Housing	Individually in LCP plastic cages from Labcare Precision Instruments Ltd., Kent, England
Temperature	16 - 20 °C
Relative humidity	40 – 70%
Photoperiod	14/10 hours light/dark cycle

In-life dates: Start 11 May 2005, End 01 June 2005

Experimental Procedure

Three groups of 25 female rabbits received Tetrahydrophthalimide by gavage at dosages of 5, 10 or 22.5 mg/kg/day from Days 6 to 28 after mating. A similarly constituted Control group received the vehicle, 0.5% w/v Tween 80 with 0.7% w/v carboxymethylcellulose prepared in water for formulation, at the same volume-dosage throughout the same period. Animals were killed on Day 29 after mating for reproductive assessment and fetal examination.

Table 3.10.3.2-1: Study design and dose levels

Group Number / Designation	Dose level (mg/kg bw/day)
1 / Control	0
2 / Low Dose	5
3 / Mid Dose	10
4 / High Dose	22.5

The homogeneity and stability was confirmed for Tetrahydrophthalimide in aqueous 0.5% w/v Tween 80 with 0.7% w/v carboxymethylcellulose formulations.

Clinical observations: Animals were inspected visually at least twice daily for evidence of ill-health or reaction to treatment. Cages and cage-trays were inspected daily for evidence of ill-health amongst the occupant(s). Any deviation from normal was recorded at the time in respect of nature and severity, date and time of onset, duration and progress of the observed condition, as appropriate.

In addition, detailed observations were recorded daily throughout the treatment period, at the following times in relation to dose administration:

- Immediately before dosing;
- Immediately after dosing on return of the animal to its cage;
- On completion of dosing of each group;
- Between one and two hours after completion of dosing of all groups or, when dosing was protracted;
- As late as possible in the working day.

Mortality and abortion: Debilitated animals were observed carefully and animals judged ***in extremis*** were killed to prevent unnecessary or prolonged suffering. A complete necropsy was performed as described in the Necropsy section.

Bodyweight: The weight of each rabbit was recorded weekly during acclimatisation, on the day of mating (Day 0), and thereafter daily until necropsy.

Food consumption: The weight of food supplied to each animal, that remaining and an estimate of any spilled was recorded on a daily basis from Day 1 after mating.

Pathology: Animals surviving until the end of the scheduled study were killed on Day 29 after mating, by overdose of sodium pentobarbitone by intravenous injection. Fetuses were killed by subcutaneous injection of sodium pentobarbitone. The sequence in which the animals were killed after completion of the study was chosen to allow inter-group comparison unbiased by chronological sequence of sacrifice.

Macroscopic pathology: All animals were subject to a detailed necropsy.

Fetal examination and processing: All fetuses and placentae were dissected from the uterus and weighed individually. Fetuses were individually identified within the litter, using a coding system based on their position in the uterus. Each fetus and placenta was externally examined and any abnormalities were recorded.

The neck and thoracic and abdominal cavities of all fetuses from each litter were dissected, the contents examined for visceral abnormalities and sex recorded. Following examination, one half of the fetuses in each litter were decapitated and their heads initially stored in Bouin's fluid. The remaining intact fetuses and torsos were eviscerated and fixed in industrial methylated spirits prior to processing and staining with Alizarin Red.

Histology: For those animals in Groups 1 and 4 the duodenum including the sphincter of Oddi was subject to histological processing

Statistics: Statistical analyses were applied where there was indication of possible meaningful intergroup differences. All statistical analyses were carried out using the individual animal (or litter) as the basic experimental unit.

II. RESULTS AND DISCUSSION

Mortality

One Control female (Group 1 Female Number 7) was found dead on Day 12 of gestation. The animal had maloccluded upper incisors from Day 8 of gestation and a broken upper incisor on Day 12 of gestation. Food consumption appeared unaffected by the maloccluded teeth as was bodyweight. The cause of death could not be established.

One female (Group 3 Female Number 70) treated at 10 mg/kg/day of Tetrahydrophthalimide was killed *in extremis* on Day 14 of gestation. On Day 14 of gestation it was underactive, thin, had reduced temperature of the pinnae and had eaten little hay and had low water intake. The animal also had few faeces which were reduced in size. Bodyweight loss of 0.14

kg had been recorded from Day 12 along with low food intake. Necropsy revealed a distended stomach with clear gelatinous substance on the wall lining, clear viscous fluid in the duodenum, green viscous fluid in the jejunum and green/brown gelatinous contents in the colon. The animal was pregnant with twelve implantations and an early resorption. Histopathology did not reveal any changes in the duodenum or sphincter of Oddi. This decline in condition leading to the animal being killed in extremis was not attributed to treatment due to the isolated nature of this death.

Six Control females, one female receiving 5 mg/kg/day, three females receiving 10 mg/kg/day and one female receiving 22.5 mg/kg/day of Tetrahydrophthalimide were found to be not pregnant at macroscopic examination on Day 29 after mating. These incidences were unrelated to treatment.

Clinical observations

There were no clinical signs observed that were considered to be related to treatment with Tetrahydrophthalimide.

An isolated observation recorded as a sign associated with dosing was fast respiration on Day 13 of gestation for one animal (Group 3 Female Number 70) treated at 10 mg/kg/day and killed *in extremis* on Day 14 of gestation. The decline in condition of this animal was not attributed to treatment.

Body weight

At 22.5 mg/kg/day mean bodyweight was the same as controls but mean bodyweight gain during Days 6-11 of gestation was fractionally lower compared to the control group. This difference reflected minor bodyweight loss was recorded for 11/24 females during this period compared with 1/18 controls. However, the magnitude of the difference from controls (mean weight gain of 0.01 kg versus 0.05 kg in controls) was very small, and in view of this and the natural variation in rabbit bodyweight performance, was concluded to be of no toxicological importance. Overall mean bodyweight gain during Days 11-29 of gestation was similar to controls.

There was no effect of treatment at 10 or 5 mg/kg/day on bodyweight gain.

Food consumption

Food consumption was considered to be unaffected by treatment.

Macropathological findings

The group mean weight of the gravid uterus and the mean adjusted body weight change unaffected by treatment.

Macroscopic examination did not reveal any treatment-related observations

Histopathology

There were no treatment-related findings seen in the duodenum or at the sphincter of Oddi of rabbits given Tetrahydrophthalimide at 22.5 mg/kg/day.

LITTER RESPONSES

The following assessment is based on the 18, 24, 21 and 24 females with live young on Day 29 of gestation in Groups 1-4 respectively. Six Control females, one female receiving 5 mg/kg/day of Tetrahydrophthalimide, three females receiving 10 mg/kg/day and one female receiving 22.5 mg/kg/day were found to be not pregnant at macroscopic examination on Day 29 after mating.

Litter data

Litter parameters as assessed by the numbers of corpora lutea, implantations, resorptions, live young and the extent of pre- and post-implantation losses were considered unaffected by treatment.

The mean number of live females in the groups treated with Tetrahydrophthalimide appeared slightly high. This was due to the number of live females in the control group being slightly lower than expected when compared with contemporary studies. The low number of live females in the control group resulted in an associated slightly higher than expected sex ratio value in this group, as the values are presented as the percentage of males in the litters. Sex ratio was, therefore, considered to be unaffected by treatment.

Placental, litter and fetal weights

Placental and fetal weight were considered to be unaffected by treatment with Tetrahydrophthalimide.

Histopathology

The incidence of major and minor abnormalities and skeletal variants showed no relationship to treatment.

III. CONCLUSION

In this study the influence of Tetrahydrophthalimide on prenatal development was assessed in New Zealand White rabbits. Three groups of female rabbits received Tetrahydrophthalimide by oral gavage administration from Days 6 to 28 after mating, covering the organogenesis and fetal phases of gestation. A similarly constituted control group received the vehicle at the same volume dosage throughout the same period. Animals were killed on Day 29 after mating for reproductive assessment and fetal examination.

There were no treatment-related deaths or any other indicators of maternal toxicity at the dosages administered on this study, up to 22.5 mg/kg/day Tetrahydrophthalimide.

Prenatal development was also considered to be unaffected by treatment with Tetrahydrophthalimide at dosages up to 22.5 mg/kg/day.

3.10.3.3 Study 13 (2012a)

Reference:	Captan: Estrogen Receptor Binding Assay Using Rat Uterine Cytosol
Author(s), year:	Anonymous (2012a)
Report/Doc. number:	Document No. R-28284
Guideline(s):	OPPTS 890.1250 (2009)
GLP:	Yes, with the exception that dose concentrations of test substance and control substances were not verified using analytical methods.
Deviations to OECD 493 (2015):	No major deviations. The guidelines are different in the setting of performance criteria (for the reference substances estrogen and norethynodrel), especially regarding the parameter for (Hill) slope ($log_{10}(M)^{-1}$).
Acceptability:	Yes

Summary

An estrogen receptor binding assay was conducted to evaluate the ability of captan to interact with the estrogen receptors (ERs) isolated from rat uteri. Three independent experiment were conducted with captan at concentrations of 10^{-11} to 10^{-4} along with the positive control 17β-estradiol, negative control octyltriethoxysilane and weak positive control 19-norethindrone.
Controls gave the expected results.
Captan was classified as a “non-interacting” in all three valid independent runs and thus has a final classification of “non-interacting”.

Material and Methods

1. Test Material: Captan (Merpan technical)
   - Description: White powder
   - Lot/Batch #: 91138103
   - Purity: 93%
   - Stability: Expiry date 14 February 2013
   - Storage conditions: At room temperature
   - Solvent used: Dimethylsulfoxide (DMSO)

2. Control Material:
   - Solvent control: DMSO at 4% (Vehicle)
   - Negative control: Octyltriethoxysilane (Sigma, 99.34% purity, Lot No. 24996KK)

Positive control: 19-norethindrone (Sigma, 99 % purity, Lot No 030M1359V)

3. Radioligand: [^{3}H]-17β-estradiol
Specific Activity: 130.2 Ci/mmol
Adjusted specific activity 126.6 Ci/mmol

4. Rat Uterine Cytosol
Supplier: Harlan Laboratories
Batch No: 210007463
Rat Strain: Sprague-Dawley
Age: 12-13 weeks
Days after ovariectomy: 7 days
Shipment: Dry ice- overnight
Protein concentration: 1.10 mg/mL
Buffer: TEDG buffer +PI buffer
Centrifugation: 105,000 × g

5. Assay Conditions
Buffer: TEDG Buffer (10 mM Tris, 1.5 mM EDTA, 0.5% protease inhibitor, 10% glycerol and 1 mM DTT [added immediately before use], pH 7.4)
Concentration of radioligand: 1 nM
Concentration of receptor: Sufficient to bind 10-15% of radioligand
Temperature: 4 ± 2°C
Incubation time: 16-20 hours

6.Test concentrations:
Captan: $1 \times 10^{-3} – 1 \times 10^{-10}$ M
Octyltriethoxysilane: $1 \times 10^{-3} – 1 \times 10^{-10}$ M
19-norethindrone: $1 \times 10^{-4} – 3.16 \times 10^{-9}$ M
17β-estradiol: $1 \times 10^{-7} – 1 \times 10^{-11}$ M

TEST PERFORMANCE:
03.11.2011 – 03.12.2011at CeeTox Inc., Kalamazoo, MI, USA

1. Experimental Procedure
12 x 75 mm siliconized tubes were used for the assay. A master mixture of radioligand and buffer (390 µL) was added to all tubes, followed by standards, weak positive, negative or test chemicals (10 µL). After the addition of the chemicals, 100 µL of cytosol was added to each tube for a final volume of 500 µl, vortexed and incubated in a rotator at 4±2°C for 16 to 20 hours.
Following incubation 250 µL of ice cold Hydroxyapatite slurry (HAP, 60% in TEDG+PI) was added to each tube.
The tubes were vortexed for approximately 10 seconds at approximately 5 minute intervals for a total of approximately 15 minutes with tubes remaining in the ice-water bath between vortexing. Two mL of cold [^{3}H]-17β-estradiol was added followed by vortexing and centrifugation. The supernatant containing the free [^{3}H]- 17β-estradiol was decanted. The HAP pellet contained the ER bound [^{3}H]- 17β-estradiol was washed (resuspended, vortexed and centrifuged) two more times with 2 mL TEDG+PI buffer.
Bound [^{3}H]- 17β-estradiol was extracted with 2 mL of absolute ethanol by repeated vortexing at room temperature followed by centrifugation and radioactivity was determined by scintillation counting.

2. Evaluation Criteria
Increasing concentrations of unlabelled 17β-estradiol displaced [^{3}H]-17β-estradiol from the receptor in a manner consistent with one-site competitive binding.
The ligand depletion was held below 15%.
The parameter values (top, bottom, and slope) for 17β-estradiol and the concurrent positive control (19-norethindrone) were within the tolerance bounds (with minor deviations).
The solvent control substance did not alter the sensitivity or reliability of the assay.
The negative control substance (octyltriethoxysilane) did not displace more than 25% of the radioligand from the ER on average across all concentrations.

Findings

The suitable top concentration of captan for use in the binding assays was 10^{-4} M.

Table 3.10.3.3-1 Estrogen Receptor (ER) Binding

	9. 1st Experiment	10. 2nd Experiment	11. 3rd Experiment	12. Relative binding affinity (RBA)
Captan (mean specific binding)	> 75%	> 75%	> 75%	- (non-interacting)
17β-estradiol ($LogIC_{50}$)	-9.1 M	-9.0 M	-8.9 M	1
19-norethindrone ($LogIC_{50}$)	-5.5 M	-5.5 M	-5.4 M	0.6

In the first valid independent run, the specific binding was > 75% at every concentration tested for captan, classifying it as "non-interacting" for this run. The weak positive control 19-norethindrone had a $LogIC_{50}$ of -5.5 M while the $LogIC_{50}$ of 17β-estradiol was -9.1 M.

In the second valid independent run, the mean specific binding was > 75% at every concentration tested for captan, classifying it as "non-interacting" for this run. The weak positive control 19-norethindrone had a $LogIC_{50}$ of -5.5 M while the $LogIC_{50}$ of 17β-estradiol was -9.0 M.

Finally, in the third valid independent run, the mean specific binding was > 75% at every concentration tested for captan, classifying it as "non-interacting" for this run. The weak positive control 19-norethindrone had a $LogIC_{50}$ of -5.4 M while the $LogIC_{50}$ of 17β-estradiol was -8.9 M.

The mean relative binding affinity (RBA; calculated by dividing the $LogIC_{50}$ of the control/test material by the $LogIC_{50}$ of the positive control 17β-estradiol) was 0.6 for 19-norethindrone.

As captan was classified as a "non-binder" for all three runs, the RBA could not be calculated.

Conclusions

Captan was classified as "non-interacting" in all three valid independent runs and thus has a final classification of "non-interacting" for the estrogen receptor.

3.10.3.4 Study 14 (2012b)

Reference:	Captan: Androgen Receptor Binding Assay Using Rat Prostate Cytosol
Author(s), year:	Anonymous (2012b)
Report/Doc. number:	Document No. R-28282
Guideline(s):	OPPTS 890.1150 (2009)
GLP:	Yes, with the exception that dose concentrations of test substance and control substances were not verified using analytical methods.
Deviations to OPPTS 890.1150 (2011):	None
Acceptability:	Yes

Summary

In an androgen receptor binding assay using rat prostate cytosol the ability of captan to interact with the androgen receptors (ARs) was evaluated. Three independent experiment were conducted with captan at concentrations of 10^{-10} to

10^{-3} (first valid independent run) or 10^{-11} to 10^{-4} in (second and third valid independent runs) competing with [³H]-R1881 at the androgen receptor.
Positive controls, R1881 and dexamethasone, gave the expected results.
Captan was classified as a "non-binder" in all three valid independent runs and thus has a final classification of "non-binder."

Material and Methods

1. Test Material: Captan (Merpan technical)
   - Description: White powder
   - Lot/Batch #: 91138103
   - Purity: 93%
   - Stability: Expiry date 14 February 2013
   - Storage conditions: At room temperature
   - Solvent used: Dimethylsulfoxid (DMSO)

2. Control Materials:
   - Negative (solvent): DMSO at 3.2%
   - Positive: R-1881 (Lot No 060M4638)
   - Weak positive: Dexamethasone (Sigma, 99% purity, Lot No 077K1050)

3. Radioligand: [³H]-R1881
   - Source: Perkin Elmer (Boston, MA, USA)
   - Lot No.: 653698
   - Specific Activity: 85.1 Ci/mmol (24 Feb 2011)
   - Adjusted specific activity: 82.0 /81.9 Ci/mmol

4. Rat Prostate Cytosol
   - Supplier: Charles River Laboratories
   - Batch No: 210007463
   - Rat Strain: Sprague-Dawley
   - Age: 90 days
   - Days after castration: < 1
   - Shipment: Overnight on dry ice
   - Protein concentration: 8.8 mg/mL
   - Buffer: TEDG buffer
   - Centrifugation: 30000 g (30 min)

Assay Conditions
- Buffer: TEDG Buffer (10 mM Tris (pH 7.4), 1 mM sodium molybdate, 1.5 mM EDTA, 10% Glycerol and 1 mM DTT [added immediately before use], pH 7.4 [cooled to 4 ± 2°C before adjusting], stored at 4 ± 2°C up to 3 months)
- Concentration of radioligand: 1 nM
- Concentration of receptor: Sufficient to bind 10-15% of radioligand
- Temperature: 4 ± 2°C
- Incubation time 16 - 20 hours

Test concentrations:
- Captan: $1 \times 10^{-3} – 1 \times 10^{-10}$ M
- R1881: $1 \times 10^{-6} – 1 \times 10^{-11}$ M
- Dexamethasone: $1 \times 10^{-3} – 1 \times 10^{-10}$ M

TEST PERFORMANCE:
24.10. – 01.11.2011 at CeeTox Inc., Kalamazoo, MI, USA

1. Experimental Procedure
12 x 75 mm siliconized tubes were used for the assay. 30 µL of 10 nM [^{3}H]-R1881 (1 x 10^{-8} M) and 50 µl triamcinolone acetonide (60 µM working solution) were added to all tubes. For the 3 tubes at the beginning of assay and at the end of

assay, 100X inert R1881 (30 µL of 1 µM) was also added. These were the nonspecific binding tubes. The tubes were placed in a speed-vac and dried. An aliquot of cytosol was thawed on ice and diluted to the predetermined optimal protein concentration.

For the assay tubes, 10 µL of each concentration of test chemical or control was added, followed by 300 µL of the diluted cytosol. The assay tubes were vortexed after additions and incubated at 4±2°C for 16 to 20 hours on a rotator. 100 µL of each incubation tube was transferred to the appropriate labelled tubes containing 500 µL of ice cold Hydroxyapatite slurry (HAP, 60% in 50 mM Tris buffer). The tubes were vortexed for approximately 10 seconds at approximately 5 minute intervals for a total of approximately 20 minutes with tubes remaining in the ice-water bath between vortexing.

Following the vortexing step, approximately 2 mL of the cold 50 mM Tris buffer was added, quickly vortexed, and centrifuged at 4±2°C for approximately 10 minutes at 700 x g. After centrifugation, the supernatant containing the free [^{3}H]-R1881 was immediately decanted and discarded. The HAP pellet contained the AR bound [^{3}H]-R1881. Approximately 2 mL of ice cold 50 mM Tris buffer was added to each tube and vortexed to resuspend the pellet. The tubes were centrifuged again at 4±2°C for approximately 10 minutes at approximately 700 x g. The supernatant was quickly decanted and discarded. The wash and centrifugation steps were repeated three more times. After the final wash, the supernatant was decanted. The assay tubes were allowed to drain briefly for approximately 30 seconds.

Bound [^{3}H]-R1881 was extracted with 2 mL of absolute ethanol by repeated vortexing at room temperature followed by centrifugation and radioactivity was determined by scintillation counting.

2. Evaluation Criteria

Increasing concentrations of unlabelled R1881 displaced [^{3}H]-R1881 from the receptor in a manner consistent with one-site competitive binding.
The ratio of total binding in the absence of competitor to the total amount of [^{3}H]-R1881 added per assay tube was no greater than 15%.
The parameter values (top, bottom, and slope) for R1881 and the concurrent positive control (dexamethasone) were within the tolerance bounds.
The solvent control substance did not alter the sensitivity or reliability of the assay. Specifically, the acceptable limit of DMSO concentration was ≤ 10%.

Findings

The suitable top concentration of captan for use in the binding assays was 10^{-4} M. There was precipitation observed at 10^{-3} M in the first valid independent run, so the concentration range was shifted for the second and third valid independent runs
All three runs resulted in very similar results indicating the assay was functioning properly.

Table 3.10.3.4-1 Androgen Receptor (AR) Binding

	13. 1st Experiment	**14. 2nd Experiment**	**15. 3rd Experiment**	**16. Relative binding affinity (RBA)**
Captan (mean specific binding)	>75%	>75%	>75%	- (non-binder)
R1881 ($LogIC_{50}$)	-9.1 M	-9.0 M	-9.0 M	1
Dexamethasone ($LogIC_{50}$)	-4.7 M	-4.6 M	-4.6 M	0.5

In the first valid independent run, the mean specific binding was > 75% at every soluble concentration tested for captan classifying it as a "non-binder" for this run. The mean specific binding was 11.4% at 10^{-3} M, however precipitation of captan was observed at this concentration so the data was not assessed. The weak positive control dexamethasone had a $LogIC_{50}$ of -4.7 M while the $LogIC_{50}$ of R1881 was -9.1 M.

In the second valid independent run, the mean specific binding was > 75% at every soluble concentration tested for captan classifying it as a "non-binder" for this run. The weak positive control dexamethasone had a $LogIC_{50}$ of -4.6 M while the $LogIC_{50}$ of R1881 was -9.0 M.

Finally, in the third valid independent run, the mean specific binding was > 75% at every soluble concentration tested for captan, classifying it as a "non-interacting" for this run. The weak positive control dexamethasone had a $LogIC_{50}$ of -4.6 M while the $LogIC_{50}$ of R1881 was -9.0 M.

The mean relative binding affinity, or RBA (Relative binding affinity; calculated by dividing the $LogIC_{50}$ of the control/test material by the $LogIC_{50}$ of the positive control R1881) was 0.5 for dexamethasone.

As captan was classified as a "non-binder" for all three runs, the RBA could not be calculated.

Conclusions

Captan was classified as a "non-binder" in all three valid independent runs and thus has a final classification of "non-binder."

3.10.3.5 Study 15 (2012c)

Reference:	Captan: Estrogen Receptor Transcriptional Activation (Human Cell Line (HeLa-9903))
Author(s), year:	Anonymous (2012c)
Report/Doc. number:	Document No. R-28285
Guideline(s):	OPPTS 890.1300 (2009), OECD 455 (2009)
GLP:	Yes
Deviations to OECD GD 455 (2015):	None
Acceptability:	Yes

Summary

An estrogen receptor transcriptional activation assay was performed to evaluate the ability of captan to act as an agonist of human estrogen receptor alpha (hERα) using the hERα-HeLa-9903 cell line. Two independent runs of the transcriptional activation assay were conducted with captan at concentrations ranging from 10^{-12} to 10^{-4} M.
Captan did not result in an increase in luciferase activity (RPC_{max} < 10%) at any of the viable concentrations tested. It is thus not an agonist of human estrogen receptor alpha (hERα) in the HeLa-9903 model system.

Material and Methods

1. Test Material: Captan (Merpan technical)
   - Description: White powder
   - Lot/Batch #: 91138103
   - Purity: 93%
   - Stability: Expiry date 14 February 2013
   - Storage conditions: At room temperature
   - Solvent used: Dimethylsulfoxide (DMSO)

2. Control Materials:
   - Negative (solvent): DMSO at 0.1%
   - Positive: hERα antagonist ICI 182,780
   - Reference: 17β-estradiol (1 nM) (Sigma, 100% purity, Lot No 110M0138V)
     17α-estradiol (weak estrogen) (Sigma, 99.7% purity, Lot No 041M4065V)
     17α-methyltestosterone (very weak agonist) (Sigma, 99% purity, Lot No 060M1543V)
     Corticosterone (negative compound) (Sigma 99.2% purity, Lot No BCBC6322V)

3. Cell line: hERα-HeLa-9903

Source: Japanese Collection of Research Bioresources (JCRB) Cell Bank
Cell culture medium: Eagle's Minimum Essential Medium (EMEM) without phenol red, with 60 mg/L of Kanamycin and 10% fetal bovine serum
Description: Human cervical tumor cell line stably expressing : hERα construct and firefly luciferase reporter construct with five tandem repeats of a vitellogenin estrogen-responsive element driven by a mouse metallothionein (MT) promoter TATA element

4. Assay Conditions
Luciferase assay reagent: Trisma Base
Magnesium Chloride
EDTA
Dithiothreitol
ATP
Coenzyme A
AMP
Luciferin
Glycerol
Triton-X100
Bovine Serum Albumin
CDTA

Test concentrations:
Captan: First run: $1 \times 10^{-4} – 1 \times 10^{-11}$ M
Second run: $1 \times 10^{-5} – 1 \times 10^{-12}$ M
17β-estradiol: $1 \times 10^{-8} – 1 \times 10^{-15}$ M
17α-estradiol: $1 \times 10^{-6} – 1 \times 10^{-13}$ M
17α-methyltestosterone: $1 \times 10^{-12} – 1 \times 10^{-5}$ M
Corticosterone: $1 \times 10^{-10} – 1 \times 10^{-4}$ M

TEST PERFORMANCE:
05.07. – 26.08.2011 at CeeTox Inc., Kalamazoo, MI, USA

1. Experimental Procedure
Cell seeding:
Cells were plated into wells of a 96 well cell culture plate at a density of ~1 x 10^4 cells/100 µL/well and incubated at 5% CO_2 and 37±1°C for at least 3 hours prior to chemical exposure.

Preliminary range finding:
Preliminary cytotoxicity and precipitation assays were conducted with captan, to identify a suitable top concentration for use in the transcriptional activation assays. A reduction in cell viability of ≥ 20% was considered evidence of cytotoxicity.

Transcriptional Activation Assay:
Reference chemicals and captan were dissolved in DMSO and serially diluted. Post-seeding incubation the chemicals were added and the plates were incubated in a 5% CO_2 incubator at 37 ± 2°C for 24 ± 2 h. The luminescence data from each plate were analysed.
All concentrations were tested in replicates of 6/plate. In addition, for each concentration, 2 replicates/plate were prepared that incorporated the hERα antagonist ICI 182,780, to allow for the identification of non-specific (i.e., non-hERα-mediated) induction of the luciferase gene.

2. Evaluation Criteria
In order to determine the relative transcriptional activity as compared to the positive control (PC), 1 nM 17β-estradiol, the luminescence data from each plate were analyzed according to the steps outlined in the study report (Point 3.6, page 14). Wells incorporating ICI 182,780 were analyzed in an identical fashion to wells not incorporating ICI 182,780, except that the data were normalized by subtracting the mean value for the ICI 182,780-containing vehicle control (VC) wells.

Findings

The suitable top concentration of captan for use in the transcriptional activation assays was 10^{-6} M, based on excessive cytotoxicity at concentrations ≥ $10^{-5.5}$ M identified in the range finder and both valid independent runs.

Table 3.10.3.5-1 Transcriptional Activation Assay, RPC_{max} values*

	17. 1st Experiment	18. 2nd Experiment
Captan (10^{-6} M) (*Relative transcriptional activation- % of positive control)	4.3 ± 1.4	4.9 ± 1.2

In both independent runs of the assay, the mean luciferase activity of the positive control (1 nM 17β-estradiol) was greater than 4-fold that of the mean luciferase activity of the vehicle control on each plate.
$LogPC_{50}$, $LogPC_{10}$, $LogEC_{50}$ and Hill slope values for the 4 reference compounds (17β-estradiol, 17α-estradiol, 17α-methyltestosterone and corticosterone) were within the acceptable ranges, with minor exceptions.

In two independent runs of the assay, captan did not result in an increase in luciferase activity at any of the concentrations tested as the RPC_{max} values (Relative transcriptional activation- % of positive control) in both independent runs of the assay were < 10% (mean values of 4.3 ± 1.4% and 4.9 ± 1.2% in the first and second independent runs of the assay, respectively).

Conclusions

Captan is not an agonist of human estrogen receptor alpha (hERα) in the HeLa-9903 model system.

3.10.3.6 Study 16 (2012)

Reference:	Captan: Human Recombinant Aromatase Assay
Author(s), year:	Anonymous (2012)
Report/Doc. number:	Document No. 9141V-100374AROM, R-28283
Guideline(s):	OPPTS 890.1200
GLP:	Yes
Deviation	Purity off by 0.15% (92.85% used instead of 93%).
Deviations to OPPTS 890.1200 (2009):	None. The limit established for the mean background control activity is ≤10% in the guideline, and not ≤15% as written in the study report. The values obtained from the study fulfil this criterion.
Acceptability:	Yes

Summary

In an aromatase inhibition assay employing androstenedione as a substrate, which is converted by an aromatase to estrone, the ability of captan to inhibit aromatase activity was determined, using human CYP19 (aromatase) and P450 reductase supersomes. Three independent experiment were conducted with captan at concentrations of 10^{-11} to 10^{-5} along with the positive control inhibitor 4-hydroxyandrostenedione.
The positive control gave the expected results.
Captan was classified to be "equivocal" for aromatase activity inhibition as it had a mean value of 60.3% control activity ± 2.1% SD at 10^{-5} M.

Material and Methods

1. Test Material: Captan (Merpan Tech)
   - Description: Powder
   - Lot/Batch #: 91138103
   - Purity: 93%
   - Stability: Expiry date 14 February 2013
   - Storage conditions: At room temperature

Solvent used: Dimethylsulfoxide (DMSO)

2. Control Materials:
Solvent control: DMSO at 1%
Positive: 4-hydroxyandrostendione (4-OH ASDN) (Formestane), Sigma, 99.6% purity, Lot No 081K2133

3. Non-radiolabelled substrate: Androstenedione (4-Androstene-3,17-dione)
Source: Steraloids, Inc.
Lot No.: L1712
Purity: 99.8%

4. Radiolabelled substrate: [1β-3H]-Androstenedione
Supplier: Perkin Elmer
Batch No: 619344
Specific Activity: 26.3 Ci/mmol
Adjusted specific activity: 0.3-0.5 Ci/mmol

5. Human recombinant microsome
Product name: Human CYP19 (Aromatase) and P450 reductase supersomes
Supplier: GentestTM (Woburn, MA)
Lot No.: 19701

6. Assay Conditions
Buffer: Sodium phosphate assay buffer
0.1 M sodium phosphate monobasic
0.1 M sodium phosphate dibasic
pH 7.4
Propylene glycol (5%)
NADPH (β-nicotinamide adenine dinucleotide phosphate, prepared fresh in assay buffer, stock solution 6 mM)
Temperature: 37 ± 2 °C
Incubation time: 15 minutes
Total assay volume: 2 mL

7. Test concentrations:
Captan: $1 \times 10^{-3} – 1 \times 10^{-11}$ M
4-OH ASDN: $1 \times 10^{-5} – 1 \times 10^{-10}$ M

TEST PERFORMANCE
06.06.2011 – 04.01.2012 at CeeTox Inc., Kalamazoo, MI, USA

1. Experimental Procedure
Propylene glycol, [^{3}H]-Androstenedione (ASDN), NADPH, and assay buffer were combined in the test tubes, with or without captan. The test tubes and microsomal suspensions were placed at 37°C in the water bath 5 minutes prior to the initiation of the assay. The reactions were terminated by the addition of ice-cold methylene chloride and vortex-mixed to extract the unreacted ASDN. The methylene chloride layer (bottom layer) was discarded and the aqueous layer was extracted two more times. The top aqueous layers were then transferred to liquid scintillation vials and analysed using Packard TriCarb LSC 2910TR.
Radioactivity found in the aqueous fractions is from the ^3H_2O formed upon hydrolysis of [^{3}H]-ASDN. One H_2O molecule is released per molecule of ASDN converted to estrogen in a stereospecific reaction. Therefore the amount of estrogen product formed was determined by dividing the total amount of ^3H_2O formed by the specific activity of the [^{3}H]-ASDN substrate (expressed in dpm/mL). Results are presented as the activity of the enzyme reaction and expressed in nmol per (mg product) per minute.

2. Evaluation Criteria
In three independent runs of the positive control assay (4-OH ASDN), the mean Hill slope, IC_{50}, bottom curve (%), and top curve (%) were within the acceptable ranges.

In three independent runs of the assay, the average of the four background activity controls within a run was within an acceptable range of -5 to +6% and the average of the four full activity controls within a run was within an acceptable range of 90 – 110%.
The minimum level of mean aromatase activity in the full activity control samples was 0.6 nmol/mg protein/min, well above the minimum acceptable activity limit of 0.100 nmol/mg protein/min.
The mean background control activity was ≤ 15% of the full activity control, the limit established in the guidelines.
The solvent control substance did not alter the sensitivity or reliability of the assay. Specifically, the acceptable limit of DMSO concentration was ≤ 10%.

Findings

In three independent runs of the assay, captan caused a decrease in aromatase activity at concentrations between 10^{-6} and 10^{-3} M. However, the test substance showed visible insolubility at 10^{-3} M and 10^{-4} M. Since these insoluble concentrations were not used in the final analysis, the highest level of inhibition occurred at 10^{-5} M and captan was determined to be 60.3% of control. This placed the value within the 50-75% range of the EDSP guideline (< 50%: inhibitor; > 75%: non-inhibitor). Thus, captan was classified as an equivocal of aromatase activity inhibition.

Table 3.10.3.6-1 Aromatase activity (% of vehicle control)

	19. 1st Experiment	20. 2nd Experiment	21. 3rd Experiment
Captan (10^{-5} M)	58.8± 0.8%	62.7± 0.6%	59.5 ± 1.2%

Conclusions

Captan was classified as an "equivocal" of aromatase activity inhibition in all three valid independent runs.

3.10.3.7 Study 9 (2011)

Reference:	The Uterotrophic Assay (OPPTS 890.1600); Captan
Author(s), year:	Anonymous (2011)
Report/Doc. number:	Document No. C198-100, R-28291
Guideline(s):	OPPTS 890.1600
GLP:	Yes
Deviation:	Purity off by 1.9% (93% used instead of 94.9%).
Deviations to OECD 440 (2007):	Administration time is shorter in the OPPTS guideline (3 days) than in the OECD guideline (7 days).
Acceptability:	Yes.

Summary

An uterotrophic assay was conducted to investigate captan for potential estrogenic activity using the ovariectomized female rat model.
Animals were orally administered captan (313 or 1000 mg/kg bw/day), vehicle control (1% CMC) or 17α-ethinyl estradiol (positive control) for three consecutive days. Body weights and clinical observations were performed daily. At termination, animals were euthanized and uteri were excised and wet and blotted weights recorded.
Final body weights of animals administered 313 or 1000 mg/kg bw/day captan were 89 and 85% of control body weights, respectively. Uterine weights (wet and blotted) were unaffected by the administration of captan at either dose level. Based on these findings, oral administration of captan up to the limit dose of 1000 mg/kg bw/day showed no evidence of estrogen activity in the Uterotrophic Assay (OPPTS 890.1600) using the ovariectomized female rat model.

Material and Methods

1. Test Material:	Captan (Merpan Captan Technical)
Description:	Fine-white powder
Lot/Batch #:	91138103
Purity:	93%
Stability:	Expiry date 14 February 2012

Storage conditions:	At room temperature
Solvent used:	Carboxymethylcellulose (CMC)

2. Control Materials:

Solvent control:	1% CMC in deionised water (Sigma, Lot No. 100M0113V) Corn oil (MP Biomedicals, Lot No. 7862K)
Reference chemicals:	17α-Ethinyl Estradiol (positive control) (Sigma (98%), Lot No. 090M1241V, vehicle: corn oil)

2. Test System

Species:	Rat, Rattus norvegicus
Strain:	Sprague-Dawley Crl:CD (SD) IGS
Number/Sex:	32 ovariectomized females
Age:	58 days
Weight:	200.4 –274.6 g
Supplier:	Charles River Laboratories International, Inc., NC, USA
Acclimatisation period:	9 days
Diet:	Teklad Global 16% Protein Rodent Diet ad libitum

3. Test Conditions

Housing:	Animals were housed in pairs in polycarbonate cages, furnished with hardwood bedding (Northeastern Products Corp., Warrensburg, NY)
Temperature:	22-25°C
Relative humidity:	39-65%
Photoperiod:	12/12 hour light/dark cycle

TEST PERFORMANCE:
10.09.2011 – 13.09.2011 at Integrated Laboratory Systems Inc., Durham, NC, USA

1. Experimental Procedure
Captan (313 or 1000 mg/kg), 1% CMC (vehicle control) or 17α-ethinyl estradiol (0.05 mg/kg) dose formulations were administered to 8 rats per dose group by oral gavage at a dose volume of 5 mL/kg body weight for three consecutive days.

2. Observations
Animals were observed for mortality twice daily on weekdays and once daily on weekends. Cage side observations were made 1 hour post dose administration. Clinical observations and body weights were for allocation of animals to study groups, daily prior to dose administration, and prior to euthanasia.

3. Statistics
Descriptive statistics (mean, standard deviation and count), final body weight, body weight gain, and tissue weights were analyzed using SAS version 9.2. Studentized residual plots were used to detect possible outliers and Levene's test was used to assess homogeneity of variance. Final body weight, body weight gain, and uterine weights were analyzed by one-way ANOVA followed by pair wise comparisons using a Dunnett's one tailed t test (uterine weights) and Dunnett's two tailed t test (final body weight and body weight gain). Statistically significant effects were reported when $p < 0.05$.

4. Evaluation Criteria
Mean blotted uterine weight of animals administered the vehicle (1% CMC) was less than 0.04% of body weight, meeting the performance criteria.

Findings

Mortality
All animals administered 0 (vehicle control) and 313 mg/kg captan survived to the scheduled study termination with no animals showing signs of moribundity. One animal administered 1000 mg/kg captan was euthanized moribund on study day 3.

Clinical observations
No clinical signs of toxicity were noted.

Body weight
Final body weight of animals administered 1000 mg/kg captan and body weight gain of animals administered 313 or 1000 mg/kg captan were significantly decreased compared to the vehicle control group. Mean final body weights of animals administered 313 and 1000 mg/kg were 89 and 85% of control animals, respectively.

Table 3.10.3.7-1 Group Mean initial, final, and body weight changes

22. Dose group	23. Test/Reference substance dose level (mg/kg bw/day)	24. Initial Mean Body Weight(g) ± SD	25. Final Mean Body Weight (g) ± SD	26. Mean Body Weight Gain (g) ± SD	27. Final Body Weight 28. % of 29. Control
Vehicle Control (1% CMC)	0	237.1 ± 26.5	252.9 ± 28.7	15.8 ± 4.4	
Captan	313	235.9 ± 21.4	225.7 ± 28.8 -	-10.2 ± 14.5*	89.2
Captan	1000	235.1 ± 20.6	215.6 ± 23.5*	-18.3 ± 12.3*	85.3

* Statistically significant compared to the vehicle control mean

Necropsy
The animal dosed with 1000 mg/kg captan and euthanized on day 3 showed left lobe of lung mottled black, renal foci (left and right kidneys) proximal to hilus, gastric and intestinal dilatation due to air. No gross observations were seen in rats surviving to the scheduled euthanasia on day 4.

Uterine Weights
17α-ethinyl estradiol, resulted in significantly increased wet and blotted uterine weights compared to the vehicle control group. Administration of captan did not cause any significant change.

Table 3.10.3.7-2 Serum hormone levels

Dose group	30. Test/Reference substance (mg/kg bw/day)	31. Wet Uterine Weight (mg)	32. Blotted Uterine Weight (mg)
Vehicle Control (1% CMC)	0	71.1 ± 11.2	67.2 ± 10.5
Captan	313	79.7 ± 11.9	74.8 ± 12.7
Captan	1000	65.0 ± 8.52	60.9 ± 7.4
17α-Ethinyl estradiol	0.05	337.8 ± 13.1*	197.4 ± 38.2*

* Statistically significant compared to the vehicle control mean

Conclusions

Administration of captan significantly decreased final body weight (1000 mg/kg bw/day) and body weight gain (313 and 1000 mg/kg bw/day) when compared to the control animals. Final body weights of animals administered 313 or 1000 mg/kg bw/day captan were 89 and 85% of control body weights, respectively. Uterine weights (wet and blotted) were unaffected by the administration of captan at either dose level. Based on these findings, oral administration of captan up to the limit dose of 1000 mg/kg bw/day showed no evidence of estrogen activity in the Uterotrophic Assay (OPPTS 890.1600) using the adult ovariectomized female rat model.

3.10.3.8 Study 10 (2012a)

Reference:	The Hershberger Bioassay (OPPTS 890.1400); Captan
Author(s), year:	Anonymous (2012a)
Report/Doc. number:	Integrated Laboratory Systems Inc., Durham, NC, USA; document No. C198-200, R-28287
Guideline(s):	OPPTS 890.1400
GLP:	Yes
Deviations to OECD 441 (2009):	Animal husbandry: lower limit of humidity slightly lower (27%) than the recommended minimum for animal welfare (at least 30%; in both OECD and OPPTS test guidelines). The guidelines (OECD and OPPTS) do not foresee to sacrifice obviously healthy animals in order to prevent the potential onset of distress or clinical signs in case of mortality or moribundity of other animals in the same dose group. Because of that, only one dose group could be used for analysis of androgen-dependent tissue.
Acceptability:	Yes, however with limitations, as only one dose group in each assay (agonist and antagonist) was examined for changes in androgen dependent tissues.

Summary

A Hershberger Bioassay was conducted to investigate captan for its potential androgen agonist/antagonist activity and 5α-reductase inhibition properties using a castrated rat model.
Oral administration of captan at a dose level of 200 mg/kg showed no evidence of any androgen agonist activity and oral administration of captan at a dose level of 100 mg/kg showed no evidence of any androgen antagonist activity.

Material and Methods

1. Test Material: Captan (Merpan Captan Technical)
   - Description: Fine-white powder
   - Lot/Batch #: 91138103
   - Purity: 93%
   - Stability: Expiry date 14 February 2012
   - Storage conditions: At room temperature
   - Solvent used: Carboxymethylcellulose (CMC)

2. Control Materials:
   - Solvent control: 1% CMC in deionised water (Sigma, Lot No. 100M0113V)
     Corn Oil (MP Biomedicals, Lot No. 7862K)
   - Reference chemicals: Testosterone Propionate (agonist positive control, antagonist negative control), Sigma, 100% purity, Lot No. 048K1328
     Flutamide (antagonist positive control), Sigma, > 99% purity, Lot No 107K1293

2. Test System
   - Species: Rat, Rattus norvegicus
   - Strain: Sprague-Dawley Crl:CD (SD) IGS
   - Number/Sex: 64 castrated males
   - Age: 59-60 days
   - Weight: 268.4– 353.6 grams
   - Supplier: Charles River Laboratories International, Inc., NC, USA
   - Acclimatisation period: 7-8 days
   - Diet: Teklad Global 16% Protein Rodent Diet ad libitum
   - Housing: Polycarbonate cages and hardwood bedding

3. Test Conditions
   - Temperature: 22-24°C
   - Relative humidity: 27-57%
   - Photoperiod: Alternating 12-hour light and dark cycles

TEST PERFORMANCE:
26.09 – 07.10.2011 at Integrated Laboratory Systems Inc., Durham, NC, USA

1. Experimental Procedure

A dose range finding study was conducted at ILS to select a dose to meet the maximum tolerated dose (MTD) as defined in the protocol. Four male rats (PND 36) per dose level were orally administered either 1% CMC (vehicle) or captan at dose levels of 200, 400, 600, 800 and 1000 mg/kg bw/day for 14 days (Study 9, 2011).

To evaluate the test substance for agonist properties, animals (8/group) were administered one of two dose levels (200 and 400 mg/kg bw/day) of captan, or 1% CMC (vehicle), or an agonist reference substance.
To evaluate captan for antagonist properties, animals (8/group) were co-administered one of three dose levels (100, 200, and 400 mg/kg bw) of captan or flutamide (3 mg/kg) with testosterone propionate (0.4 mg/kg).
Captan, flutamide and CMC dose formulations were administered by oral gavage at a dose volume of 5 mL/kg bw. Testosterone propionate dose formulations were administered by subcutaneous injection into the dorsoscapular region at a dose volume of 0.5 mL/kg bw. In co-administered animals, oral gavage preceded subcutaneous injections. The dose formulations were administered for 10 consecutive days.

2. Observations
Animals were observed for mortality twice daily on weekdays and once daily on weekends. Cage side observations were made 1 hour post administration. Clinical observations and body weights were collected within 2 days of arrival, again for allocation of animals to study groups, daily prior to dose administration, and prior to euthanasia.

3. Statistics
Descriptive statistics (mean, standard deviation and count) were calculated using MS Excel. Final body weight, body weight gain, and tissue weights were analyzed using SAS version 9.2. Studentized residual plots were used to detect possible outliers and Levene's test was used to assess homogeneity of variance. Final body weight, body weight gain, and tissue weights were analyzed by one-way ANOVA followed by pair wise comparisons using a Dunnett's one tailed t test (tissue weights) or Dunnett's two tailed t-test (final body weight and body weight gain). Statistically significant effects were reported when $p < 0.05$.

Findings

Dose range finding study
Based on the above mentioned range finding study, the current test guideline dosing regimen (10 days of dosing), and animal model (castrated adult male), and an oral LD_{50} in rat of > 5000 mg/kg body weight (captan material data safety sheet), a high dose of 400 mg/kg bw/day was selected to meet the dose selection requirements in the Hershberger Bioassay (OPPTS 890.1400) of approaching the MTD.
All male rats administered 1% CMC, 200 or 400 mg/kg captan survived until study termination. A single rat in each of the remaining dose groups (600, 800, or 1000 mg/kg captan) was euthanized prior to the scheduled termination due to body weight loss (> 20% decrease from animal's maximum weight) and abnormal breathing. No obvious signs of dosing error were observed during the necropsies. After 10 consecutive days of dosing, mean body weights of animals administered 200, 400, 600, 800, and 1000 mg/kg captan were 94%, 86%, 93%, 84%, and 78% of control body weight, respectively.

A. Mortality
Androgen agonist:
All animals administered the vehicle control (Group 1) survived to scheduled euthanasia with none showing signs of moribundity. Two animals administered 200 mg/kg bw/day (Group 2) died before study termination: one animal was found dead on day 3 (suspected dosing error), and one animal was euthanized on day 4 because of breathing difficulties.
All animals administered 400 mg/kg captan (Group 3) were euthanized prior to study termination: four were euthanized between days 3 and 5 for abnormal breathing and body weight loss. Based on abnormal clinical observations, severe body weight loss, and gross necropsy observations of 4 of the rats in this group, the remaining four animals, absent of abnormal clinical observations were, with the study monitor's approval, humanely euthanized prior to the potential onset of breathing difficulties and initiation of or continued weight loss that was observed.

Androgen Antagonist:
All animals administered TP (testosterone propionate) alone (Group 4) survived to scheduled euthanasia with none showing signs of moribundity. One animal co-administered FT (flutamide) and TP (Group 8) was humanely euthanized on day 6 due to a dosing error. One animal co-administered 100 mg/kg captan and TP (Group 5) was found dead on day 1 with no obvious sign of dosing error present.
All animals co-administered 200 mg/kg captan and TP (Group 6) were humanely euthanized prior to study termination; two were euthanized on day 4 for abnormal breathing and body weight loss. On study 4/5 the remaining six animals were humanely euthanized. Four of the six animals were euthanized due to body weight loss ranging between 16 and 50 g from

their previous maximum weight. A ruptured esophagus (dosing error) was observed in one rat. Based on abnormal clinical observations, severe body weight loss and gross necropsy observations found among 6 rats in this group, the remaining two rats, absent of abnormal clinical observations, were euthanized prior to the potential onset of breathing difficulties and weight loss, with the study monitor's approval.
All animals co-administered 400 mg/kg captan and TP (Group 7) were euthanized or found dead prior to study termination; one animal was found dead on day 4, and four were euthanized on day 3 or 5 for abnormal breathing and body weight loss. The remaining 3 animals were, absent of abnormal clinical observations and with the study monitor's approval, humanely euthanized on days 4/5 prior to the potential onset of breathing difficulties and severe weight loss. Of these 3 rats, a ruptured esophagus was observed in one rat and white particulate matter, consistent with the test substance, was found in the lavage fluid of another.

B. Clinical observations

Androgen Agonist

No clinical signs of toxicity were observed in any animals administered vehicle control (Group 1) at 1 or 24 hours (± 30 minutes) post dose administration. Two animals (# 10 and 15) administered 200 mg/kg captan (Group 2) were noted with abnormal breathing (gasping) 1 hour after dosing; this symptom persisted to 24 hours post dose in one rat (#15) and it was humanely euthanized on day 4. Animal #10 survived to study termination. Four animals administered 400 mg/kg captan (Group 3) exhibited abnormal breathing post dose and body weight loss prior to humane euthanasia (# 20 - 23).

Androgen Antagonist

At 1 hour post dose, abnormal breathing was noted (day 2) in one animal administered TP alone (Group 4) and did not persist 24 hours after dosing. At 1 and 24 hour(s) post dose administration on day 2 rales or abnormal breathing (wheezing) was noted in one animal co-administered FT and TP (Group 8). No other animals from Groups 4 and 8 exhibited any clinical signs of toxicity. One animal co-administered 100 mg/kg captan and TP (Group 5) exhibited clinical signs of toxicity: lethargy (day 6; 1 hour post dose), wheezing and thin (24 hours post dose from days 7, 9, 10, and prior to termination). Two animals (# 47 and 48) co-administered 200 mg/kg captan and TP (Group 6) were noted with abnormal breathing (wheezing or rales) 1 hour after dosing; this was accompanied by lethargy and a clear nasal discharge in one animal (#48). Animal #47 continued to exhibit abnormal breathing 24 hours post dose. Absent of abnormal clinical observations, body weight loss was observed in four of the remaining six animals and with the study monitor's approval, along with the remaining two rats were humanely euthanized prior to the onset of breathing difficulties and continued or further weight loss. Two animals (#49 and 50) co-administered 400 mg/kg captan and TP (Group 7) were noted to be lethargic and to have abnormal breathing 1 hour post dose (days 3 and 4); abnormal breathing persisted to 24 hours after dosing in animals #49.

C. Body weight

In the androgen agonist assay, the mean final body weight and body weight gain of animals administered 200 mg/kg bw/day captan (Group 2) was significantly decreased compared to vehicle controls (Group 1); the final body weight of Group 2 animals was 88% of controls.
In the androgen antagonist assay, no significant difference in mean final body weight or body weight gain was noted for animals co-administered 100 ,g/kg captan and TP (Group 5) as compared to TP alone (Group 4).

Table 3.10.3.8-1 Change in body weight, agonist and antagonist assay

Group number	Test/reference substance / control	Dose level (mg/kg bw/day)	Number of animals[A]	Final mean body weight (g) ± S.D.	Mean body weight gain (g) ± S.D.
1	Vehicle control (1% CMC)	0	8 (8)	357.8 ± 21.7	37.7 ± 11.4
2	Captan	200	8 (6)	315.2 ± 20.*	-6.7 ± 12.3*
3	Captan	400	8 (0)	N.A.[B]	N.A.[B]
4[C]	Vehicle control+ TP	0 + 0.4	8 (8)	381.8 ± 27.8	60.7 ± 14.2
5	Captan + TP	100 + 0.4	8 (8)	351.4 ± 55.1	31.2 ± 42.7
6	Captan + TP	200 + 0.4	8 (0)	N.A.[B]	N.A.[B]
7	Captan + TP	400 + 0.4	8 (0)	N.A.[B]	N.A.[B]
8	Flutamide + TP	3.0 + 0.4	8 (7)	365.4 ± 31.2	43.2 ± 22.8

[A] Number of animals at start of study (in brackets: animals surviving to study termination)
* Statistically significant compared to the vehicle control mean (Dunnett's test; $p < 0.05$)
[B] All animals were euthanized prior to study termination
[C] Group 4 served as the positive control for the agonist assay and as the negative control in the antagonist assay

D. Necropsy

The following gross observations were noted at necropsy:

200 mg/kg (Group 2)
Animal 9: fibrinous pleurititis, left lung small and adhered to heart
Animal 15: gastrointestinal dilatation

400 mg/kg (Group 3)
Animals 20, 22: gastrointestinal dilatation
Animal 21: gastrointestinal dilatation; multifocal, diffuse mucosal inflammation of glandular stomach
Animal 23: mucosal inflammation of glandular stomach, red area on thymus

200 mg/kg + TP (Group 6)
Animal 41: dark red area on cardiac lobe of the lung
Animal 44: ruptured esophagus
Animal 47: gastrointestinal dilatation, foci on left lobe of lung
Animal 48: gastrointestinal dilatation

400 mg/kg + TP (Group 7)
Animals 49, 50: gastrointestinal dilatation, read areas in the lung
Animal 51: 3-4 pinpoint petechiae on mucosal surface of glandular stomach
Animal 52: white particulate in lung lavage
Animals 54, 55: gastrointestinal dilatation
Animal 56: ruptured esophagus

Group 8
Animal 57: white discharge from glans penis
Animal 58: ruptured esophagus, gelatinous salivary glands

E. Tissue Weights

Table 3.10.3.8-2 Androgen dependent tissue weights, agonist and antagonist assay

Group	Dose level (mg/kg bw/day)	N. of animals[A]	33. Mean tissue weight (mg) ± SD				
			34. Glans penis	35. Cowper's gland	36. LABC	37. Ventral prostrate weight	38. Seminal vesicle

Vehicle control (1% CMC)	0	8 (8)	71.1 ± 7.4	10.3 ± 4.7	200.3 ± 74.9	24.2 ± 6.7	82.9 ± 20.6
Captan	200	8 (6)	68.3 ± 10.9	10.1 ± 3.4	168.2 ± 44.0	24.5 ± 3.3	79.5 ± 21.1
Captan	400	8 (0)	NA	NA	NA	NA	NA
Vehicle control + TP [B]	0 + 0.4	8 (8)	111.9 ± 6.6*	57.5 ± 10.5*	566.2 ± 71.1*	243.3 ± 28.3*	1107.0 ± 116.4*
Captan + TP	100 + 0.4	8 (7)	112.0 ± 4.9	58.4 ± 15.9	552.8 ± 73.8	290.7 ± 61.3	1084.2 ± 92.5
Captan + TP	200 + 0.4	8 (0)	N.A.	N.A.	N.A.	N.A.	N.A.
Captan + TP	400 + 0.4	8 (0)	N.A.	N.A.	N.A.	N.A.	N.A.
Flutamide + TP	3.0 + 0.4	8 (8)	84.9 ± 7.0*	20.5 ± 5.2*	225.1 ± 52.3*	66.4 ± 14.1*	234.3 ± 72.9*

Abbreviations: SD - standard deviation; LABC-levator ani plus bulbocavernous muscle complex; TP – testos terone propionate, FT - flutamide, NA –not applicable
[A] Number of animals at start of study (in brackets: animals surviving to study termination)
[B] Positive Control for agonist, negative control for antagonist assay
* Statistically significant compared to the vehicle control mean ($p < 0.05$)

Androgen Agonist
No significant effect was observed on glans penis, Cowper's glands, LABC, ventral prostate, and seminal vesicle weights in animals administered 200 mg/kg captan (Group 2) compared to vehicle control (Group 1). The five androgen-dependent tissues weights were significantly increased in the positive control group (TP - Group 4) as compared to vehicle control group (Group 1).

Androgen Antagonist
No significant effect was observed on glans penis, Cowper's glands, LABC, and seminal vesicle weights in animals co-administered 100 mg/kg captan and TP (Group 5) compared to TP alone (Group 4). While not statistically significant, a decrease in ventral prostate weight was noted ($p > 0.05$ and < 0.1) in Group 5 animals compared to those administered TP alone (Group 4). The five androgen-dependant tissues weights were significantly decreased in the positive control group, (FT and TP; Group 8) compared to the TP control group (Group 4).

F. Evaluation
In this Hershberger bioassay dose levels deemed appropriate from the range finding study resulted in a number of deaths in male castrated rats. Moribund animals all exhibited abnormal breathing (gasping, wheezing or rales) that was severe enough to result in rapid weight loss and at necropsy both the stomach and/or intestines were severely distended with air. Similar, but less severe, symptoms were noted during the range finder study.

Based on a recent publication (Damsch et al., 2011) and the information gathered during this study it appears that captan in 1% CMC resulted in gavage-related reflux in selected animals which led to severe morbidity and mortality at high dose levels. These deaths were not anticipated based on company captan study reports and the range finding study conducted at ILS. The recent publication by Damsch et al. (2011) on gavage-related reflux suggests that adverse respiratory effects and mortality arise in gavage studies as a consequence of a combination of factors including the characteristics of the test formulation e.g. irritancy of captan, viscosity of the 1% CMC, the volume of test formulation administered, and possibly the length of the gavage needle. Although, the needle size used for dose administration in the Hershberger assay was within published guidelines based on animal weight, it is possible that it might have played a role in what appeared to be a more sensitive response in this model compared to the other animal models used for the EDSP Tier I mammalian studies. Since the adverse response seen in this study was likely a reflection of the irritancy of the test substance when administered by oral gavage in a viscous vehicle rather than systemic toxicity of the compound, the actual MTD was not believed to be exceeded in this evaluation.

In the agonist assay, dose related adverse respiratory effects and morbidity resulted in the death or humane/moribund euthanasia (respiratory distress and body loss weight) of two animals administered 200 mg/kg captan and all animals administered 400 mg/kg captan.

Mean final body weight and body weight gain was significantly decreased in animals administered 200 mg/kg captan (n=6) compared to vehicle controls. As none of the animals administered 400 mg/kg captan survived to study termination, they could not be evaluated for the potential agonist properties of captan. However, a dose level of 200 mg/kg captan did not increase androgen dependent tissue weights compared to the vehicle control group.

In the antagonist assay, dose related adverse respiratory effects and morbidity resulted in the death or humane/moribund euthanasia (respiratory distress and body loss weight) of one animal co-administered 100 mg/kg captan and TP and all animals co-administered 200 or 400 mg/kg captan and TP. Body weights of animals co-administered 100 mg/kg captan and TP (n=7) were unchanged compared to control animals (TP alone). As none of the animals co-administered 200 or 400 mg/kg captan and TP survived to study termination, they could not be evaluated for the potential antagonist properties of captan. However, captan co-administered with TP at a dose level of 100 mg/kg did not decrease androgen dependent tissue weights compared to TP alone.

Conclusions

Oral administration of captan at a dose level of 200 mg/kg showed no evidence of any androgen agonist activity and oral administration of captan at a dose level of 100 mg/kg showed no evidence of any androgen antagonist activity.

3.10.3.9 Study 11 (2012b)

Reference:	Pubertal Development and Thyroid Function in Intact Juvenile/Peripubertal Male Rats (OPPTS 890.1500); Captan
Author(s), year:	Anonymous (2012b)
Report/Doc. number:	Document No. C189-301, R-28289
Guideline(s):	OPPTS 890.1500
GLP:	Yes
Deviations:	Animal husbandry: Room temperature and humidity lower (13-24°C, 19-66%) than recommended by international animal welfare standards (22±3°C, 30-70%). However, as only on one day during the study the animals experienced slight discomfort regarding the temperature (18°C on PND25), this is considered a minor deviation.
Acceptability:	Yes

Summary

The purpose of this assay was to identify the potential of captan to interact with the endocrine system by identifying effects on pubertal development and thyroid function in the intact juvenile/peripubertal male rat. Animals were orally administered captan (100 or 200 mg/kg bw/day) or the vehicle control (1% Carboxymethylcellulose, CMC) for 31/32 days. Two hours following the final dose administration, the animals were humanely euthanized and examined.
Administration of captan to intact juvenile/peripubertal male rats at a dose level of 100 or 200 mg/kg bw/day resulted in no changes in thyroid gland weight or TSH levels. There were no histopathological findings in the thyroid gland. However, several androgen-dependent tissue weights and serum testosterone were significantly decreased.

Material and Methods

1. Test Material: Merpan Captan Technical
   - Description: Fine-white powder
   - Lot/Batch #: 91138103
   - Purity: 94.4%
   - Stability: Expiry date: 27 April 2012
   - Storage conditions: At room temperature
   - Solvent used: Carboxymethylcellulose (CMC)

2. Control Materials:

Solvent control:	1% CMC in deionised water Sigma-Aldrich (St. Louis, MO) 100M0113V

3. Test System

Species:	Rat, Rattus norvegicus
Strain:	Sprague-Dawley Crl:CD (SD) IGS
Number/Sex:	F1: 48 males
Age:	PND 23 days
Weight:	51.4-72.8 g
Supplier:	Charles River Laboratories International, Inc., Raleigh, USA
Acclimatisation period:	PND 0 – PND 22
Diet:	Teklad Global 16% Protein Rodent Diet ad libitum
Housing:	Polycarbonate cages and hardwood bedding

4. Test Conditions

Housing:	Animals were housed in pairs in polycarbonate cages, furnished with hardwood bedding (Northeastern Products Corp., Warrensburg, NY)
Temperature:	13-24°C
Relative humidity:	19-66%
Photoperiod:	14/10 hour light/dark cycle
Enrichment:	none

TEST PERFORMANCE:
14 January 2012 – 10 April 2012 at Integrated Laboratory Systems Inc., Durham, NC, USA

1. Experimental Procedure
A dose range finding study was conducted, where four immature male Sprague-Dawley rats (PND 23) per dose level were orally administered 1% CMC or captan at dose levels of 200, 400, 600, 800, and 1000 mg/kg/day for 14 days (Study 10, 2012a).
Captan (100 or 200 mg/kg bw/day) or 1% CMC (vehicle control) dose formulations were administered by oral gavage at a dose volume of 5 mL/kg bw, for 31/32 consecutive days (PND 23 through 53 or 54) to groups of 16 young male SD-rats. Dosing formulations were analysed and of satisfactory concentrations.

2. Observations
Animals were observed for mortality twice daily on weekdays and once daily on weekends or holidays. Cage side observations were made 1 hour (± 30 min) post dose administration. Clinical observations and body weights were made for allocation of animals to study groups, daily prior to dose administration, and prior to euthanasia. Preputial separation (PPS) was observed daily.

3. Statistics
Descriptive statistics (mean, standard deviation, coefficient of variance and count) were calculated using MS Excel. Final body weight, body weight gain, and tissue weights were analysed using SAS version 9.2. Studentized residual plots were used to detect possible outliers and Levene's test was used to assess homogeneity of variance. Heterogeneous data were transformed and if still heterogeneous, analysed using the non-parametric Kruskal-Wallis and Dunn's test. Homogeneous data were analysed by one-way ANOVA followed by pair wise comparisons using a Dunnett's two tailed t test (final body weight and body weight gain). Statistically significant effects were reported when $p < 0.05$.

Findings

Dose range finding study:
All male rats administered 0 (vehicle control), 200 or 400 mg/kg bw/day captan survived until study termination. One animal in each group administered 600, 800, or 1000 mg/kg bw/day was euthanized due to moribundity before study termination. No signs of dosing error were observed during necropsy. Male body weights of animals administered 200, 400, 600, 800, or 1000 mg/kg bw/day were 94.1%, 85.8%, 91.0%, 87.8%, or 79.9%, respectively, of control body weights. No clinical signs of toxicity were observed 3 or 24 hours post-dose administration for animals administered 0 (vehicle control), 200 or 400 mg/kg bw/day captan. Abnormal breathing/gasping/wheezing were the most common clinical observation in animals administered 600 mg/kg/day (1 rat), 800 mg/kg/day (1 rat) or 1000 mg/kg bw/day (3 rats) captan and in four of these animals this observation was coincident with body weight loss. There was no change in food

consumption (measured weekly) between control and captan administered male rats, with no changes in relative liver or kidney weight. At necropsy, no gross lesions within the stomach or the jejunum (small intestine) were observed.

A. Mortality

All male rats administered the vehicle control (1% CMC) or 100 mg/kg bw/day captan (Group 2) survived to scheduled euthanasia with no animals showing signs of moribundity. Two rats administered 200 mg/kg bw/day captan (Group 3) were euthanized before scheduled euthanasia due to moribundity: one rat was euthanized on study day 18 (PND 40), and one rat was euthanized on study day 27 (PND 49). No signs of dosing error were observed at necropsy. Gastrointestinal dilatation was observed in both animals.

B. Clinical observations

No clinical observations were observed in animals administered the vehicle control. 24 hours post dose administration on study days 22 and 23, one rat administered 100 mg/kg bw/day captan was observed with rales; a second animal in the same group exhibited alopecia of the dorsal area on study days 31 and 32. No other abnormal findings were observed in animals administered 100 mg/kg bw/day captan. One rat administered 200 mg/kg bw/day captan was ungroomed 24-hours post dose administration on study days 27 and 28. Two animals administered 200 mg/kg bw/day captan were observed with abnormal breathing (gasping): one rat on study day 18 and one rat on study day 27. Both animals were euthanized due to moribundity.

C. Body weight

A significant decrease in mean final body weight and body weight gain was observed in males administered 100 or 200 mg/kg bw/day captan compared to the vehicle control group. Terminal body weights of male rats administered 100 or 200 mg/kg bw/day captan were 91.3% or 86.8%, respectively, of mean control body weight.

Table 3.10.3.9-1 Bodyweight data

Test Substance/Control	Vehicle Control (1% CMC), n=16	Captan (100 mg/kg bw/day), n=16	Captan (200 mg/kg bw/day)	
	Mean ± SD	Mean ± SD	Mean ± SD	n
Body weight at PPS (g)	250.4 ± 26.6	**230.8 ± 18.3*** **-7.8%**	**218.5 ± 17.7*** ** **-12.7%**	**15**
Initial body weight (PND 23, g)	63.9 ± 6.3	64.4 ± 5.2	64.1 ± 4.6	16
Final body weight (g)	326.1 ± 26.7	**297.6 ± 24.5** ** **-8.7%**	**283.0 ± 29.3*** ** **-13.2%**	**14**
Body weight gain (g)	262.2 ± 22.2	**233.2 ± 21.6** ** **-11.1%**	**219.3 ± 27.1*** ** **-16.4%**	**14**

*p < 0.05, **p < 0.01 and ***p < 0.001, significantly different from control, performed with Dunnett's test

D. Preputial separation

No significant differences were noted in the age of males at complete PPS for animals administered 100 or 200 mg/kg bw/day captan compared to the vehicle control group. A significant decrease in mean body weight at complete PPS was observed for animals administered 100 or 200 mg/kg bw/day captan compared to the vehicle controls.

Table 3.10.3.9-2 Age at complete and partial preputial separation (PPS)

Test Substance/Control	Vehicle Control (1% CMC), n=16	Captan (100 mg/kg/day), n=16	Captan (200 mg/kg/day), n=15
	Mean ± SD	Mean ± SD	Mean ± SD
Age at PPS (PND)	44.9 ± 2.2	44.8 ± 2.4 n.s.	43.7 ± 1.5 n.s.

Age at partial PPS (PND)	40.3 ± 1.7	40.3 ± 1.1 n.s.	41.0 ± 1.4 n.s.

n.s. = not significantly different than vehicle control, performed with Dunnett's test

E. Necropsy

Observations at necropsy revealed testis enlargement, intestinal or kidney dilation in some animals. Findings were considered incidental and not related to dosing.

Table 3.10.3.9-3 Gross obervations at necropsy

39. Dose group	40. Animal #	41. Macroscopic observation
42. 0 (control)	1	Cyst on left kidney
43. 200	40	Intestinal dilation
44. 200	46	Right testis enlarged

F. Tissue weights

A statistical dose dependent decrease in unadjusted liver weight was noted with captan administration; however, adjusted and relative liver weights in captan administered animals were not significantly different from vehicle controls. Relative kidney and adrenal weights were increased in rats administered 100 or 200 mg/kg bw/day captan compared to the vehicle control group. There were no significant changes in the pituitary gland or thyroid weights compared to the vehicle control group. Ventral prostate weight was decreased in rats administered 100 or 200 mg/kg bw/day captan compared to the vehicle control group. LABC weight was significantly decreased in animals administered 200 mg/kg bw/day captan compared to the vehicle control group. Administration of captan did not change seminal vesicle plus coagulating gland with or without fluid, dorsolateral prostate, epididymis, or testis weights compared to the vehicle control group.
Apparent decreasing dose trends were seen for seminal vesicles, both with and without fluid, the ventral prostate, LABC, as well as the epididymides (stronger trend in the left than in the right one).

Table 3.10.3.9-4 Tissue Weights (Mean ± SD)

Tissue	Vehicle Control (1% CMC) (n=16)	Captan (100 mg/kg/day) (n=16)	Captan (200 mg/kg/day) (n=14)
Liver (g)	14.11 ± 1.84	13.02 ± 1.97 -7.7%	12.70 ± 2.41 -10.0%
Relative to bw	4.25 ± 0.23	4.28 ± 0.31 +0.7%	4.36 ± 0.51 +2.6%
Kidneys (g)	2.18 ± 0.19	2.14 ± 0.23 -1.8%	2.16 ± 0.24 -0.9%
Relative to bw	0.66 ± 0.03	**0.71 ± 0.04**** **+7.6%**	**0.75 ± 0.05***** **+13.6%**
Adrenals (mg)	44.4 ± 5.5	46.0 ± 5.2 +3.6%	43.5 ± 7.3 -2.0%
Relative to bw	13.41 ± 1.39	**15.24 ± 1.51*** **+13.7%**	**15.14 ± 2.56*** **+12.9%**
Seminal vesicles with fluid (mg)	588.0 ± 92.6	575.8 ± 138 -2.1%	559.1 ± 120.8 -4.9%
Without fluid (mg)	353.9 ± 37.7	332.8 ± 51.8 -6.0%	325.7 ± 54.2 -8.0%
Ventral prostate (mg)	256.9 ± 52.0	**218.4 ± 46.3*** **-15.0%**	**206.2 ± 40.4**** **-19.7%**
LABC (mg)	422.7 ± 63.1	386.1 ± 47.4 -8.7%	**361.6 ± 38. 3**** **-14.5%**
Epididymis left (mg)	212.6 ± 28.2	208.8 ± 30.3 -1.8%	201.2 ± 23.5 -5.4%
Right (mg)	215.8 ± 37.1	214.6 ± 27.6 -0.6%	209.4 ± 23.4 -3.0%

*p < 0.05, **p < 0.01, significantly different than control, performed with Dunnett's test

G. Histopathology

Administration of 100 mg/kg bw/day or 200 mg/kg bw/day captan for 31/32 days to male rats was not associated with histologic alterations in the left testis, left epididymis, thyroid glands, or left kidney. No changes in follicular cell height or follicular colloid area were observed in the thyroid gland in captan-administered animals compared to the vehicle controls.

H. Hormone and clinical chemistry analysis

Administration of 200 mg/kg bw/day captan significantly decreased serum T4 and testosterone concentrations compared to the vehicle control group. No change in serum TSH concentration was detected following administration of 100 or 200 mg/kg bw/day captan compared to the vehicle control group. In rats administered 100 or 200 mg/kg captan, serum alkaline phosphatase (ALP) concentration was significantly decreased and sorbitol dehydrogenase (SDH) concentration was significantly increased compared to the vehicle control group. Blood urea nitrogen (BUN) concentrations were significantly decreased in animals administered 100 mg/kg bw/day captan compared to the vehicle control group. Administration of 200 mg/kg bw/day captan significantly decreased alanine aminotransferase concentrations compared to the vehicle control group. There were no changes in serum sodium, potassium, chlorine, calcium, phosphorous, aspartate aminotransferase, gamma-glutamyl-transferase, creatinine, total bilirubin, total protein, or albumin levels following administration of captan.

Table 3.10.3.9-5 Serum hormone and clinical chemistry levels

Hormone	45. Vehicle Control (1% CMC)	46. Captan 100 47. mg/kg bw/day	48. Captan 200 49. mg/kg bw/day	50. Normal range
Serum T4 (µg/dL)	4.24 ± 0.81*	**4.08 ± 0.65*** **-3.8%**	**3.49 ± 1.01*** **-17.7%**	3-7
Serum TSH (ng/mL)	3.48 ± 1.60	2.76 ± 1.36 -20.7%	3.63 ± 2.19 +4.3%	0.57-3.41
Serum Testosterone (ng/mL)	2.04 ± 1.26*	**1.57 ± 0.80*** **-23.0%**	**1.18 ± 0.88*** **-42.2%**	
ALP (U/L)	346 ± 54	**281 ± 50**** **-18.8%**	**257 ± 56***** **-25.7%**	17-183
BUN (mg/dL)	14 ± 2	**12 ± 2*** **-14.3%**	13 ± 3 -7.1%	12-23
SDH (U/L)	31 ± 2	**34 ± 3**** **+9.7%**	**36 ± 3***** **+16.1%**	13-37

Abbreviations: T4 = thyroxine, TSH = thyroid-stimulating hormone, T = testosterone, ALP = alkaline phosphatase, BUN = blood-urea nitrogen, SDH = sorbitol dehydrogenase
* p < 0.05, ** p < 0.01 and *** p < 0.001, significantly different than control, performed with Dunnett's test

I. Discussion

According to the study author, the mortality of two rats administered 200 mg/kg bw/day may have been a result of oral gavage-related reflux that results in serious respiratory effects and mortality. Adverse respiratory effects and mortality can arise in gavage studies as a consequence of a combination of factors, e.g. irritancy of test article (which is the case for captan), viscosity of vehicle (1% CMC), etc. The body weight loss and the adverse clinical signs noted with captan in these two rats were likely attributed to gavage-related reflux rather than to the systemic toxicity of captan.
No adverse clinical signs were observed in surviving males administered 200 mg/kg/day captan. According to the study author, the lack of adverse clinical observations, or signs of systemic toxicity indicate that for animals administered 100 or 200 mg/kg/day captan, the MTD was approached but not exceeded.

After 31/32 days of captan administration there were no changes in thyroid gland weight; or TSH concentration, however; circulating T4 concentrations were significantly decreased with administration of 200 mg/kg bw/day captan compared to controls. If a thyroid toxicant induces enzymes involved in thyroid metabolism, changes in serum hormones would be reflected as a decrease in T4 and a concomitant increase in TSH; the latter was not observed in this study, nor was hepatic enzyme induction observed. As noted by O'Connor et al. (1999), potential thyroid modulators induce one or more of the following responses: elevation in TSH, decrease in T3/T4, increased thyroid weight and/or altered thyroid histology. In

general, organ weight measurements are useful endpoints for detecting thyroid modulators with increased relative liver weight typically observed with hepatic-enzyme inducers, a secondary mechanism for peripheral metabolism of thyroid hormones (McClain et al., 1989). No changes in histopathological findings in the thyroid glands were noted with captan administration.
Overall, the study author concluded that administration of 100 or 200 mg/kg bw/day captan to intact juvenile/peripubertal male rats does not appear to show thyroid gland disruption.
However, several androgen-dependent tissue weights (ventral prostate, LABC) and serum testosterone were significantly decreased following captan administration.

Conclusions

Administration of 200 mg/kg bw/day captan to intact juvenile/peripubertal male rats resulted in significantly decreased circulating T4 concentrations. However, the study author concluded that administration of 100 or 200 mg/kg bw/day captan does not appear to show thyroid gland disruption, as there were no changes in histopathological findings in the thyroid glands, in thyroid gland weight or TSH concentration.
Several androgen-dependent tissue weights (ventral prostate, LABC) and serum testosterone were significantly decreased after administration of 100 mg/kg bw/day captan and above.

3.10.3.10 Study 12 (2012c)

Reference:	Pubertal Development and Thyroid Function in Intact Juvenile/Peripubertal Female Rats (OPPTS 890.1450); Captan
Author(s), year:	Anonymous (2012c)
Report/Doc. number:	Document No. C198-300, R-28288
Guideline(s):	OPPTS 890.1450
GLP:	Yes
Deviations:	Animal husbandry: Room temperature and humidity much lower (13-24°C, 19-66%) than recommended by international animal welfare standards (22±3°C, 30-70%). However, as the temperature and humidity were corrected one week prior to experiment start, this is considered a minor deviation.
Acceptability:	Yes

Summary

The purpose of this assay was to identify the potential of captan to interact with the endocrine system by identifying effects on pubertal development and thyroid function in the intact juvenile/peripubertal female rat (OPPTS 890.1450). Animals were orally administered captan (300 or 600 mg/kg/day) or the vehicle control (1% Carboxymethylcellulose, CMC) for 21/22 days. Approximately two hours following the final dose administration, the animals were humanely euthanized.
Administration of captan approached, but did not exceed, the MTD. A significant delay in vaginal opening, day of first estrus, decreased number of corpora lutea, and decreased pituitary gland, ovarian, and uterine weights suggest an effect on pubertal development. In addition, there were a decrease in serum T4 concentrations, without concomitant increases in TSH concentrations, and histopathological changes in the thyroid following administration of captan.

Material and Methods

1. Test Material: Merpan Captan Technical
   - Description: Fine-white powder
   - Lot/Batch #: 91138103
   - Purity: 94.9%
   - Stability: Expiry date: 27 April 2012
   - Storage conditions: At room temperature
   - Solvent used: Carboxymethylcellulose (CMC)

2. Control Materials:
   - Solvent control: 1% CMC in deionised water (Sigma-Aldrich (St. Louis, MO), 100M0113V)

3. Test System

Species:	Rat, Rattus norvegicus
Strain:	Sprague-Dawley Crl:CD (SD) IGS
Number/Sex:	F1:48 females
Age:	PND 22
Weight:	46.8-67.6 g
Supplier:	Charles River Laboratories International, Inc., NC, USA
Acclimatisation period:	PND 0 – PND 21
Diet:	Teklad Global 16% Protein Rodent Diet, ad libitum

4. Test Conditions

Housing:	Animals were housed in pairs in polycarbonate cages, furnished with hardwood bedding (Northeastern Products Corp., Warrensburg, NY)
Temperature:	13-24°C
Relative humidity:	19-65%
Photoperiod:	14/10 hour light/dark cycle

TEST PERFORMANCE:
13 January – 10 April 2012 at Integrated Laboratory Systems Inc., Durham, NC, USA

1. Experimental Procedure
A dose range finding study was conducted to select a dose to meet the MTD as defined in the protocol. Four immature female SD rats (PND 22) per dose level were orally administered 1% CMC or captan at dose levels of 200, 400, 600, 800 or 1000 mg/kg bw/day for 14 days.
The test substance or 1% CMC (vehicle control) dose formulations were administered by oral gavage at a dose volume of 5 mL/kg body weight. The dose formulations were administered for 21/22 (PND 22 through 42 or 43) consecutive days. Dosing occurred 24 hours (± 2 hours) from the previous dose.

2. Observations
Animals were observed for mortality twice daily on weekdays and once daily on weekends. Cage side observations were made 1 hour post dose administration. Clinical observations and body weights were for allocation of animals to study groups, daily prior to dose administration, and prior to euthanasia. Vaginal opening was examined daily following dosing and following the formation of complete vaginal opening. Vaginal smears were prepared daily to evaluate the overall cycling pattern.

3. Termination
Unscheduled and scheduled deaths were necropsied, trunk blood was collected, collected tissues (adrenals, kidneys, liver, thyroid, ovaries, uterus, pituitary) were grossly observed and weighed. Hormone concentrations (T4 and TSH) and clinical chemistry were analysed. Histopathology of uterus, ovary, kidney and thyroid was evaluated.

4. Statistics
Descriptive statistics (mean, standard deviation and count) were calculated using MS Excel. Final body weight, body weight gain, and tissue weights were analysed using SAS version 9.2. Studentized residual plots were used to detect possible outliers and Levene's test was used to assess homogeneity of variance. Homogeneous data were analysed by one-way ANOVA followed by pair wise comparisons using a Dunnett's one tailed t test (tissue weights) or Dunnett's two tailed t test (final body weight and body weight gain). Heterogeneous data were analysed using Kruskal-Wallis and Dunn's test. Statistically significant effects were reported when $p < 0.05$.

Findings

Dose-range finding study

All female rats survived until study termination except for two rats administered 1000 mg/kg bw/day captan that were euthanized due to body weight loss coincident with abnormal breathing after 12-14 days on study. No signs of dosing error were observed in these animals at necropsy. Female terminal body weights for animals administered 200, 400, 600, 800 and 1000 mg/kg bw/day captan were 92%, 88%, 91% and 93% of control body weight, respectively.

No abnormal cage-side or clinical observations performed 3 or 24 hours post-dose respectively were noted for females administered 0 (vehicle control), 200, 400, 600, or 800 mg/kg bw/day captan. Two animals administered 1000 mg/kg bw/day captan were observed with abnormal breathing (wheezing, gasping) and a distended abdomen. These findings

were coincident with body weight loss and both animals were euthanized prior to study termination. There were no changes in absolute or relative liver or kidney weights. At necropsy no gross lesions within the stomach or jejunum (small intestine) were observed.

Based on these data, a high dose level of 600 mg/kg bw/day was selected to meet the requirements of achieving an MTD in female rats.

A. Mortality

All female rats administered 0 or 300 mg/kg/day captan survived to scheduled euthanasia with no animals showing signs of moribundity. Two animals administered 600 mg/kg bw/day captan were euthanized prior to scheduled termination due to moribundity: one rat was euthanized on study day 5 (PND 26), and one was euthanized on study day 17 (PND 38). No signs of dosing error were observed at necropsy: gastrointestinal dilatation was observed in both animals.

B. Clinical observations

At 1 hour (± 30 minutes) post-dose administration, one rat administered 300 mg/kg bw/day captan was observed with decreased movement on study day 21 and one rat was observed with abnormal breathing on study day 5. No abnormal findings were observed 1 hour (± 30 minutes) post-dose in animals administered 600 mg/kg bw/day captan. At approximately 24 hours post-dose, one rat administered 300 mg/kg bw/day captan was observed with abnormal breathing (mild gasping) on study day 5, but recovered the following day. Two animals administered 600 mg/kg bw/day captan were observed with abnormal breathing (gasping) 24 hours post-dose: one rat on study day 5 and one rat on study day 17. Both animals were euthanized due to moribundity.

C. Body weight

A significant decrease in mean final body weight and body weight gain was observed in female rats administered 300 or 600 mg/kg bw/day captan compared to the vehicle control group. Final body weights of female rats administered 300 or 600 mg/kg bw/day captan were 90.6% and 90.2% of mean control body weight.

D. Vaginal Opening

Age of complete VO was significantly delayed for females administered 300 or 600 mg/kg bw/day captan compared to the vehicle control group. Mean age at completion of VO was PND 31.8 (vehicle controls), PND 35.8 (300 mg/kg bw/day captan) and PND 37.1 (600 mg/kg bw/day captan). Body weight at completion of VO was significantly increased for females administered 600 mg/kg bw/day captan compared to the vehicle control group.

Table 3.10.3.10-1 Vaginal opening and general growth

Parameter	51. Vehicle control (1% CMC)	52. Captan 300 53. mg/kg bw/day	54. Captan 600 55. mg/kg bw/day
Age at VO (PND)	31.8 ± 1.6	**35.8 ± 3.4[d]** **+12.6%**	**37.1 ± 1.9[d]** **+16.7%**
Body weight at VO (g)	114.4 ± 10.7	123.2 ± 16.2[d] +7.7%	**131.2 ± 8.1[d]** **+14.7%**
Initial body weight (PND 22, g)	58.3 ± 3.7	57.7 ± 5.1 -1.0%	58.2 ± 5.7 -0.2%
Final body weight (g)	171.2 ± 8.2	**155.1 ± 15.3**** **-9.4%**	**154.3 ± 12.2**** **-9.8**
Body weight gain (g)	112.8 ± 8.2	**97.3 ± 12.3***** **-13.7%**	**96.3 ± 9.6***** **-14.6%**

VO = vaginal opening,
PND = postnatal day,
d = Dunn's test (no p-value given), statistically significant if bold
** p < 0.01, *** p < 0.001 statistically different than vehicle control (Dunnet's test)

E. Estrous Cyclicity

Mean age at first estrus was significantly delayed in animals administered 300 or 600 mg/kg bw/day captan compared to the vehicle control group: PND 33.2 (vehicle control), 35.7 (300 mg/kg bw/day captan) and 38.4 (600 mg/kg bw/day captan). Animals administered 0 (vehicle control), 300 or 600 mg/kg bw/day captan exhibited similar cycle regularity and mean cycle length. However, a decreased trend was observed for regularly cycling animals.

Table 3.10.3.10-2 Estrous cyclicity and cycle status (number of animals)

Hormone	56. Vehicle Control (1% CMC)	57. 58. Captan 300 mg/kg bw/day	59. 60. Captan 600 mg/kg bw/day
Mean Age at First Estrus (PND)	33.2 ± 2.9	**35.7 ± 3.1*** **+7.5%**	**38.4 ± 2.3***** **+15.7%**
Mean Cycle Lenth (d)	4.5 ± 0.6	4.4 ± 0.7 -2.2%	4.8 ± 0.8 +6.7%
Cycling (%)	100	100	100
Regularly cycling (%)	100	94	87
Diestrus	9	3	8
Proestrus	1	3	3
Estrus	6	8	2
No cycling	0	0[a]	0[a]

* $p < 0.05$, *** $p < 0.001$ statistically different than vehicle control
[a] cycle of one animal undetermined

F. Necropsy

Small intestinal dilatation was observed in one animal administered 300 mg/kg bw/day captan and 12 animals administered 600 mg/kg bw/day captan. It is believed these findings are a result of a combination of the vehicle, test substance, and route of administration resulting in gavage-related reflux.

G. Tissue Weights

Absolute liver weight was significantly decreased in rats administered 300, but not 600 mg/kg bw/day captan and relative kidney weight was significantly increased in captan administered. Pituitary gland (relative weight at 600 mg/kg bw/day only) and ovary weights were significantly decreased in females administered 300 or 600 mg/kg bw/day captan and uterus (wet, adjusted weight only and blotted) weight significantly decreased in animals administered 600 mg/kg bw/day captan compared to the vehicle controls. There were no changes in adrenal gland and thyroid gland weights in captan administered animals.

Table 3.10.3.10-3 Tissue weights at necropsy

Tissue	Vehicle Control (1% CMC) (n=16)	Captan (300 mg/kg/day) (n=16)	Captan (600 mg/kg/day) (n=14)
Liver (g)	7.34 ± 0.51	**6.46 ± 1.00[d]** **-12.0%**	6.87 ± 1.03[d] -6.4%
Relative to bw	4.24 ± 0.17	4.11 ± 0.35 -3.1%	4.38 ± 0.36 +3.3%
Kidneys (g)	1.34 ± 0.1	1.26 ± 0.13 -6.0%	1.3 ± 0.13 -3.0%
Relative to bw	0.77 ± 0.04	**0.81 ± 0.03*** **+5.2%**	**0.83 ± 0.04***** **+7.8%**
Pituitary (mg)	9.8 ± 1.2	**8.0 ± 1.8**** **-18.4%**	**7.2 ± 1.3***** **-26.5%**
Relative to bw	5.7 ± 0.8	5.1 ± 0.9 -10.5%	**4.6 ± 0.8**** **-19.3%**
Adrenals (mg)	35.1 ± 6.0	35.7 ± 6.8 +1.7%	32.2 ± 7.4 -8.7%
Relative to bw	20.3 ± 3.5	22.9 ± 4.1 +12.8%	20.6 ± 3.9 +1.5%
Ovaries (mg)	62.7 ± 9.4	**51.3 ± 9.7**** **-18.2%**	**48.3 ± 7.8***** **-23.0%**

Uterus wet (mg) blotted	327.7 ± 127.2 289.3 ± 69.7	308.7 ± 169.4 -5.8% 255.5 ± 90.4 -11.7%	216.1 ± 83.5 -34.1% **192.1 ± 43.0**** **-33.6%**
Thyroid (mg)	10.71 ± 3.04	11.72 ± 2.09 +9.4%	10.04 ± 1.93 -6.3%

d = Dunn's test (no p-value given), statistically significant if bold
* p < 0.05, ** p < 0.01, *** p < 0.001 statistically different than vehicle control

H. Histopathology

Administration of captan to female rats was not associated with histopathologic changes in the uterus, left ovary, or left kidney. In ovary, the number of corpora lutea in animals administered 300 or 600 mg/kg bw/day captan was significantly decreased compared to vehicle controls, however; the number of small, medium, antral and atretic follicles, and follicular cysts were not significantly changed. Thyroid gland follicular cell height was statistically increased and colloid area decreased in animals administered 600 mg/kg bw/day captan.

Table 3.10.3.10-4 Number of ovary follicles, follicular cystas and corpora lutea

Test substance/control	**61. Vehicle Control (1% CMC)**	**62. Captan 300 63. mg/kg bw/day**	**64. Captan 600 65. mg/kg bw/day**
Small Follicles	29 ± 17	28 ± 15	29 ± 17
Medium Follicles	14 ± 3.6	13 ± 4.8 -7.1%	12 ± 4.8 -14.3%
Antral Follicles	9.8 ± 2.8	11 ± 4.8	10 ± 2.8
Atretic Follicles	31 ± 7.4	28 ± 10	33 ± 8.6
Follicular Cysts	0	0	0
Corpora Lutea	9.1 ± 1.9	**6.4 ± 2.4*** **-29.7%**	**6.5 ± 3.3*** **-28.6%**

* Significantly different compared to the vehicle control mean (Dunnett's test)

Table 3.10.3.10-5 Thyroid gland: Follicular cell height and colloid area

Test substance/control	**66. Vehicle Control (1% CMC)**	**67. Captan 300 68. mg/kg bw/day**	**69. Captan 600 70. mg/kg bw/day**
Follicular cell height			
1	0	0	0
2	11 (68.8%)	6 (37.5%)	**3 (21.4%)***
3	4 (25.0%)	8 (50.0%)	**3 (21.4%)***
4	1 (6.3%)	2 (12.5%)	**8 (57.1%)***
5	0	0	0
Follicular colloid area			
1	0	0	0
2	0	0	0
3	1 (6.3%)	1 (6.3%)	**8 (57.1%)***
4	15 (93.8%)	15 (93.8%)	**6 (42.9%)***
5	0	0	0

* Statistically significantly different compared to the vehicle control (Dunn's test, no p-value given)

Table 3.10.3.10-6 Uterus: Summary of histologic findings

Test substance/control	71. Vehicle Control (1% CMC)	72. Captan 300 73. mg/kg bw/day	74. Captan 600 75. mg/kg bw/day
No. examined	16	16	14[1]
Lumen dilatation			
Not present	15	15	14
Minimal	1	1	0
Mild	0	0	0
Endometrial folding			
Not present	7	6	11
Minimal	8	10	2
Mild	1	0	1
Cells or Debris in Endometrial Glands or Lumen			
Not present	13	16	13
Minimal	3	0	1
Endometrial Edema			
Not present	16	15	12
Minimal	0	1	2

[1] two rats were euthanized prior to study termination, and no tissues were collected for histologic evaluation

I. Hormone and Clinical Chemistry Analysis

The serum concentration of T4 was significantly decreased below the normal range in female rats administered 300 or 600 mg/kg bw/day captan compared to the control group. No significant changes in serum TSH concentrations were detected following administration of either 300 or 600 mg/kg bw/day captan compared to the vehicle control group. Mean group serum hormone and clinical chemistry concentrations results are shown in the following table.

Table 3.10.3.10-7 Serum hormone and clinical chemistry levels

Test	76. Vehicle Control (1% CMC)	77. Captan 300 mg/kg bw/day	78. Captan 600 mg/kg bw/day	79. Normal range
Serum T4 (µg/dL)	3.69 ± 0.68	**2.68 ± 0.79*** **-27.4%**	**2.15 ± 0.50*** **-41.7%**	**3-7**
Serum TSH (ng/mL)	1.65 ± 0.46	1.35 ± 0.36 -18.2%	1.39 ± 0.40 -15.8%	0.57-3.41
Na^+ (MEQ/L)	145 ± 4	147 ± 1 +1.4%	**150 ± 2*** **+3.4%**	**141-151**
K^+ (MEQ/L)	8.2 ± 0.4	8.0 ± 0.7	8.3 ± 0.6	5-7.6
Cl^- (MEQ/L)	109 ± 4	113 ± 1 +3.7%	**115 ± 1*** **+5.5%**	**84-109**
Calcium (mg/dL)	9.7 ± 0.5	9.3 ± 0.7	9.4 ± 0.8	9.4-13.8
Phosphorous (mg/dL)	10.2 ± 1.4	10.8 ± 1.3 +5.9%	11.1 ± 0.9 +8.8%	4.7-9.5
AST (U/L)	250 ± 0.49	275 ± 37 +10.0%	279 ± 73 +11.6%	68-192
ALT (U/L)	47 ± 12	53 ± 15 +12.8%	55 ± 18 +17.0%	20-56
GGT (U/L)	0 ± 0	0 ± 0	0 ± 0	0-4
ALP (U/L)	252 ± 50	275 ± 37 +9.1%	279 ± 73 +10.7%	17-183
BUN (mg/dL)	12 ± 5	12 ± 3	13 ± 3	12-23
Creatinine (mg/dL)	0.4 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	0.5-1.1
Total Bilirubin (mg/dL)	0.03 ± 0.06	0.01 ± 0.03 -66.7%	0.00 ± 0.00 -100.0%	0-0.5
SDH (U/L)	27 ± 2	27 ± 1	**29 ± 2*** **+7.4%**	**13-37**
Total Protein (g/dL)	6.2 ± 0.4	6.0 ± 0.4	6.1 ± 0.3	4.8-9.2
Albumin (g/dL)	5.1 ± 0.3	5.1 ± 0.5	4.8 ± 0.4	2.9-6

Abbreviations: T4 = thyroxine, TSH = thyroid-stimulating hormone, T = testosterone Na+ = sodium, K+ = potassium, Cl- = chloride, AST = aspartate aminotransferase, ALT = alanine aminotransferase, GGT = gamma glutamyltransferase, ALP = alkaline phosphatase, BUN = blood urea nitrogen, SDH = sorbitol dehydrogenase
* Statistically significant compared to the vehicle control mean

Conclusions

Administration of captan to intact juvenile/peripubertal female rats at dose levels of 300 or 600 mg/kg/day approached, but did not exceed, the MTD. A significant delay in vaginal opening, day of first estrus, decreased number of corpora lutea, and decreased pituitary gland, ovarian, and uterine weights suggest an effect on pubertal development. In addition, there was a decrease in serum T4 concentrations, without concomitant increases in TSH concentrations (both dose levels), and histopathological changes in the thyroid were observed (statistically significant at 600 mg/kg bw/day) following administration of captan.

3.10.3.11 Study 17 (2012)

Reference:	Captan: Steroidogenesis (Human Cell Line – H295R)
Author(s), year:	Anonymous (2012)
Report/Doc. number:	CeeTox Inc., MI, USA; document no. 9141V-100374STER, R-28290
Guideline(s):	OPPTS 890.1550 (2009)
GLP:	Yes
Deviations to OECD 456 (2011):	No moderate and weak inducer/inhibitor used in the proficiency test performed (only the strong inducer forskolin and the strong inhibitor prochloraz were tested); in case of positive first run, test should be repeated by refining the selected test concentrations using ½-log concentration spacing, whereas here the test was repeated without refining the test concentrations; invalid confirmatory Run 4
Acceptability:	Limited

Summary

In a steroidogenesis assay using H295R human adrenocortical carcinoma cell line, the ability of captan to affect the steroidogenic pathway, beginning with the sequence of reactions occurring after the gonadotropin hormone receptors through the production of testosterone and estradiol/estrone, was investigated.
Four independent experiments (Run 1 was not analysed) were conducted with captan at concentrations of 0.0001, 0.001, 0.01, 0.1, 1, 10 and 100 µM along with the reference and solvent chemicals. Captan exposure was associated with a statistically significant decrease in estradiol concentration at 0.001, 1, and 10 µM captan concentrations. In Run 3, a statistically significant decrease in testosterone was observed at the 10 µM. No other statistically significant changes in testosterone or estradiol were observed in any of the independent runs of the assay at any of the captan concentrations that could be analysed.

Material and Methods

1. Test Material: Captan, Merpan Tech
   - Description: White powder
   - Lot/Batch #: 91138103
   - Purity: 93%
   - Stability: Expiry date 14 February 2013
   - Storage conditions: At room temperature
   - Solvent used: Dimethylsulfoxide (DMSO)

2. Control Materials:
   - Solvent control: DMSO at 0.05% (Sigma, Lot No. RNBB7617)
     Ethanol at 0.025% (solvent for 22R-hydroxycholesterol)
   - Reference chemicals: Forskolin (inducer of steroidogenesis) (Sigma, 98% purity, Lot No 109K50571V)
     Prochloraz (inhibitor of steroidogenesis) (Sigma, 99.1% purity, Lot No SZE6220X)
   - Medium supplement: 22R-Hydroxycholesterol (to increase basal hormone production) (Sigma, 99% purity, Lot No 089K4132, 060M4098)

4. Test concentrations:
   - Captan: 0.0001, 0.001, 0.01, 0.1, 1, 10, and 100 µM
   - Forskolin: 1 and 10 µM
   - Prochloraz: 0.1 and 1 µM

TEST PERFORMANCE:
06.06.2011 - 12.09.2011 at CeeTox Inc., Kalamazoo, MI, USA

1. Experimental Procedure
Cell seeding & chemical exposure
Cells were plated into wells of a 24 well cell culture plate at a density of ~300,000 cells/mL and incubated at 5% CO_2 and 37 ± 1°C for at least 24 hours prior to chemical exposure. With each independent run of captan, a concurrent QC plate was included containing background, blank, solvent control and reference control wells.
After addition of the reference / test chemical dosing solutions, the plates were incubated in a 5% CO_2 incubator at 37°C for 48 hours. Following incubation, each well was observed microscopically and the media was collected from all wells

in two equal aliquots and stored at -80°C until for hormone measurement. After media removal, cell viability was measured in all plates by MTT assay.

Cytotoxicity Assay
Cell viability was monitored by MTT assay after the 48 hour exposure. The change in cell viability was determined by comparing treated wells to the solvent control wells. A greater than 20% reduction in cell viability was considered evidence of cytotoxicity.

Precipitation Assay
Observations for precipitation were made after the final dilutions of test chemical in media and after the 48 hour incubation period using the microscope. If precipitation was observed, the concentration was considered insoluble and was excluded from further analysis.

Hormone Measurement System
Testosterone and estradiol levels were measured using HPLC/MS-MS. The method detection limit is 100 pg/mL for testosterone and 10 pg/mL for estradiol.

2. Evaluation Criteria
In all three independent runs of the assay, the basal production of testosterone and estradiol on the quality control plates was above the required levels. The fold change required after induction with 10 µM forskolin and inhibition with 1 µM prochloraz met the requirements.
The coefficients of variation for solvent control replicate wells within a plate (based on absolute concentrations) and the coefficients of variation for the solvent control wells between plates (based on fold change) for testosterone and estradiol were less than 30%.

Findings

The suitable top concentration of captan that could be analysed was 10 µM in Runs 2 and 3 because of cytotoxicity greater than 20% at the 100 µM concentration. The top concentration of captan that could be analysed in Run 4 was 0.01 µM because of precipitation observed at the 0.1, 1, 10, and 100 µM concentrations.

Table 3.10.3.11-1 Results for testosterone and estradiol concentrations

Captan concentration (µM)	Fold change over SC Run 2		Fold change over SC Run 3		Fold change over SC Run 4	
	Mean	SD	Mean	SD	Mean	SD
Testosterone						
0.0001	1.03	0.03	1.00	0.03	0.93	0.02
0.001	0.95	0.02	0.97	0.03	0.92	0.04
0.01	0.96	0.01	0.98	0.06	0.97	0.04
0.1	0.99	0.01	1.01	0.04	N/A	N/A
1	1.00	0.03	1.04	0.02	N/A	N/A
10	0.94	0.02	0.91*	0.05	N/A	N/A
100	N/A	N/A	N/A	N/A	N/A	N/A
Estradiol						
0.0001	1.00	0.01	0.97	0.02	0.94	0.01
0.001	0.88*	0.03	1.00	0.04	0.93	0.02
0.01	0.96	0.02	0.99	0.03	0.96	0.05
0.1	0.97	0.00	1.00	0.02	N/A	N/A
1	0.91*	0.03	0.99	0.03	N/A	N/A
10	0.75*	0.02	0.94	0.03	N/A	N/A
100	N/A	N/A	N/A	N/A	N/A	N/A

SC = Solvent Control
SD = Standard Deviation
N/A = concentration not analyzed because of cytotoxicity and/or precipitation
* Statistical significance ($p \leq 0.05$)

In Run 2, statistically significant decreases in estradiol were observed at the 0.001, 1 and 10 µM captan concentrations. According to testing guideline OECD 456 (2011), the results of a run are positive if the fold-change is statistically

significant from the solvent control at two or more adjacent conentrations, which is the case for Run 2. In case of a positive first test run, the test should be repeated by refining the selected test concentrations using ½ log concentration spacing (e.g. 0.1, 0.3, 1, 3, 10, 30, 100 µM). In contrast to the recommendations of OECD 456, the test was repeated with the original concentrations.
In Run 3, a statistically significant decrease in testosterone was observed at the 10 µM captan concentration. According to OECD 456, the results of a run are equivocal if the fold-change is statistically significant from the solvent control at only one concentration.
No statistically significant changes in testosterone or estradiol concentration were observed in Run 4. However, only concentrations up to 0.01 µM captan were evaluated as precipitation was observed at 0.1 µM and above. Therefore, the results of Run 4 cannot be used to confirm the results of Runs 2 and 3, where decreases in hormone levels were observed at higher concentrations. Furthermore, it is unclear why precipitation occurred in Run 4 at concentrations of 0.1 µM and above, but did not occur in the two previous runs at concentrations up to 100 µM.

Conclusions

Statistically significant decreases in estradiol concentration were observed at three of the captan concentrations (0.001, 1 and 10 µM) analysed in one run and a statistically significant decrease in testosterone was observed at the 10 µM captan concentration analysed in another run. Another (confirmatory) run was considered invalid since concentrations of 0.1 µM captan and above were excluded from analysis due to precipitation, which did not occur in the first two runs at concentrations up to 100 µM. No refinement of test concentrations was done after the positive result of the first run.

According to testing guideline OECD 456 (2011), the H295R steroidogenesis assay is considered positive in case of one equivocal and one positive run. In the steroidogenic pathway, conversion of androgenic substances into estrogenic substances occurs: Androstenedione is converted to testosterone by 17β-HSD (17β-hydroxysteroid dehydrogenase). The enzyme CYP19 (aromatase) converts testosterone into estradiol and androstenedione into estrone, which is converted into estradiol by 17β-HSD.

If results for estradiol and testosterone are considered separately, a valid third confirmatory run is missing in order to interpret the experimental results conclusively (OECD 456: “A chemical is judged to be negative following two independent negative results, or in three runs, comprising two negative runs and one equivocal or positive run.”).
Therefore, the experimental results are not interpretable.

3.11 Specific target organ toxicity – single exposure

3.11.1 Animal data

No relevant studies.

3.11.2 Human data

No relevant studies.

3.11.3 Other data

No relevant studies

3.12 Specific target organ toxicity – repeated exposure

3.12.1 Animal data

3.12.1.1 Study 1 (1987)

Reference:	Four week oral range-finding study in Beagle dogs with Captan
Author(s), year:	Anonymous (1987)
Report/Doc. number:	International Research and Development Corporation, report No. IRDC 153-197 (Company file: R-4689/TMN-0869)
Guideline(s):	Not applicable, an OECD guideline for 28 day studies with non-rodents is not available; the study generally met requirements of EEC Method B.7 except that functional observations were not recorded
GLP:	Yes
Deviations:	Not applicable
Acceptability:	Yes as supplemental due to low animal number (2/group/sex)

Material and Methods

In a four week study, Captan (purity 90.4%; batch number WRC 4921-26-15) was administered in gelatine capsules to beagle dogs (2 animals/sex/dose) at dosage levels of 30, 100 300, 600 and 1000 mg/kg bw/day in order to establish dosage levels for a one year study. A control group was administered empty gelatine capsules (2 animals/sex). Parameters evaluated were clinical signs (daily), body weight and food intake (weekly), haematology and clinical chemistry (after 4 weeks in fasted animals), organ weights (kidney, liver), gross pathology and histopathology (duodenum, kidney, liver).

Findings

There were no mortalities throughout the study. Emesis was noted for all animals treated with the test substance and was observed most frequently at 300 mg/kg bw/day and above, sometimes twice daily. The emesis was noted as yellow in colour (the test material is white). Reduced food consumption was noted for most treated groups compared to controls, with two males, each one at 600 and 1000 mg/kg bw/day, showing marked inappetence.
Test substance related and dose related effects on body weights were observed in this study. By week four, animals at 600 and 1000 mg/kg bw/day showed overall weight loss (16.0% and 14.8% of pre-test weight in males and females at 600 mg/kg bw/day, and 27.0% and 18.9% of pre-test weight in males and females at 1000 mg/kg bw/day. Animals at 300 mg/kg bw/day did not exceed the pre-test weight at any interval during the study. Animals at lower doses gained very little weight during the study.

Table 3.12.1.1-1 Group mean bodyweights and bodyweight change

Dosage level (mg/kg bw/day)	**Mean bodyweight (kg)**					
	Males			**Females**		
	Pre-test	**Week 4**	**% change[1]**	**Pre-test**	**Week 4**	**% change[1]**
0	10.2	11.2	+9.8	8.9	9.3	+4.5
30	9.4	10.0	+6.4	9.4	9.6	+2.1
100	10.7	10.9	+1.9	8.6	9.3	+8.1
300	10.3	9.9	-3.9	8.2	8.0	-2.4
600	10.0	8.4	-16.0	8.8	7.5	-14.8
1000	10.6	7.7	-27.0	9.0	7.3	-18.9

[1] percent difference from pre-test bodyweights

Table 3.12.1.1-2 Average food consumption (g/kg bw/day) from weeks 1 through 4

Dosage level (mg/kg bw/day)	Males		Females	
	Average food consumption (g/kg bw/day)	% difference from control	Average food consumption (g/kg bw/day)	% difference from control
0	28.3		25.3	
30	25.5	-9.9	20.8	-17.8
100	22.1	-21.9	25.2	-0.4
300	24.3	-14.1	21.2	-16.2
600	22.1	-21.9	15.5	-38.7
1000	15.7	-44.5	16.3	-35.6

Table 3.12.1.1-3 Incidence of emesis per sex and dose group (2 anmials/sex/dose)

Dosage level (mg/kg bw/day)	Males		Females	
	Incidence of emesis	Study days	Incidence of emesis	Study days
0	0	-	0	-
30	3	D1-11	6	D1-20
100	5	D3-19	8	D3-28
300	10	D1-15	11	D2-15
600	25	D1-18	10	D1-18
1000	9	D1-11	13	D1-17

There were no treatment-related effects on haematological parameters. The few variations that were observed in clinical chemistry (see Table 3.12.1.1-4) were considered probably related to the emesis, poor food consumption and loss in body weight at higher dose levels.
No treatment-related gross pathological changes were observed. Absolute organ weights were unaffected by treatment. Regarding the microscopic changes, one male at 1000 mg/kg bw/day showed mild, multifocal fatty changes in the liver and mild, multifocal, bilateral fatty changes in the collecting tubules (cortex) of the kidneys. However, due to the small number of animals per group, the toxicological significance of this lesion is equivocal.

Table 3.12.1.1-4 Mean biochemical values (number of animals = 2/group/sex)

Parameter measured, Week of study	Dosage level (mg/kg bw/day)					
	0	30	100	300	600	1000
Males						
Chloride [mEq/L], pre-test	115	113	115	113	114	115
Chloride [mEq/L], week 4	116	116	117	116	124	121
Calcium [mg/dL], pre-test	10.6	10.3	10.6	10.7	10.5	10.4
Calcium [mg/dL], week 4	11.0	10.8	10.6	10.7	9.9	9.6
Cholesterol [mg/dL], pre-test	163	175	199	170	179	193
Cholesterol [mg/dL], week 4	168	183	193	171	165	257
Glucose [mg/dL], pre-test	121	116	118	122	124	113
Glucose [mg/dL], week 4	124	118	109	118	114	83
Total protein [g/dL], pre-test	5.1	5.0	5.2	5.5	5.2	5.1
Total protein [g/dL], week 4	5.0	4.7	4.6	4.9	4.0	3.8
Albumin [g/dL], pre-test	3.1	3.0	3.1	3.3	3.2	3.1
Albumin [g/dL], week 4	3.3	3.1	3.0	3.0	2.5	2.3
Females						
Chloride [mEq/L], pre-test	111	113	115	115	112	116
Chloride [mEq/L], week 4	115	120	118	120	121	122
Calcium [mg/dL], pre-test	10.7	10.5	10.6	10.9	10.5	10.2
Calcium [mg/dL], week 4	11.2	11.0	11.0	10.7	10.2	10.2
Cholesterol [mg/dL], pre-test	203	186	155	191	208	143
Cholesterol [mg/dL], week 4	202	185	156	198	197	164
Glucose [mg/dL], pre-test	111	116	113	108	112	109
Glucose [mg/dL], week 4	110	122	115	118	109	109
Total protein [g/dL], pre-test	5.1	5.1	5.0	5.1	5.1	5.0
Total protein [g/dL], week 4	4.9	4.7	4.7	4.4	4.0	4.1
Albumin [g/dL], pre-test	3.2	3.3	3.4	3.3	3.4	3.2
Albumin [g/dL], week 4	3.3	3.3	3.3	3.0	2.7	2.6

Conclusion

The study recommended a top dose level for the one year dog study of 100 mg/kg bw/day, based on marked emesis, poor food consumption and actual body weight loss over the course of four weeks seen at 300 mg/kg bw/day and above.
The NOAEL was set at 100 mg/kg bw/day.

3.12.1.2 Study 2 (1988)

Reference:	One year oral toxicity study in dogs with Captan technical
Author(s), year:	Anonymous (1988)
Report/Doc. number:	International Research and Development Corporation, report No. IRDC 153-198 (Company file: R-5284 / TMN-0764)
Guideline(s):	USEPA Guideline 83-1
GLP:	Yes
Deviations from OECD 452 (2009) and OECD 409 (1998):	The essential criteria of OECD testing guidelines are met.
Acceptability:	Yes

Materials and Methods

In a one year study, captan (purity 90.4%) was administered in gelatine capsules to beagle dogs at dosage levels of 12.5, 60 and 300 mg/kg bw/day (5/sex/dose). A control group was administered empty gelatine capsules (5/sex). Parameters evaluated were clinical signs (twice daily), body weight and food intake (pretest, weekly until week 14 and every four weeks thereafter), ophthalmoscopic examination (pretest and at 6 and 12 months of the study), physical examination (pretest and at 3, 6, 9 and 12 months), haematology, clinical chemistry and urinalysis (before test initiation and at 3, 6, 9

and 12 months in fasted animals), organ weights (adrenal, brain, eye, heart, kidney, liver, ovary, pituitary, testis, prostate, thyroid/parathyroid), gross pathology and histopathology.

Findings

There were no mortalities throughout the study. A higher incidence of emesis and soft/mucoid stool was sporadically noted in 300 mg/kg bw/day animals compared to the control, although these were also noted in other groups including controls. These observations were considered to be related to the administration of the test substance.

There were no significant differences in group mean body weights at any interval in males and females. Food consumption values for females administered test substance appeared lower than control females. However, food consumption in the control group females was considered higher than normal and therefore no toxicological significance is attributed to the apparently low food consumption in the 60 and 300 mg/kg bw/day animals.

No treatment-related ophthalmological abnormalities were detected. Physical examination findings revealed no significant findings. There were no treatment-related effects on haematological or clinical chemistry parameters, and urinalysis was considered normal. Occasional statistical differences between test substance treated groups and controls were considered to be of no toxicological significance by the study authors, since differences were small and were considered within the range of normal biological variations.

No treatment-related gross pathological changes were observed. A significant increase in the relative liver weight was observed in 300 mg/kg bw/day males compared to the control. This was considered to be related to the lower body weight observed in this group compared to the control and not treatment-related. Apart from that, no further statistically significant organ weights changes were observed in any of the treatment groups. However, absolute liver weights were slightly increased in the top dose group (+11% and +11.7% compared to controls in males and females, respectively). No treatment-related histopathological changes were observed.

Table 3.12.1.2-1 Mean body weights, average group mean food consumption from weeks 1 through 52, frequency of emesis, soft/mucoid stool or slight diarrhea per sex and group throughout the study, mean absolute and relative liver weights and clinical chemistry parameters

Dosage level (mg/kg bw/day)	**Males**				**Females**			
	0	**12.5**	**60**	**300**	**0**	**12.5**	**60**	**300**
Bodyweight ± SD (kg) pretest	12.2 ± 3.33	11.7 ± 2.08	11.1 ± 1.36	11.7 ± 2.24	9.0 ± 1.99	9.3 ± 1.23	9.3 ± 1.39	9.4 ± 1.43
Bodyweight ± SD (kg) 52 weeks	13.3 ± 3.95	12.9 ± 2.36	11.8 ± 1.51	12.6 ± 2.91	9.4 ± 2.53	10.1 ± 1.96	10.8 ± 1.89	10.0 ± 1.28
% difference from pretest	+9.0	+10.3	+6.3	+7.7	+4.4	+8.6	+16.1	+6.4
Food consumption (g/kg bw/d)	30.1	33.8	38.0	34.5	44.4	31.5	32.1	36.5
% difference from control	-	+12.3	+26.2	+14.6	-	-29.1	-27.7	-17.8
Emesis[1]	3 (2/5)	4 (3/5)	3 (2/5)	15 (5/5)	7 (4/5)	3 (2/5)	11 (3/5)	17 (5/5)
Soft or mucoid stool[1]	4 (3/5)	5 (3/5)	6 (2/5)	14 (4/5)	2 (2/5)	2 (2/5)	3 (1/5)	6 (4/5)
Diarrhea[1]	-	-	-	1 (1/5)	3 (2/5)	-	-	2 (2/5)
combined	4 (3/5)	5 (3/5)	6 (2/5)	15 (4/5)	6 (3/5)	2 (2/5)	3 (1/5)	8 (4/5)
Liver weight ± SD (g)	277.82 ± 48.398	274.81 ± 47.972	280.50 ± 57.223	308.41 ± 85.617 *(+11%)*	220.56 ± 43.836	236.54 ± 51.600	226.38 ± 44.387	246.33 ± 32.778 *(+11.7%)*
Liver/body	2.13	2.13	2.34	**2.46***	2.40	2.36	2.15	2.49

Dosage level (mg/kg bw/day)	Males				Females			
	0	12.5	60	300	0	12.5	60	300
weight (%)								
Total protein (xxx/dL) pretest	5.1	5.1	5.1	4.9	5.1	4.9	5.0	4.9
Total protein (xxx/dL) 12 months	6.3	6.4	6.3	**5.8***	6.2	5.7	6.2	6.1
Albumin (xxx/dL) pretest	3.3	3.3	3.3	3.3	3.3	3.4	3.3	3.4
Albumin (xxx/dL) 12 months	3.6	3.5	3.5	**3.2****	3.5	3.5	3.3	3.3
Alkaline phosphatase (xxx/L) pretest	71	74	65	63	61	59	63	76
Alkaline phosphatase (xxx/L) 3 months	38	38	45	48	37	32	55	**63***
Alkaline phosphatase (xxx/L) 6 months	31	31	32	36	28	27	38	**52****
Alkaline phosphatase (xxx/L) 9 months	26	27	30	37	30	23	36	49
Alkaline phosphatase (xxx/L) 12 months	27	24	34	41	30	23	43	50

[1] Frequency of observation per group throughout the study (animals affected)
* Significantly different from control group; $p < 0.05$
** Significantly different from control group; $p < 0.01$

Conclusion

In conclusion, captan was well tolerated by dogs when administered orally in gelatine capsules at dose levels up to 300 mg/kg bw/day for 52 weeks. In accordance with the EFSA conclusion on the peer review of captan (EFSA Scientific Report (2009) 296, 1-90), the NOAEL for this study is set at 60 mg/kg bw/day based on liver weight increase greater than 10% and statistically significant decreases in total plasma protein and albumin in males dosed with 300 mg/kg bw/day and increased alkaline phosphatase activity in females dosed with 300 mg/kg bw/day, and based on a slightly higher incidence of emesis and soft/mucoid stool at 300 mg/kg bw/day in both sexes.

3.12.1.3 Study 3 (1987)

Reference:	Twenty-one day dermal toxicity study in rabbits with technical Captan
Author(s), year:	Anonymous (1987)
Report/Doc. number:	International Research and Development Corp., report No. 415-046 (Company file: R-4666/TMN-0870)
Guideline(s):	US EPA guideline 82-2
GLP:	Yes
Deviations from OECD 410 (1981):	No
Acceptability:	Yes

Material and Methods

The local and systemic tolerance of captan (purity and batch no. not specified in report) was examined in a subacute dermal toxicity study on New Zealand White rabbits (5/sex/dose group). The test substance was applied as a paste (vehicle: deionized water) to a shaved area on the dorsal trunk at dosage levels of 12.5, 110 and 1000 mg/kg bw covering an area of approximately 5, 10 and 90% of the application site, respectively. The control animals were administered 2.2 mL vehicle (equal to the amount of vehicle applied to the high dose group). The test area was covered with an occlusive dressing. The dressing was removed after 6 hours and the test site was washed in tepid tap water. Animals were treated five days per week, over a period of 21 days.

Parameters evaluated were clinical signs (twice daily), general appearance, behaviour and pharmacotoxic signs and dermal irritation (pretest and on days 2, 4, 8, 11, 15, 18 and 21), body weight (pretest and twice weekly), food intake (weekly), haematology and clinical chemistry (on day 21), gross pathology, organ weights (adrenal, brain, kidney, liver, ovary, testis) and histopathology (adrenal, brain, kidney, liver, lung, ovary, testis, skin, spleen and gross lesions).

Findings

General observations:
One female from the 110 mg/kg bw group was found dead on day 9, but the death was considered to be unrelated to exposure of captan. No test substance related clinical signs were observed. In females, the incidence of no stool, or diarrhea was higher in the 1000 mg/kg group compared to control, but was considered not test article related.

Table 3.12.1.3-1 Subacute dermal toxicity in rabbits: summary of clinical findings (number of animals)

Observation	0 mg/kg bw	12.5 mg/kg bw	110 mg/kg bw	1000 mg/kg bw
Males				
Diarrhea	1	0	0	0
No stool	1	0	0	0
Females				
Diarrhea	0	0	0	1
No stool	0	0	1	3

Body weight gain and food consumption in 12.5 and 110 mg/kg bw animals were comparable to the control animals. The body weight of 1000 mg/kg females was significantly lower than the control on day 22. The high dose group consistently consumed less food than the control group throughout the study. Significant differences in food consumption were noted between the 1000 mg/kg bw dose group and the control in weeks 2 and/or 3.

Table 3.12.1.3-2 Subacute dermal toxicity in rabbits: summary of female body weights (mean ± sd)

Day	Body weight (g)			
	0 mg/kg bw	**12.5 mg/kg bw**	**110 mg/kg bw**	**1000 mg/kg bw**
0	2422 ± 113.1	2441 ± 135.8	2414 ± 175.4	2407 ± 175.3
3	2430 ± 137.9	2450 ± 140.4	2429 ± 199.7	2302 ± 158.2
7	2433 ± 281.2	2534 ± 140.1	2522 ± 250.0	2290 ± 215.3
10	2588 ± 126.8	2585 ± 143.1	2664 ± 264.0	2336 ± 167.5
14	2680 ± 156.4	2684 ± 170.7	2742 ± 304.2	2387 ± 220.1
17	2745 ± 156.6	2736 ± 179.4	2830 ± 324.7	2387 ± 201.9
22	2793 ± 125.6	2819 ± 171.4	2893 ± 333.8	**2429 ± 163.0***

* Significantly different from the control ($p < 0.05$).

Table 3.12.1.3-3 Subacute dermal toxicity in rabbits: summary of food consumption (mean ± sd)

Week	Food consumption (g/animal/day)			
	0 mg/kg bw	**12.5 mg/kg bw**	**110 mg/kg bw**	**1000 mg/kg bw**
males				
1	127.9 ± 59.57	163.8 ± 85.35	144.4 ± 20.99	81.1 ± 13.93
2	170.7 ± 35.82	159.2 ± 33.12	161.3 ± 11.05	**110.3 ± 26.10***
3	132.3 ± 23.58	129.9 ± 41.78	134.9 ± 11.34	110.9 ± 12.18
females				
1	124.0 ± 52.41	150.3 ± 17.75	128.7 ± 41.98	76.5 ± 35.90
2	159.7 ± 27.28	161.3 ± 18.86	160.0 ± 23.14	**107.4 ± 15.84****
3	137.4 ± 15.75	137.1 ± 16.33	135.0 ± 14.96	**92.3 ± 22.23****

* Significantly different from the control ($p < 0.05$); ** significantly different from the control ($p < 0.01$).

Local findings:
There were no signs of dermal irritation observed at the application sites in the control animals. Animals in the 12.5 mg/kg bw and 110 mg/kg bw were also normal apart from slight desquamation noted in one female at 12.5 mg/kg bw on day 21 and slight desquamation observed in two females at 110 mg/kg bw on day 21. In the 1000 mg/kg bw group, very slight erythema, very slight oedema and desquamation were observed starting at day 4 in some animals.

Table 3.12.1.3-4 Summary of dermal findings in the 1000 mg/kg bw dose group

Dermal Sign		Days													
		2		4		8		11		15		18		21	
		m	f	m	f	m	f	m	f	m	f	m	f	m	f
Erythema:	None	5	5	5	3	5	4	5	5	5	4	2	2	2	2
	Very slight	0	0	0	**2**	0	**1**	0	0	0	**1**	**3**	**3**	**3**	**3**
	Well defined	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Moderate to severe	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Severe	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Oedema:	None	5	5	5	5	5	5	5	5	5	5	2	3	2	3
	Very slight	0	0	0	0	0	0	0	0	0	0	**3**	**2**	**3**	**2**
	Slight	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Moderate	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Severe	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Atonia:	Normal	5	5	5	5	5	5	5	5	5	5	5	4	5	5
	Slight	0	0	0	0	0	0	0	0	0	0	0	**1**	0	0
	Moderate	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Marked	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Desquamation:	None	5	5	5	5	5	5	5	5	5	3	1	1	1	1
	Slight	0	0	0	0	0	0	0	0	0	**2**	**4**	**4**	**4**	**3**
	Moderate	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**
	Marked	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Number of animals		5	5	5	5	5	5	5	5	5	5	5	5	5	5

m=males, f=females

Haematology and clinical chemistry: There were no treatment-related effects on haematological or clinical chemistry parameters.

Postmortem examination: Treatment-related changes noted at autopsy were observed at the dermal application sites and were limited to mild desquamation in one 12.5 mg/kg bw female and in one male and three females from the 1000 mg/kg bw dose group. Microscopic changes to the site of application occurred in all dose groups, however there was no apparent dose-relationship.
Organ weight changes were not considered to be related to treatment. In males of the mid dose group there was a statistically significant decrease in mean brain weight, relative to body weight, and a statistically significant increase in mean right kidney weight, relative to brain weight, both without dose-reponse relationship and therefore not considered test article related. In females of the 1000 mg/kg group, there was a statistically significant increase in mean brain weight, relative to body weight, which however was attributed to the statistically significant decrease in the final mean body weight of this group.

Table 3.12.1.3-5 Subacute dermal toxicity in rabbits: Organ weight findings (n=4-5; mean ± standard deviation)

Organ	**0 (mg/kg bw/day)**	**12.5 (mg/kg bw/day)**	**110 (mg/kg bw/day)**	**1000 (mg/kg bw/day)**
Males				
Body Weight	2.802 ± 0.3075	2.865 ± 0.2518	2.915 ± 0.1454	2.642 ± 0.1455
Brain (g)	9.13 ± 0.621	8.96 ± 0.555	8.08 ± 0.963	9.11 ± 0.275
Relative (to body, % x 10)	3.27 ± 0.273	3.15 ± 0.314	2.77 ± 0.336*	3.45 ± 0.170
Liver (g)	104.22 ± 21.209	116.24 ± 20.600	115.44 ± 8.652	92.79 ± 13.201
Relative (to body, %)	3.72 ± 0.625	4.04 ± 0.401	3.96 ± 0.263	3.50 ± 0.341
Relative (to brain, % x 10^{-2})	11.44 ± 2.258	13.05 ± 2.796	14.48 ± 2.212	10.17 ± 1.228
Adrenal, Left (g)	0.15 ± 0.074	0.12 ± 0.037	0.13 ± 0.018	0.14 ± 0.030
Relative (to body, % x 10^3)	5.33 ± 2.408	4.11 ± 1.271	4.54 ± 0.686	5.38 ± 1.184
Relative (to brain, % x 10)	16.28 ± 7.255	13.01 ± 3.340	16.64 ± 3.700	15.67 ± 3.780
Adrenal, Right (g)	0.15 ± 0.030	0.12 ± 0.027	0.13 ± 0.022	0.13 ± 0.025
Relative (to body, % x 10^3)	5.26 ± 1.199	4.08 ± 0.981	4.42 ± 0.885	5.00 ± 0.964
Relative (to brain, % x 10)	15.99 ± 2.990	12.91 ± 2.734	16.04 ± 3.167	14.56 ± 3.124
Kidney, Left (g)	8.76 ± 1.035	8.57 ± 1.305	8.98 ± 0.822	7.87 ± 0.936
Relative (to body, %)	0.31 ± 0.035	0.30 ± 0.028	0.31 ± 0.030	0.30 ± 0.023
Relative (to brain, %)	95.80 ± 6.693	96.30 ± 18.923	111.74 ± 10.166	86.32 ± 8.640
Kidney, Right (g)	8.18 ± 0.892	8.43 ± 0.972	8.70 ± 0.833	7.67 ± 0.695
Relative (to body, %)	0.29 ± 0.028	0.29 ± 0.017	0.30 ± 0.032	0.29 ± 0.015
Relative (to brain, %)	89.61 ± 6.984	94.54 ± 14.620	108.53 ± 13.688*	84.18 ± 6.134
Testis, Left (g)	1.87 ± 0.246	2.01 ± 0.172	1.95 ± 0.317	1.78 ± 0.236
Relative (to body, %)	0.07 ± 0.011	0.07 ± 0.011	0.07 ± 0.010	0.07 ± 0.009
Relative (to brain, %)	20.60 ± 3.320	22.51 ± 2.747	24.69 ± 6.638	19.53 ± 2.755
Testis, Right (g)	1.85 ± 0.277	2.02 ± 0.194	1.98 ± 0.319	1.76 ± 0.108
Relative (to body, %)	0.07 ± 0.010	0.07 ± 0.011	0.07 ± 0.009	0.07 ± 0.006
Relative (to brain, %)	20.39 ± 3.463	22.66 ± 2.845	24.99 ± 6.467	19.38 ± 1.625
Females				
Body Weight	2.829 ± 0.1457	2.837 ± 0.1709	2.920 ± 0.3197	2.445 ± 0.1411*
Brain (g)	8.68 ± 0.601	8.88 ± 0.820	8.65 ± 0.651	9.16 ± 0.193
Relative (to body, % x 10)	3.07 ± 0.194	3.15 ± 0.431	2.97 ± 0.196	3.76 ± 0.236**
Liver (g)	93.66 ± 10.086	106.57 ± 8.734	96.03 ± 9.605	81.07 ± 12.889
Relative (to body, %)	3.31 ± 0.283	3.75 ± 0.133	3.31 ± 0.402	3.32 ± 0.560

Relative (to brain, % x 10^{-2})	10.83 ± 1.395	12.13 ± 1.887	11.16 ± 1.540	8.87 ± 1.549
Adrenal, Left (g)	0.16 ± 0.023	0.12 ± 0.047	0.17 ± 0.029	0.16 ± 0.046
Relative (to body, % x 10^3)	5.65 ± 0.690	4.38 ± 1.662	5.71 ± 0.402	6.55 ± 1.903
Relative (to brain, % x 10)	18.40 ± 1.856	14.39 ± 6.888	19.36 ± 2.640	17.47 ± 5.084
Adrenal, Right (g)	0.13 ± 0.028	0.13 ± 0.030	0.15 ± 0.032	0.12 ± 0.022
Relative (to body, % x 10^3)	4.72 ± 0.795	4.49 ± 0.963	5.19 ± 0.555	4.98 ± 0.772
Relative (to brain, % x 10)	15.36 ± 2.330	14.74 ± 4.763	17.60 ± 2.971	13.32 ± 2.383
Kidney, Left (g)	7.79 ± 0.948	8.77 ± 0.838	8.30 ± 0.586	7.40 ± 0.765
Relative (to body, %)	0.28 ± 0.033	0.31 ± 0.034	0.29 ± 0.017	0.30 ± 0.034
Relative (to brain, %)	89.86 ± 10.220	99.23 ± 10.692	96.24 ± 7.065	80.93 ± 9.426
Kidney, Right (g)	7.61 ± 0.648	8.31 ± 0.677	8.12 ± 0.447	7.35 ± 0.742
Relative (to body, %)	0.27 ± 0.024	0.29 ± 0.029	0.28 ± 0.018	0.30 ± 0.034
Relative (to brain, %)	87.91 ± 8.336	94.09 ± 10.108	94.24 ± 7.341	80.30 ± 8.752
Ovary, Left (g)	0.14 ± 0.027	0.18 ± 0.088	0.19 ± 0.043	0.11 ± 0.029
	5.0 ± 0.79	6.3 ± 3.04	6.4 ± 1.14	4.5 ± 1.01
Relative (to brain, %)	16.40 ± 3.123	20.85 ± 12.170	21.50 ± 3.583	12.01 ± 3.139
Ovary, Right (g)	0.14 ± 0.031	0.16 ± 0.064	0.16 ± 0.048	0.11 ± 0.034
Relative (to body, %)	4.8 ± 1.02	5.7 ± 2.18	5.5 ± 1.72	4.5 ± 1.12
Relative (to brain, %)	15.76 ± 3.995	18.77 ± 8.812	18.37 ± 4.999	12.03 ± 3.721

Statistically significant difference from control (* $p < 0.05$, ** $p < 0.01$)

Conclusion

The NOAEL for systemic effects was 110 mg/kg bw in both males and females, based on reduced food consumption (males and females) and reduced body weights (females).

The NOEL for local effects was less than 12.5 mg/kg bw in both males and females, based on dermal irritation changes to the skin observed at the macroscopic and microscopic level in the lowest dose group tested.

3.12.1.4 Study 4 (1989)

Reference:	Captan: 90-day inhalation toxicity study in the rat
Author(s), year:	Anonymous (1989)
Report/Doc. number:	ICI Central Toxicology Laboratory, report No. CTL/P/2543 (Company file: R-5603/TMN-0865)
Guideline(s):	US EPA Guideline 82-4; 87/302/EEC Method B29
GLP:	Yes
Deviations from OECD 413 (2009):	The study met the essential criteria of the test guideline. One female of the high dose group was returned accidentally to the wrong cage and became pregnant. Subsequently female animal 116 was terminated in week 6 at which time pregnancy was confirmed.
Acceptability:	Yes

Material and Methods

In a 13-week inhalation (nose only) study with captan (purity 88.7%, batch number 11240-37-1), ALpk:APfSD (Wistar derived) rats were exposed to nominal concentrations of 0.1, 0.5 and 5.0 µg/L captan (10/sex/dose) and 15 µg/L captan (20/sex) for six hours per day, 5 days per week. Test atmospheres were generated by the suspension of captan particulate in air using a Wright dust feed generator. A concurrent control group of 20 males and 20 females was exposed to air only. At the end of the exposure period 10 males and 10 females from each group were sacrificed from the main study group. The remaining animals (10/sex/dose - control and 15 µg/L) were sacrificed after a 4-week 'recovery' period and are referred to as the satellite group. Parameters evaluated were mortality and clinical signs (daily and frequently during exposure), body weight and food consumption (weekly), ophthalmoscopy (pretest, week 13 (0 and 15 µg/L) and week 18 ('recovery' group)), haematology and clinical chemistry (study termination), urinalysis (males in week 13 and females in

week 14), gross pathology, organ weights (lungs with trachea attached but larynx removed, heart, liver, kidneys, brain, adrenal glands and testes) and histopathology. Kidney sections from control and high dose animals were taken for immunocytochemical detection of α-2μ-globulin.

Results

Physical parameters - test atmosphere: The nominal and achieved concentrations, along with the particle diameter are summarised in Table .

Table 3.12.1.4-1 Summary of nominal and achieved concentrations and MMAD

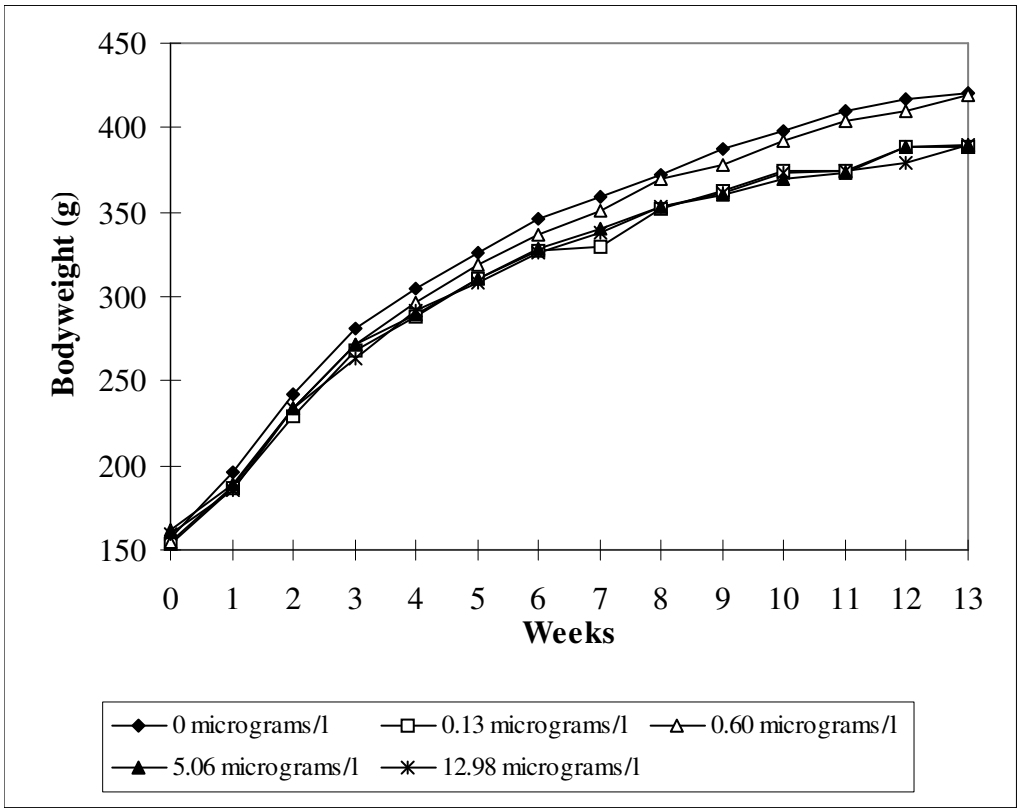
Nominal concentration (µg/L)	Achieved concentration (µg/L)	MMAD ± SD (µm)[1]
0.10	0.13	0.95 ± 0.2
0.50	0.60	1.22 ± 0.3
5.00	5.06	1.57 ± 0.3
15.00	12.98	1.60 ± 0.4

[1] MMAD ± SD = mass median aerodynamic diameter ± standard deviation.

General observations: Four males exposed to 12.98 µg/L captan were found dead over weeks 5 to 13 and another male from the same dose group was killed *in extremis* in week 11. One 0.13 µg/L female and one 5.06 µg/L female were killed *in extremis* in week 6 and week 10, respectively, but were considered to be not treatment-related. Any observed clinical signs were associated with restraint and occurred equally in test animals and controls. Respiratory noise was present towards the end of the study, primarily in those animals in the higher dose groups. Mucus nasal secretion was noted in a few females and may have been related to treatment.

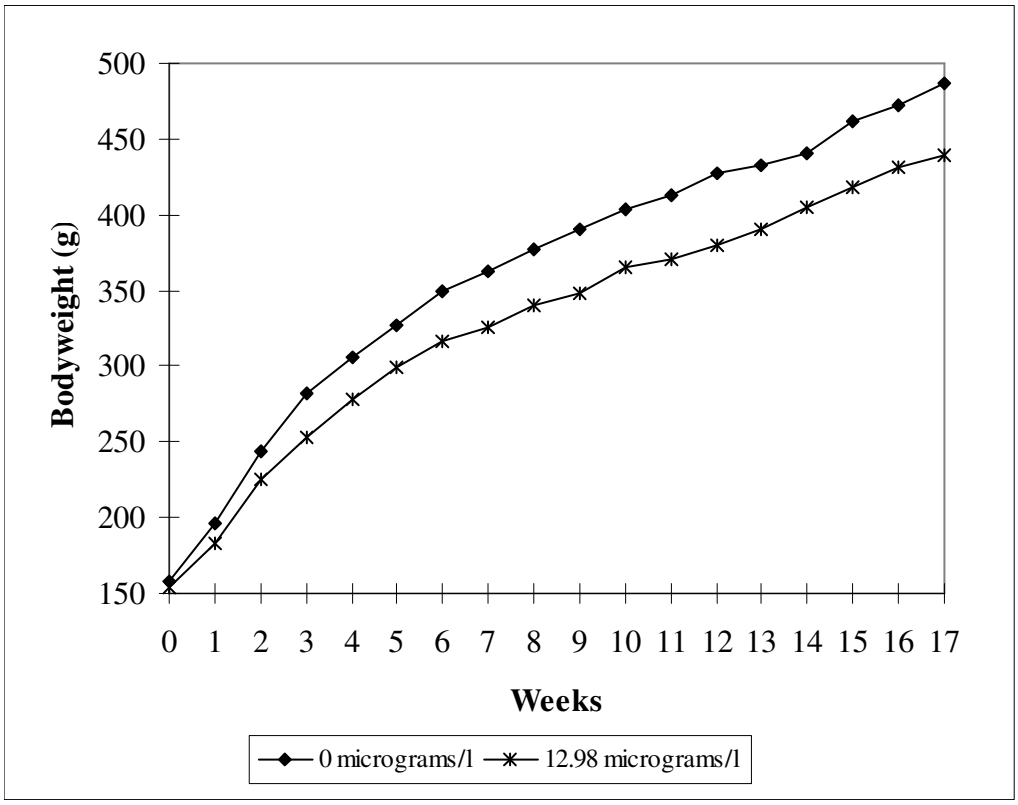
Bodyweight gain: Bodyweight gain in females was similar in all dose groups throughout the course of the study. A statistically significant reduction in the bodyweight of males from all test groups compared to the control was noted during week 1. This divergence from the control group continued for the duration of the study for all test groups except for the 0.60 µg/L dose group, which returned to control levels from week 2 onward. By week 13 final bodyweights were statistically decreased by (rounded) 8, 7 and 8% compared to the control in the 0.13, 5.06 and 12.98 µg/L dose groups, respectively. This was not considered to be a toxicologically significant effect. The bodyweights of males in the main study are summarised in Figure 3.12.1.4-1.

Figure 3.12.1.4-1 Mean bodyweights (g) – males (main study)



A similar picture was seen in the male satellite group but during the four week recovery period the rate of bodyweight gain of the high dose males was similar to controls. Final bodyweight of males in the satellite group was significantly lower in the high dose group compared to the control group. However, the reduction in final bodyweight was less than 10% (Figure 3.12.1.4-2).

Figure 3.12.1.4-2 Mean bodyweights (g) - males (satellite study)



Food intake in 5.06 and 12.98 µg/L males from the main group was reduced by approximately 10% versus controls, although these effects were not consistent and showed no dose relationship. Food efficiency was not affected by treatment in either sex.

Ophthalmoscopy, haematology and clinical chemistry: No treatment-related effects were observed.

Gross pathology, organ weights and histopathology:
There were no macroscopic changes related to treatment with captan. Gross findings in the lungs of animals found dead or sacrificed prior to study termination (red discolouration and partial deflation) were considered to be agonal and not treatment-related.

No toxicologically significant changes were seen in organ weights, or organ weights adjusted for bodyweights. Animals in the satellite group exposed to captan had statistically significantly reduced liver weights in absence of any pathological effects. These are not considered toxicologically significant.

Microscopic changes related to inhalation of captan were seen in the lung, larynx, nasal passages and trachea and occurred in both sexes. Treatment-related laryngeal changes were seen in male and female rats at all exposure levels (Table 3.12.1.6-2). The study author considered effects seen at 12.98 and 5.06 µg/L to be of toxicological significance, whereas effects seen at lower concentrations were considered to be an adaptive response to irritants. Following a 4-week recovery period the lung and nasal passage effects had resolved, but the laryngeal effects (squamous metaplasia and squamous hyperplasia) were still present in the high dose group (low and mid dose groups were not examined).
There was no evidence of any treatment-related increase in α-2µ-globulin in the kidney in either males or females from the 12.98 µg/L dose group.

The factor contributing to death in intercurrent males exposed to 12.98 µg/L captan was considered to be necrosis of the bronchial/bronchiolar epithelium. This effect was also seen in 5.06 and 12.98 µg/L captan animals that survived to terminal sacrifice. Other treatment-related effects were consistent with repeated exposure to an irritant particulate and are considered to have no toxicological significance in the context of captan exposure to humans.

Table 3.12.1.4-2 Summary of microscopic larynx findings

Removal reason / sex	Squamous metaplasia (total)	Body weight (g)				
		0 µg/L	0.13 µg/L	0.60 µg/L	5.06 µg/L	12.98 µg/L
Terminal / males	Examined	10	10	10	9	6
	Squamous metaplasia (total)	0	0	0	9	6
	- Slight	0	0	0	2	4
	- Moderate	0	0	0	7	2
	Squamous hyperplasia (total)	0	1	5	9	6
	- Minimal	0	0	2	4	0
	- Slight	0	1	3	5	6
Terminal / females	Examined	10	9	10	9	9
	Squamous metaplasia (total)	0	0	0	7	9
	- Slight	0	0	0	1	4
	- Moderate	0	0	0	6	5
	Squamous hyperplasia (total)	0	4	7	8	9
	- Minimal	0	2	2	0	1
	- Slight	0	2	5	8	8
Recovery / males	Examined	10	0	0	0	9
	Squamous metaplasia (total)	0	-	-	-	8
	- Slight	0	-	-	-	3
	- Moderate	0	-	-	-	5
	Squamous hyperplasia (total)	0	-	-	-	7
	- Minimal	0	-	-	-	6
	- Slight	0	-	-	-	1
Recovery / females	Examined	10	0	0	0	10
	Squamous metaplasia (total)	2	-	-	-	9
	- Minimal	2	-	-	-	0
	- Slight	0	-	-	-	1
	- Moderate	0	-	-	-	8
	Squamous hyperplasia (total)	0	-	-	-	7
	- Minimal	0	-	-	-	1
	- Slight	0	-	-	-	6

Conclusion

Exposure of rats for 90 days to captan aerosols at atmospheric concentrations of 0.13, 0.60, 5.06 and 12.98 µg/L captan resulted in five treatment-related mortalities in males exposed to the highest concentration, triggering classification for **STOT RE 1 – H372** ("Causes damage to organs through prolonged or repeated exposure if inhaled.") due to significant toxic effects (mortality) occurring at concentrations below the guidance value of 0.02 mg/L/6h/day for 90-day inhalation toxicity studies in rats exposed to dust/mist/fume, according to Regulation (EC) No 1272/2008.

Treatment-related effects were confined to the respiratory tract and in all cases were consistent with the effects of exposure to an irritant particulate. Effects in the lung, considered to be responsible for the mortalities at 12.98 µg/L captan, were present at 5.06 and 12.98 µg/L captan with 0.60 µg/L being a systemic NOAEC.

The NOAEC for toxicological effects was 0.60 µg/L captan in both male and female rats, based on effects in the lung at doses of 5.06 µg/L and above.
In addition, during the peer review meeting (PREV 13, September 2019) a local LOAEC was set at 0.13 µg/L based on the observed hyperplasia in the larynx mucosa.

3.12.2 Human data

No relevant studies.

3.12.3 Other data

3.12.3.1 Position Paper (2018)

Reference:	Captan: Immunotoxicity Data Requirement Waiver Request
Author(s), year:	Anonymous (2018)
	New data, submitted for purpose of review
Report/Doc. number:	ALS Reference Number: 2018-269, ADAMA Reference Number: 000101214
Guideline(s):	Not applicable
GLP:	Not applicable
Deviations:	Not applicable
Acceptability:	Yes

Executive summary

The Sponsor Companies believe that immunotoxicity studies for captan are not warranted, according to Reg. (EU) 283/2013, based on the following weight of evidence (WOE):

- There are no toxicological alerts from subchronic and chronic mammalian studies with captan that suggest an immunologic mode of action. Similarly, there are no such alerts for the structural analog of captan, folpet.
- THPI and phthalimide, the primary and systemically available metabolites of captan and folpet respectively, are not immunotoxic.
- There is no systemic exposure to captan regardless of dose route.
- A 1982 study which suggested captan had immunotoxic effects was conducted at a dose that was significantly higher than the point of departure (POD) (i.e. 25 mg/kg body weight (bw)) used to derive the proposed Acceptable Daily Intake (ADI) of 0.25 mg/kg bw.
- Immunotoxic effects, even if occurring secondary to the primary irritation of captan, will not affect the overall human health risk assessment for captan.

Based on the information presented and discussed within this document it is the position of the Sponsor Companies that additional toxicological testing on captan to evaluate its immunotoxic potential is unnecessary. This additional data will not lead to a more robust data for human health risk assessments and will lead to the unnecessary use of laboratory animals.

Background

No immunotoxicity studies are available for captan. The Sponsor Companies believe that immunotoxicity studies for captan are not warranted, according to Reg. (EU) 283/2013. There is no indication within the toxicological database for captan of an immunotoxic potential.
Additionally, a retrospective analysis of the value of immunotoxicity testing with regard to deriving Points of Departure (PODs) for human health risk assessments has been conducted (Gehen et al., 2014; Glenn, 2011a,b; Rowland, 2013). The conclusion of this analysis was that immunotoxicity study outcomes had little, if any, relevance for altering risk assessments made by regulatory authorities.
Therefore, the Sponsor Companies request waiver of immunotoxicity testing with captan based on review of the following information.

The toxicological profile of captan, noting immune-specific endpoints:

- Hematology (decreased WBC and/or differential counts);
- Clinical Chemistry (A/G ratio);
- Histopathology of immune-related tissues (spleen, thymus, lymph nodes, bone marrow, Peyer's patches; and,
- Increase in tumors in immune-related tissues.

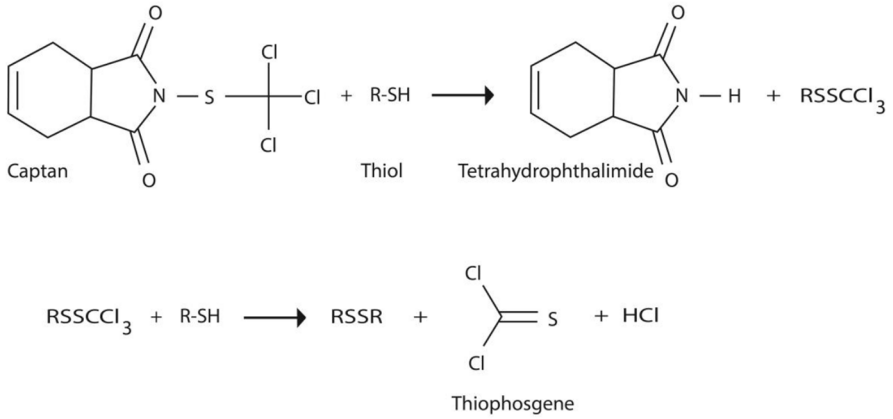
PODs and endpoints used for risk assessment and the rationale supporting the use of these PODs for immunotoxicity.

The overall WOE conclusion is that additional data generation is not warranted and would lead to the unnecessary sacrifice of laboratory animals without providing a more robust human health risk assessment.

Captan Toxicological Profile

Captan degrades by hydrolysis or reaction with thiols into the two initial degradates Tetrahydrophthalimide (THPI) and thiophosgene. THPI is relatively stable, while thiophosgene is very reactive and unstable. The step-wise reaction that leads to thiophosgene is shown in Figure 3.12.3.1-1.

Figure 3.12.3.1-1 Captan Stepwise Reaction with Thiols



The half-life of captan when introduced to human blood is 0.97 seconds (Gordon and Williams, 1999). The presence of thiophosgene in human blood is even more fleeting, as the half-life has been measured at 0.6 seconds (Arndt and Dohn, 2004).
THPI, while relatively stable, does undergo a series of metabolic transformations (THPAM, 3-OH-THPI, 3-OH-THPAM, 5-OH-THPI, THPI-Epoxide and 4,5-diOH THPI). THPI and all of its metabolites do not share the same toxicological properties of captan.

The trichlormethylsulfanyl-moiety convincingly explains the hazard profile for captan, due to direct interaction at the site of first contact due to the release of thiophosgene. This is based on data recently generated in a 7-day study that shows a qualitative difference in the proliferation response of captan and its metabolites, THPI and THPAM (Touati, 2018), and a comparative 28-day repeat dose study in rats with Captan and THPI (Coleman, 2018). It is demonstrated that captan's metabolites THPI and THPAM cannot induce the postulated mode of carcinogenic action of captan because they lack the chemical ability to induce proliferation, a necessary key event. Further, THPI and captan show a quantitative toxicological difference, which is demonstrated by different NOAELs in the 28-day study. THPI is significantly less toxic than captan: it produced a higher NOAEL based on nominal dose levels, which is notable because THPI has half the molar weight of captan.

The identity and chemical/physical properties of captan, THPI and thiophosgene are shown in the following table.

Table 3.12.3.1-1 Identity and Chemical/Physical properties of captan and its two initial degradates Tetrahydrophthalimide (THPI) and thiophosgene

Captan	
Chemical family	Phthalimide
IUPAC Name	1,2,3,6-Tetrahydro-*N*-(trichloromethyl thio) phthalimide
Formula	C9H8Cl3NO2S; C6H8(C=O)2N-SCCl3
Molecular weight	300.61 g/mol
Physical state	Solid
CAS Number	133-07-3
Solubility:	3.3 mg/L (25ºC)
Hydrolysis, pH 5	18.4 hours at 25ºC [1]
Hydrolysis, pH 7	4.8 hours at 25ºC [1]
Hydrolysis, pH 9	7.8 minutes25ºC [1]
Half-life in blood	0.97 seconds[2]
Ring degradate	Tetrahydrophthalimide (THPI)
Side Chain degradate	Thiophosgene
THPI	
Chemical name	1,2,3,6-Tetrahydrophthalimide
Formula	C8H9NO2
Molecular weight	151.16 g/mol
Physical state	Solid
CAS	1469-48-3
Hydrolysis, pH 4	stable at 25°C [3]
Hydrolysis, pH 7	152 days at 25°C [3]
Hydrolysis, pH 9	3.2 days at 25°C [3]
Hydrolysis	Stable
Thiophosgene	
Formula	CSCl2
Molecular weight	114.98 g/mol
Physical state	Liquid, red
CAS	463-71-8
Solubility	Decomposes, yielding CO2, HCl, and CS_2 [4]
Half-life in blood	0.6 seconds [5]

[1]: (Captan DRAR, List of endpoints, December 2017);
[2]: (Gordon and Williams, 1999)
[3]: (Captan DRAR, List of endpoints, December 2017);
[4]: (ChemicalBook, 2010);
[5]: (Arndt and Dohn, 2004)

Relevant immunotoxicity endpoints of captan were derived from Rowland, J. (2013), A Retrospective Analysis of the Immunotoxicity Study (OCSPP Test Guideline No. 870. 7800), Health Effects Division, Office of Pesticide Programs, U.S. Environmental Protection Agency. An overview of relevant immunotoxicity endpoints of captan are presented in the table below.

Table 3.12.3.1-2 Immunotoxicity Related Endpoints (according to Rowland, 2013)

Parameter	Findings
Thymus (weight)	No effects reported within the Captan database.
Spleen (weight)	No effects reported within the Captan database.
Microbial Infections	No increase reported within the Captan database.
Hematology	No effect on WBC or differential counts within the Captan database.
Clinical chemistry	A/G ratio not affected within the Captan database.
Histopathology	No effects within the Captan database on: spleen, thymus, lymph nodes, bone marrow, and Peyer's patches.
Carcinogenicity	Restricted to duodenum (MOA based on irritation).

The collective data show the following:

- Captan interacts locally with mucus membranes; systemic exposure is absent.
- Internal exposure is restricted to THPI and THPI metabolites.
- Captan has mutagenic potential, based on *in vitro* assays, but is not mutagenic *in vivo*. Its mutagenic potential *in vitro* is reduced or abolished by the addition of thiol groups, *e.g.* by metabolic activation.
- Captan induces duodenal tumors in mice, but the risk of cancer in humans is essentially absent. Tumors do not develop in rats.
- Occupational risk focuses on eye irritation and skin sensitization.
- Captan is neither a developmental nor reproductive substance.
- Captan is not an endocrine disruptor.

The collective data show the following with regard to immunotoxicity:

- Captan does not induce weight changes in the thymus or spleen.
- Long-term bioassays do not show evidence of increased susceptibility to microbial infections.
- Effects on Sheep Red Blood Cell antibody formation occur only at high dose and are not relevant to occupational exposure levels.

Captan Mode of Action

The reactivity of captan with thiols (Figure 3.12.3.1-1) essentially prevents any systemic exposure. Toxicological effects are restricted to points of initial contact such as mucus membranes and intestinal surfaces.
For mucus membranes such as those associated with the eye, there is a marked transient irritation. For the intestinal tract, captan causes damage to the villi but this occurs at doses above the known threshold (approximately 1000 ppm, dietary). Prolonged exposure to captan above this threshold results in duodenal tumors (polys leading to adenomas, eventually developing into adenocarcinomas). The non-mutagenic mode of action for theses tumors has been established (US EPA, 2004; EFSA, 2009).

Structural Analogs

The structural analog to which captan is related is folpet. Captan shares a common mechanism of toxicity with folpet (Bernard and Gordon, 2000) and as such further informs on the likelihood of immunotoxic effects of captan.
There are no indications of adverse effects on parameters relevant to immunotoxicity in subchronic and chronic studies with folpet (FAO/WHO, 1996; US EPA, 1999).

Report of Immunologic Effect

A study in which captan was fed to mice at 3000 ppm for 7, 14, 21 and 42 days was reported to have adverse effects on the immune system (Lafarge-Frayssinet and Decloître, 1982). The SRBC-antibody (Sheep Red Blood Cell) formation was depressed by about 70% in both rats and mice after 14 days of treatment. A release of inhibition occurred in mice at day 42. The dose administered, however, was three-fold higher than that necessary for disruption of the intestinal villi in mice and development of duodenal tumors (US EPA, 2004; Wilkinson, 2004). Therefore, it is likely that this study reflected general toxicity rather than immune-specific toxicity.

An estimate of the dose is 600 mg/kg/day for mice and 240 mg/kg/day for rats (Blackburn, 1988).[3]The authors also noted that captan was mutagenic, but cited an early study (Bridges, 1975) that did not accurately reflect captan's mutagenic potential (Arce et al., 2010). The authors also noted that captan was teratogenic, but cited an early study (Martin et al., 1978) that used direct application to chick embryos as the test system; this study does not accurately reflect the developmental toxicity of captan (Neal et al., 2001). Finally, the authors state that, "Captan was orally administered in such a way that the absorption, the distribution, and the metabolism of the pesticide were taken into account in the response of the animals," yet subsequent data shows that that these investigators did not realize that there was no systemic exposure to captan. Collectively, these considerations show that captan is not immunotoxic.

Points of Departure (PODs) and Estimation of Exposure

The points of departure used for deriving the Acute Reference Dose (ARfD) and Acceptable Daily Intake (ADI) are the following:

- Acute Reference Dose (ARfD) = 0.9 mg/kg bw/day
  NOAEL: 90 mg/kg bw/day, developmental toxicity study in rat, Safety Factor: 100
- Acceptable Daily Intake (ADI) = 0.25 mg/kg bw/day
  NOAEL 25 mg/kg bw/day, carcinogenicity studies in rats, Safety Factor: 100

The Theoretical Maximum Daily Intake (TMDI), the National Estimated Daily Intake (NEDI), and the International Estimated Short-Term Intake (IESTI) calculations were carried out by means of the EFSA chronic and acute risk assessment model revision 2 (Model PRIMo, August 2008). The conclusion of all analyses was that the TMDI and NEDI values were below the ADI, and the IESTI did not exceed the ARfD or ADI, and thus a long- and short-term intake of captan residues is unlikely to present a public health concern.

As the PODs for both derivation of the ARfD and ADI are driven by reduced food consumption, body weight gain and toxicological effects in the liver and kidney respectively, an immunotoxicity study will not change the currently proposed human health risk assessment for captan. Additionally, the absence of systemic exposure, coupled with the mode of action of captan and the relative low toxicity of THPI support the judgment that captan would not be immunotoxic at expected exposure levels.

Discussion

The overall toxicological profile of captan demonstrates that there are no indications of immune-related toxicity endpoints in repeat dose studies. This is true, also, for the captan analog, folpet. Immunotoxic effects are not apparent in the subchronic and chronic studies with captan and folpet. They are also not likely to occur at consumer exposure levels which are below the proposed ARfD and ADI values.

Captan's metabolism/pharmacokinetics data indicate that captan degrades quickly, resulting in the absence of systemic dose, regardless of route of exposure. While effects could be found at high doses, these effects are likely secondary to the primary irritative actions of captan.

Collectively, the WOE supports granting this waiver:

- There are no indications of immune disruption in subchronic/chronic studies;
- The physical/chemical properties prevent systemic exposure to captan;
- The current ARfD and ADI are acceptable;
- Report of immune effects with captan occur at dose levels that are associated with systemic toxicity and are well above current PODs for human health risk assessments;
- There are no reactive moieties on THPI, the systemic captan degradate.

[3] Calculations: mice consume 4 g/day and weigh 20 g; rats consume 20 g/day and weigh 250 g. The diet contained 3 mg captan/g feed (3000 ppm = 3 g/kg = 3 mg/g).
Mouse: 4 g/day x 3 mg/g = 12 mg/day. 12 mg/day ÷ 0.02 kg = 600 mg/kg/day. 600 mg/kg/day ÷ 0.13 mg/kg/day = **4615-fold higher** than the cPAD.

Rat: 20 g/day x 3 mg/g = 60 mg/day. 60 mg/day ÷ 0.25 kg = 240 mg/kg/day. 240 mg/kg/day ÷ 0.13 mg/kg/day = **1816-fold** higher than the cPAD.

Conclusion

The extensive database for captan, augmented by the database for folpet, does not suggest it present an immunotoxic potential. The rapid degradation of captan in blood results in systemic exposure to THPI and THPI metabolites only. These metabolites are less toxic than captan and do not present structural alerts, based on the absence of active moieties. Thus, the Sponsor Companies request waiver of an immunotoxicity study with captan as the collective data assure that such a study will not show immunotoxic effects and, if effects are seen they will (1) be secondary to the primary irritative action of captan, and (2) will occur well above the PODs currently proposed for human health risk assessments. Therefore, the generation of such data will not produce a better human health risk assessment and will only lead to the unnecessary sacrifice of laboratory animals.

3.12.3.2 Kluxen and Koenig *unpublished*

This is the accepted manuscript submitted to *Regulatory Toxicology and Pharmacology.*

Title: Inhalation Toxicity of Captan and Folpet

Authors: Felix M. Kluxen[a], Claire M. Koenig[b]

[a]ADAMA Deutschland GmbH, Cologne, Germany

[b]ADAMA US, Raleigh NC, USA

*Corresponding author

Abstract

The active ingredients Captan and Folpet are contact fungicides used in pesticidal products that act by the release of thiophosgene. This reaction occurs quickly and plausibly explains their hazard profile, which is driven by the direct interaction at the site of first contact.

With respect to their inhalation toxicity, it may be questioned whether adverse effects observed in repeated exposure studies are specific target organ toxicity distinct from acute effects or whether they occur due to the same aetiology? This is relevant for the chemicals' health classification, which should reflect the actual hazard profile and appropriately communicate hazard to users.

By considering exposure duration, using both Haber's rule and the Multiple-Path Particle Dosimetry (MPPD) model, we demonstrate that the compounds are acute local irritants and must be classified accordingly. Captan and Folpet do not have specific toxicity in the respiratory tract but are irritating to all bio-membranes, as observed in their toxicological data package and which is also reflected by their fungicidal activity. Exposure duration was previously not considered in the hazard assessment of Captan and Folpet's inhalation toxicity properties.

Introduction

The active ingredients Captan and Folpet are contact fungicides, which directly react with or release thiophosgene which reacts with bio-membranes (Gordon, 2010a). This reaction occurs quickly and results in local irritative effects in safety assays. This mode of action plausibly explains the related hazard profile for Captan and Folpet, driven by the direct interaction at the site of first contact.

There are several acute inhalation toxicity studies in the rat available for both compounds, due to the ownership of these substances by different companies over time, their registration in different countries and their manufacture at different production plants. There are also repeated exposure studies available in rats, a 13-week subchronic inhalation toxicity study for Captan and a 4-week subacute inhalation toxicity study for Folpet.

Adverse observations and mortality occurring in the repeated inhalation exposure studies may trigger the classification for Specific Target Organ Toxicity upon Repeated Exposure (STOT-RE) according to the

European classification, labelling and packaging (CLP) regulation (Reg. 1272/2008) (European Commission, 2008).

In the 4-week study with Folpet, moderate metaplasia, keratinization of the metaplastic epithelium and inflammatory cells in the larynx occurred at the lowest test concentration of 5 µg/L, which is below the guidance trigger value of 0.06 mg/L (60 µg/L). Further, degeneration/atrophy of the olfactory epithelium and squamous/squamoid metaplasia in the nasal turbinates and inflammatory cells in the mucosa of the trachea were observed.

Similar local effects in the respiratory tract were also observed for Captan; however, additionally 50% mortality occurred at the highest test concentration of 12.98 µg/L in the 13-week repeated inhalation study, which is below the CLP-guidance trigger value of 0.02 mg/L/6 h/day (20 µg/L). The presence of mortality is arguably the relevant effect for STOT classification due to the severity of the effect.

Based on the generic rules laid out in European Commission (2008), a classification proposal for STOT-RE appears superficially plausible only if duration of exposure and the inherent hazard properties of Captan and Folpet are ignored. Because both Captan and Folpet are irritants and acute inhalation toxicants, these characteristics should take priority with regard to classification according to EChA (2017).

The current manuscript summarizes the available relevant toxicological information for both compounds, compares the observations in the acute and repeated exposure studies and demonstrates by dosimetry that the effects observed in the repeated exposure studies are actually manifestations of repeated local acute irritation and should thus be accordingly classified. The effects in the repeated exposure studies are clearly not distinct adverse effects warranting a separate classification and are not specific to the respiratory system.

Available regulatory toxicity data

Full regulatory toxicology datasets are available for Captan and Folpet. However, in the following, only the relevant data for the manuscript's hypothesis are shortly summarized.

Acute local toxicants

Captan and Folpet have the same fungicidal mode of action and show similar hazard profiles that demonstrate acute local effects. Neither Captan nor Folpet appear to be systemically available, as their half time ($t_{1/2}$) in blood is less than 10 seconds (Gordon and Williams, 1999; Gordon et al., 2001).

Both compounds show severe damage to the eye in the available animal studies and are skin sensitisers. While irritation to the skin is observed in acute corrosion/irritation studies in rabbits, the effect is not potent enough to warrant a classification according to CLP. However, for Folpet, significant skin irritation is observed in a 4-week dermal toxicity study in rats, which tested up to 30 mg/kg bw/day in 6 animals/sex and produced a LOAEL at 1 mg/kg bw/day. The top dose had to be reduced to 20 mg/kg bw/day due to the severity of skin irritation at Day 6. The animals showed scabs, dryness/flakiness, redness and swelling from 10 mg/kg bw/day onwards; thickening and sloughing was observed in the high dose group. The irritation was shown to be reversible.

The difference in irritation potential in the rabbit and the rat studies with Folpet might be due to differences in cumulative mass per area units and differences in application method and test system. The acute irritation studies tested either moistened material (Study 1) or dry material on moistened skin (Study 2) for four hours. Whereas the repeated exposure dermal study applied Folpet in a mineral oil vehicle for 6 hours and the formulation was replaced daily 21-times over 4-weeks. The maximum possible application in the repeated exposure study was therefore about 20% of the dose used in the acute irritation studies; i.e. 30 mg/kg bw/day × 21 days × 1.5 hour exposure-ratio to acute studies × 0.25 kg assumed body wt = 236.25 / 2 application areas = ~120 mg /application area vs 500 mg/application area in the acute studies. If the actual size difference of application areas is additionally considered, 6.25 cm^2 for rabbits (OECD TG 404) and about 10% in dermal rat studies (OECD TG 410) which corresponds[4] to 2.5 cm^2, the cumulative application per square centimetres

[4] FDA, 2005 "Guidance for Industry Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers".

is substantially (>100-fold!) higher in rat (i.e. 500 mg/6.25 cm^2= 0.8 mg/cm^2 in rabbits and 236.25 mg/2.5 cm^2= 94.5 mg/cm^2 in rats). As Folpet reacts and degrades rapidly, the daily application and unhindered diffusion towards the target skin, due to lower concentration, alternating treated sites and stabilizing vehicle, presumably maximised the amount available for skin reactions in the repeated exposure study.

While Captan and Folpet are acutely toxic via the inhalation route, they are not acutely toxic via the oral or the dermal route. This may again be driven by the experimental conduct: in the respiratory tract, rapidly degraded material is quickly replenished by newly inhaled and deposited material, while a bolus gavage dose, as used in the acute oral studies, has relatively limited contact to a specific part of the gastrointestinal tract. The diffusion distance within the lower respiratory tract is also shorter than in the intestine, and there is no potential to interact with residue diet. It is further obvious that a functional disturbance in the gastrointestinal tract is of less short-term toxicological concern than in the respiratory system.

Hence, irritation potency and thus local toxicity are not driven by exposure route but by the time and amount the respective epithelia were in contact with Captan and Folpet, which is plausible according to the rapid reaction and degradation observed in the kinetic studies.

Irritative effects are also observed in the gastrointestinal tract in the repeated dose and chronic studies with oral exposure where the tissues' exposure time is increased.

Canal-Raffin et al. (2008) showed in an *in vitro* model that cytotoxicity can occur rapidly in bronchial epithelial cells upon contact with Folpet. Such direct effects also lead to positive *in vitro* results in genotoxicity assays for Captan and Folpet but not in their corresponding *in vivo* versions.

In mice, continuous high dietary concentrations lead to gastrointestinal tumors. The aetiology of this is well investigated, and driven by subsequent local irritation following repeated oral exposure and not genotoxicity (Arce et al., 2010; Cohen et al., 2010; Gordon et al., 2012; Gordon, 2010b; Thompson et al., 2017). Acceptance of this aetiology for carcinogenicity lead to a cancer classification in the United States of America as "not likely to be carcinogenic to humans" (Gordon, 2007), which is clearly different from the European classification. This well-defined aetiology of toxicity also led to a proposed Adverse Outcome Pathway, which considers local high concentrations, and subsequent cytotoxicity due to local irritation, as key events in the pathway, a pathway which does not appear to be of relevance to humans. Irritation is also seen in rats, almost all high dose rats in 90 day dietary or 2-year studies show hyperkeratosis in the oesophagus or the non-glandular stomach. However, rats do not develop small intestinal tumors.

All effects in the repeated oral dose animal studies, therefore, appear to be either subsequent to irritation in the gastrointestinal tract or due to attrition, based on reduced diet intake. Animals virtually stop feeding when initially exposed to high-concentration Captan and Folpet diets and only resume feeding in desperation following continued exposure with no alternative dietary options.

Together, the hazard profile clearly demonstrates that both Captan and Folpet are acute local irritants; a summary of proposed classifications within the pesticide registration process in Europe is shown in Table 1.

Table 1 Potential classifications for Captan and Folpet with respect to the hazard profile, based on the data base and discussions in the pesticide registration process in Europe

Potential harmonized classification	Comment
Captan: Acute tox Cat 3 [LC_{50} 0.67 mg/L] Folpet: Acute tox Cat 2 [LC_{50} 0.39 mg/L]	Neither compound is toxic via the oral route. They are also not toxic after dermal exposure. Mortality is driven by oedema subsequent to local irritation in the respiratory tract. The compounds are cytotoxic to bronchial epithelial cells *in vitro*. The effect seems to be driven by cumulative exposure time as both compounds rapidly degrade and react with bio-membranes.
Eye damage Cat 1	Generally, cytotoxicity occurs in all *in vitro* studies, i.e. genotoxicity assays or other cell-based systems. In dietary exposure studies, irritation is observed throughout the gastrointestinal tract of mice and rats.

Potential harmonized classification	Comment
	Irritation is also observed in the repeated inhalation exposure studies. These irritation properties result also in positive eye irritation studies, which indicates that the same mode of toxic action is driving the irritation. The presence of an intact *stratum corneum* seems to prevent positive results in the dedicated acute skin irritation studies.
Acute irritation Cat 2	While the observed irritation effects in the acute corrosion/irritation studies do not warrant classification, significant irritation is observed in a repeated exposure dermal rat study or chronic mouse studies where animals were exposed to a powdered diet. Since humans have a thicker *stratum corneum* than the used laboratory animals, the effect appears to be not relevant in human exposure scenarios. However, skin irritation is clearly associated with Captan and Folpet exposure in rodent studies and part of their hazard profile, with limited relevance for human hazard classification.
Skin Sensitisation Cat 1	*In vivo* Skin sensitisation assays are positive. It is unclear whether this indicates true skin sensitisation potential or is an artefact from the irritation properties. Neither compound is systemically available and the local lymph node assays for their primary systemic metabolites Tetrahydrophthalimide (THPI) and Phthalimide, for Captan and Folpet, respectively, are negative (REACh dossiers available). Further, there were no reports of skin sensitisation in the manufacturing plants or by pesticide handlers, when using fungicidal products. (NB: The products have been used for decades)
Carcinogenicity Cat 2	Carcinogenicity in mouse duodenum is driven by local irritation, subsequent cytotoxicity and regenerative proliferation (Cohen et al., 2010). Carcinogenicity does not occur systemically or in rat intestine. The usefulness of this classification for hazard communication may be scrutinized as carcinogenicity occurs only subsequent to acute local irritation and there appears to be no human relevance (Bhat et al., 2020; Gordon, 2007); life-long high dietary concentrations lead to cytotoxicity in mouse duodenum, which only occurs in an experimental set-up as test species generally avoid diet with high Captan and Folpet concentrations (either because of their distinctive "chemical" smell or because of the irritation). There is no gastrointestinal toxicity in dogs, which have a more similar stomach to humans.
STOT-RE Cat 1	Based on mortality in the 13-week inhalation toxicity study with Captan and local effects in the respiratory tract of the 4-week Folpet study, STOT-RE Cat 1 was proposed during the pesticide registration process. However, the observations in the repeated exposure studies seem to be reflections of repeated acute local irritation events, similar to the effects observed throughout the study package. The key argument for classification is that the relevant observations occur below generic trigger values for exposure concentrations. However, due to the aetiology of the effects in the repeated exposure studies, i.e. acute local irritation, it is necessary to consider the cumulative exposure leading up to the effects. Consideration of exposure duration shows that the low concentrations used in the repeated exposure studies actually lead to higher cumulative respiratory exposure than the acute inhalation toxicity studies. Thus, the observations in the repeated exposure studies are actually manifestations

Potential harmonized classification	Comment
	of repeated acute local effects that are neither specific to the respiratory tract nor a different hazard.

Acute inhalation toxicity data

Multiple acute inhalation toxicity studies are available for Captan and Folpet, see Table 2. Individual group responses are depicted in Figure 1.

The data-set for Folpet includes studies with nose-only and whole-body exposure. According to the data requirements for active substances, as laid out in Commission Regulation (EU) No 283/2013, head/nose only exposure shall be used to determine acute inhalation toxicity (European Commission, 2013). Similarly, the preferred mode of exposure is nose-only according to OECD TG 403 (2009). The reason for the preference for nose only exposure is given in OECD guidance document No. 39 on inhalation toxicity studies -- most importantly, nose-only exposure minimises exposure or uptake via non-inhalation routes (2018). For example, the acute inhalation whole-body exposure study 000092041 (1988) with Folpet reported several clinical signs that pointed to effects induced via exposure routes other than inhalation: red or colorless eye discharge, corneal opacity, swollen face (around eyes), alopecia around eyes and in the anogenital area, red/yellow/orange anogenital discharge, reduced feces, and diarrhea.

Animals that are exposed to irritant aerosols in whole-body exposure chambers, receive toxic insults via multiple routes, which increases general distress and can lead to lower LC_{50} values. Phalen et al. (1984) describe the whole-body exposure paradigm as follows, which is of course exacerbated when using irritative aerosols: "*Exposure to particles is messy; airborne material can enter animals through skin, mouth and eyes and deposits on every surface, covering fur, food, caging and chamber surfaces. Animal wastes such as ammonia, carbon dioxide, hair and dander are always present in the exposure environment.*" Therefore, whole-body exposure studies are not considered to be relevant for the determination of specifically acute inhalation toxicity.

For Folpet, there are four acute inhalation toxicity studies with nose-only exposure, 000040833 (1991), 000009988 (1993), 000041394 (1993) and 000041392 (1993).

Study 000040833 (1991) produced a MMAD of <2 µm (micronised) and resulted in LC_{50} values of 0.39 mg/L and 0.43 mg/L for males and females, respectively, with 1/5 deaths occurring first at 0.14 mg/L. Study 000009988 (1993) produced a MMAD of about 5 µm (micronised) and resulted in LC_{50} values of 1.54 mg/L and 2.89 mg/L for males and females, respectively, with 3/5 and 1/5 deaths occurring first at 1.60 mg/L. Further studies investigated different source materials and the impact of particle size. Study 000041394 (1993) produced a MMAD of <4 µm (micronised) and resulted in LC_{50} values of >4.35 mg/L and 1.08 mg/L for males and females, respectively, with a curious dose-response relationship in males, see Figure 1. Study 000041392 (1993) tested folpet in a non-micronized form (as placed on the market) which does not appear to be acutely toxic via the inhalation route and thus was not included in the dataset, though these data are more representative for the product on the market and thus a realistic human exposure scenario.

For Captan, two acute nose-only inhalation toxicity studies are available. Study 000041002 (1991) which produced a MMAD <2 µm (micronised) and resulted in LC_{50} values of 0.9 mg/L and 0.67 mg/L for males and females, respectively, with 3/5 and 4/5 deaths occurring first at 0.94 mg/L. Study 000042268 (1995) produced a MMAD of about 5 µm (micronised) and resulted in LC_{50} values of 1.21 mg/L and 1.05 mg/L for males and females, respectively, with 1/5 mortalities in females first at 0.43 mg/L.

It is well known that the LC_{50} of irritant aerosols is driven by particle size (Pauluhn, 2004). For both Captan and Folpet studies the effect of particle size may be biased by purity, which is not known for either study 000040833 (1991) or 000041002 (1991). Additionally, the previously reported aerosolic particle size in study 000040833 (1991) was not achieved in the more current studies 000009988 (1993) and 000041394 (1993), even when the test material was specifically micronized.

All of the available acute inhalation toxicity studies reported clinical observations in-line with acute irritation, for example gasping, laboured and noisy respiration. The gross necropsy findings in each study also support the presence of acute irritation, for example haemorrhage and swollen lungs, fluid in the respiratory system and increased lung weights.

Overall, Folpet appears to be less acutely toxic than Captan according to Figure 1, with the exception of the results for study 000040833 (1991), the study with material that is not used anymore. However, the study gives the worst-case LC_{50} available for Folpet.

Table 2 Acute inhalation toxicity results, n=5 /group/sex, 4 hour exposure

Active	Year	Reference number	Study type	Purity [%]	Tested concentrations [mg/L]	LC_{50} (m) [mg/L]	LC_{50} (f) [mg/L]	MMAD* [µm]
Captan	1991	000041002	Nose-only	*unknown*	0.23, 0.94, 4.81	0.9	0.67	1.8
	1995	000042268	Nose-only	98.1	0, 0.43, 0.82, 1.36,	1.21	1.05	5.6-6.5
Folpet	1979	000039795	Whole-body	*unknown*	0.64, 0.67, 2.68, 3.61	1.38	1.30	~ 2
	1988	000092041	Whole-body	89.2	0.21, 0.53, 0.95, 1.49	0.34	1.00	2.5-3 / 4.23
	1991	000040833	Nose-only	*unknown*	0.14, 0.36, 1.06, 4.35	0.39	0.43	<2
	1993	000009988	Nose-only	98.99	0.8, 1.6, 1.99	1.54	2.89	~ 5
	1993	000041394	Nose-only	95.6	M: 1.84, 2.14, 3.57, 4.35 F: 0.79, 1.11, 1.84, 2.14	>4.35	1.08	<4
	1993	000041392	Nose-only	98.99	2.14	>2.14	>2.14	14.3

*as the LC_{50} is estimated from the treatment concentrations the MMAD is stated that corresponds to the nearest treatment concentration to the LC_{50}

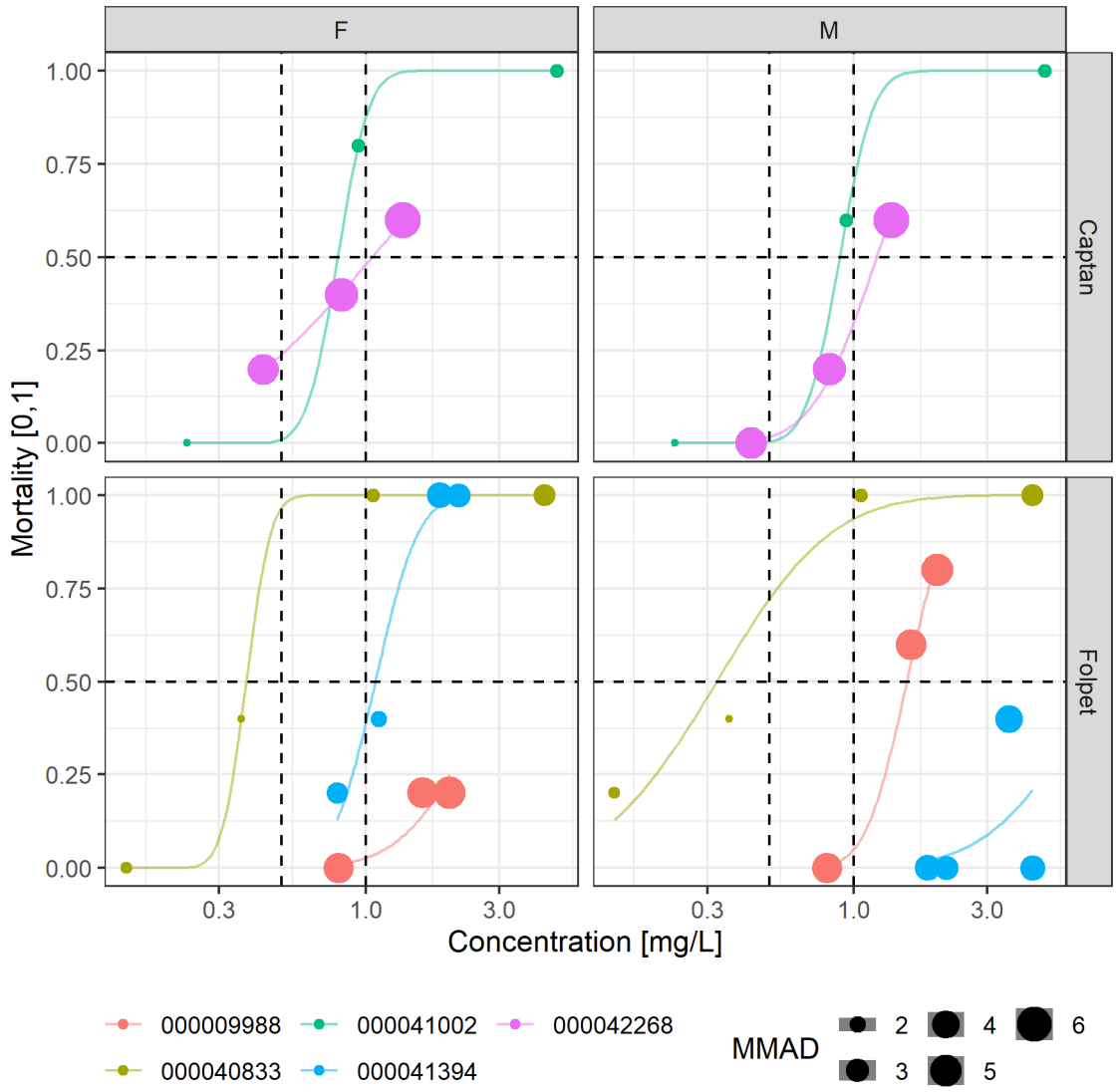


Figure 1 Results of the nose-only acute inhalation toxicity studies of Captan and Folpet for male (M) and female (F) rats, with overplotted Probit fits, using R (R Core Team, 2020) and ggplot2 (Wickham, 2016). Study 000041392 was excluded because of the MMAD physical properties of the test item. Blacked dashed lines indicate 50% mortality on the y axis and concentrations of 0.5 mg/L and 1 mg/L, which are relevant limits for classification categorisation.

Repeated exposure inhalation toxicity data

There are two repeated exposure inhalation toxicity studies available in rats, a 13-week study with Captan and a 4-week study with Folpet (see Table 3) with treatments for 6 hours/5 days/week.

The 13-week Captan study was based on a 3-week concentration-range finder study, 000040643 (1989). In the concentration-range finder study, an increase in bronchiolar associated lymphoid tissue was observed for all animals, including controls, which possibly indicates an underlying infection. According to the report, an underlying infection may have resulted in an increased sensitivity towards toxic irritation in the respiratory tract; thus, while the study results are not considered to be reliable, a short summary is provided for informational purposes. Two mortalities occurred in the 24.8 µg/L male group. Both were attributed to irritative effects on the respiratory tract. In the high concentration group, histopathological changes were observed in the respiratory tract that are consistent with effects observed for respiratory tract irritants, such as raised areas on all surfaces of the lungs and dark or red areas in various lobes, moderate loss of epithelial lining of the larynx or necrosis, alveolar oedema, congestion and haemorrhage in the lungs. According to the study

director, the histopathological findings were consistent with Captan having a direct irritant effect on the tissues where deposition had occurred. A NOEC of 5.3 µg/L was derived.

In the 13-week Captan study, 000040642 (1989), which included satellite groups with a follow-up period of four weeks, treatment-related effects were confined to the respiratory tract. The observed respiratory tract effects were consistent with exposure to an irritant particulate and, according to the study director, the effects in the lung were considered responsible for five male mortalities observed in the highest concentration group (12.98 µg/L). Lungs from animals that were allowed to recover for four weeks following exposure at this concentration were completely normal, clearly demonstrating reversibility of the effects. A dose of 0.60 µg/L was considered to be a no-effect level for the lung. The larynx was the only other organ to be affected. Effects considered to be an adaptive response to irritants were seen at the lower concentration and more details are provided below.

In the 4-week Folpet study, 000060792 (2008), all animals survived except a single male in the highest concentration group that was sacrificed on Day 13 due to poor condition, labored breathing, decreased activity and a hunched appearance. All treatment groups showed squamous metaplasia, epithelial hyperplasia and degenerative and inflammatory changes in the upper and lower respiratory tract as well as laryngeal changes. Generally, target organ toxicity was most pronounced at the highest treatment level with the effects at lower exposure levels considered to be only minor exacerbations of background inflammation (i.e. mixed inflammatory cells in the tracheal mucosa or lung). Squamous metaplasia in the nasal turbinates and larynx along with degeneration, atrophy and ulceration of the epithelium in the nasal cavity can be considered adverse responses if they compromise organ function. The laryngeal lesions, although moderate in severity and present at all exposure levels, were considered as non-adverse changes by the study director.

Table 3 Repeated exposure inhalation toxicity studies (nose-only)

Active	Year	Reference number	Study type	Purity [%]	Achieved concentrations 6 hours/Day [µg/L]	MMAD [µm]	Limit value in study report
Captan	1989	000040643	3-week concentration-range finder	89.7	0, 0.8, 5.3, 24.8 µg/L; n=5/group/sex	-, 1.87, 1.86, 2.48	NOAEC 5.3 µg/L
	1989	000040642	13-week	88.7	0, 0.13, 0.60, 5.06, 12.98 µg/L and 0, 12.98 µg/L with a 14 day follow-up; n=10/group/sex	-, 0.95, 1.22, 1.57, 1.60	NOAEC 0.60 µg/L
Folpet	2008	000060792	4-week	96.8	0, 5.2, 26, 97 µg/L/day n=5/group/sex	-, 1.9, 1.7, 2.1	NOAEC 26 µg/L NOAEC (local) 5.2 µg/L

Considering exposure duration for inhalation toxicity

To assess whether the results in the repeated exposure inhalation studies are occurring due to acute effects and are thus manifestations of multiple acute irritation insults, two main lines of evidence are considered: 1) whether the effects observed in the acute studies correspond qualitatively to effects observed in the repeated exposure studies and 2) by comparing the actually achieved cumulative doses that result in the observed effects.

Cumulative toxic effects: Haber's rule

"Haber's rule" directly follows the toxicological axiom that toxic potency is a function of dose and expands on this idea by integrating time, i.e. effects due to small doses over a long time should correspond to effects due to high doses over a short time. There has been some work done investigating whether dose and time are directly proportional (Connell et al., 2016; Rozman, 2000), which is, however, *per se* unlikely due to detoxification and regenerative capacity of the investigated bio-system. Miller et al. (2000) showed that power functions are actually more predictive than linear extrapolations, which is similar to observations on body weight progression that include age (Huxley, 1924; Trieb et al., 1976).

Haber's rule is specifically taken into account when extrapolating 28-day study data for STOT-RE generic thresholds to 90-day studies (European Commission, 2008).

"The guidance values refer to effects seen in a standard 90-day toxicity study conducted in rats. They can be used as a basis to extrapolate equivalent guidance values for toxicity studies of greater or lesser duration, using dose/exposure time extrapolation similar to Haber's rule for inhalation [highlighted by the authors], *which states essentially that the effective dose is directly proportional to the exposure concentration and the duration of exposure. The assessment shall be done on a case-by-case basis; for a 28-day study the guidance values below is increased by a factor of three."*

Conversely, both regulation (European Commission, 2008) and guidance (ECHA, 2019) ignore the potential of cumulative toxicity in their assessment criteria.

The key insight from Haber's rule is that toxicity acquired over repeated exposures may relate to toxicity observed after acute single exposures and that the function of dose and time can guide the corresponding assessment.

In the 4-week Folpet study, the lowest concentration resulting in local effects was 5.2 µg/L, or 0.005 mg/L, in study 000060792 (2008). This value of 0.005 mg/L is 78 times lower than the proposed LC_{50} of 0.39 mg/L. It should be noted, however, that respiratory signs and a single mortality were already observed at a single acute exposure of 0.14 mg/L in study 000040833 (1991), i.e. which is only 28 times above the low concentration of 0.005 mg/L. Further, it needs to be considered that the animals in the repeated study were exposed a total of 20-times and 50% longer per day (6 h/day).

In the 13-week Captan study, the lowest concentration resulting in 50% mortality was observed at 12.98 µg/L, or 0.013 mg/L, which is 52 times lower than the LC_{50} of 0.67 mg/L. However, here the animals in the repeated study were exposed a total of 62 times and 50% longer per day.

When we consider the exposure time for Folpet, the lowest cumulative concentration of 0.005 mg/L in study 000060792 (2008) would correspond to approximately 0.156 mg/L (0.005 mg/L × 4 weeks × 5 days/week × 1.5 [6 h to 4 h ratio]) as a single exposure concentration. Thus, the low dose concentration value would be about half the LC_{50} and the high dose concentration value would be approximately 3 times the LC_{50}. For Captan, the concentration of 0.013 mg/L in study 000040642 (1989) would correspond to about 1.3 mg/L (0.01298 mg/L× 13 weeks × 5 days/week × 1.5), which is about twice the LC_{50}. To further expand on this point, the single dose equivalents for all groups of the repeated exposure studies are depicted in Table 4.

The toxicological profile of the compounds, together with these simple calculations, illustrate that exposure duration needs to be considered when evaluating the observed effects in the repeated exposure studies. This point will be explored more formally below through the application of Haber's rule and through integration of refined dosimetry modelling.

Table 4 Single exposure equivalent concentrations extrapolated by exposure duration

Study type	Concentration [µg/L or mg/m^3]	Concentration [mg/L]	Single exposure equivalent concentration* [mg/L]
Captan 3-week DRF[&] 000040643 (1989)	*0.8*	*0.0008*	*0.018*
	5.3	*0.0053*	*0.119*
	24.8	*0.0248*	*0.558*
Captan 13-week 000040642 (1989)	0.13	0.00013	0.013
	0.6	0.0006	0.059
	5.06	0.00506	0.493
	12.98	0.01298	1.266
Folpet 4-week 000060792 (2008)	5.2	0.0052	0.156
	26	0.026	0.780
	97	0.097	2.910

* days exposed × Concentration × 1.5 (6 h vs 4 h acute exposure)

[&]The results are in grey as the animals in the study appeared to have an underlying infection, which may bias the results: increased sensitivity towards respiratory irritation insults.

Qualitative comparison of acute and repeated exposure studies

The difficulty of comparing acute and repeated exposure studies lies with the test guideline-required observations.

Detailed histological observations are typically missing from acute studies. However, the data presented in Table 5 demonstrate that the pattern and quality of clinical observations from the acute inhalation studies are comparable to histopathological observations in the repeated exposure studies. In both cases, exposure to an irritant particle is indicated. The histopathological observations are also concordant to the other irritation properties of Captan and Folpet described initially.

Based on the above, it is very likely that the observations in the repeated exposure studies occur due to multiple acute irritation events. The 13-week Captan study also suggests that the effects observed in the respiratory tract are reversible upon removal of the exposure.

Table 5 Comparison of respiratory effects in (nose-only) acute and repeated exposure studies

Captan studies		
Acute (2 studies)	During exposure, wet fur, decreased respiratory rate. Then hunched posture, lethargy, piloerection, ataxia, ptosis. Incidences of gasping, laboured and noisy respiration and red/brown stains around snout. Isolated signs: pallor of extremities, dehydration and frequent sneezing.	Wet fur, hypothermia, pigmented staining on snout, piloerection, underactivity, hunched posture, slow and deep respiration, gasping, rales, ungroomed appearance, pigmented orbital secretion, thin, closed eyes and pallor of skin. Incidences of hepatisation of the lungs, incomplete collapse when the trachea was cut, presence of pale or clear aerated fluid

	Haemorrhage and swollen lungs, fluid in lungs. Incidences of fluid in nasal/oral tract. Isolated pale lungs, fluid present in lung cavity.	in trachea, presence of pale brown material/fluid in nasal cavity, soft pale amorphous material in larynx, clear fluid in thoracic cavity. Increase of lung weight.
13-week	After exposure clinical abnormalities generally associated with restraint. There were no treatment-related clinical observations seen after exposure. Respiratory noise was present towards the end of the study in control and treated male rats, the incidence being increased in rats exposed to the higher concentrations of Captan. The observation of mucus secretion from the nose of a few female rats may have been related to treatment. Treatment-related effects were confined to the respiratory system. Histopathological changes seen in the lung were characteristic of exposure to irritant particulates. The factor contributing to death in all the males exposed to the highest concentration was necrosis of the bronchial/bronchiolar epithelium in the lung. Focal ulceration in the ventral meatus of the anterior section of the nasal passages (1 m and 1 f) was observed. There was also focal degeneration and atrophy of the olfactory epithelium in the dorsal meatus in two males and the female exposed to 12.98 µg/l and squamous metaplasia in the lachrymal duct of some of the males and the female exposed to the highest concentration of Captan. Two out of five males exposed to the highest concentration had a rhinitis. Larynx: The squamous epithelium overlying the projections was hyperplastic and keratinised and in two males and one female exposed to the highest concentration there was ulceration at the tips of the arytenoid projections. In the males there was also focal parakeratosis and vacuolar degeneration of the squamous epithelium on the arytenoid projections. In the ventral part of the same sections squamous metaplasia was evident in all the males and females exposed to the highest concentrations. Minimal or slight laryngitis was also seen in both sexes. Trachea: One male exposed to the highest concentration showed slight hyperplasia of the epithelium lining the trachea. Other changes affecting the airways of males were peribronchial inflammation, bronchitis hyperplasia of bronchiolar epithelium and an inflammatory proteinaceous exudate within the lumen of bronchioles. Changes in alveoli associated with inhalation of Captan were alveolitis, alveolar macrophage infiltration, alveolar epithelisation and hyaline membrane formation. In four males there was also minimal or slight cuffing of blood vessels with mixed inflammatory cells.	
Folpet studies		
Acute **(multiple studies)**	Dose-related decreased respiratory rate during exposure. Wet fur. Post exposure signs of ataxia, lethargy, hunched posture, piloerection and ptosis, whilst pallor of the extremities and decreased respiratory rate were noted in the higher concentration groups. Ataxia had, in many cases, subsided one hour after completion of exposure but respiratory effects were more severe by this time in survivors from all groups. In some individual animals the signs of toxicity observed at this time were severe with abnormal respiration, dehydration, hypothermia and pallor of the extremities being particularly noteworthy.	During exposure, irregular, slow, deep or shallow respiration and gasping. Incidence and time related to treatment. Higher concentrations showed changes in respiratory rate and pattern, gasping, rales, vocalisation, underactivity hunched or prone posture, partially closed eyes, pigmented staining of the snout, piloerection and wet fur. Signs persisted post exposure for several days, most prominently rales. Macroscopic findings at necropsy that were attributed to treatment were seen only

	Surviving animals from these two groups recovered from days 3 to 5 and appeared normal throughout the remainder of the study. Common abnormalities noted at necropsy throughout the dose groups were associated with the lungs. Haemorrhagic and swollen lungs, dark patches, pale patches, dark foci, abnormal redness and greyish discolouration.	among decedents and comprised: clear viscous fluid in the trachea, dark lungs, incomplete collapse of the lungs and occasional pale areas on the lungs. Skin and fur staining were also evident for a number of these animals. Also, the lung weights increased with treatment.
4-week	Treatment-related effects were confined to the respiratory system. One male exposed to the highest test concentration was euthanized in moribund condition on Day 13 from the start of exposures with labored breathing, decreased activity and a hunched appearance. Grossly, the animal exhibited red distended lungs that microscopically corresponded to slight vascular congestion and moderate amounts of fibrin and edema within alveoli and airways. Fibrin and edema were also present within the trachea and the cause of death is considered pulmonary edema related to test substance exposure. Findings in the larynx consisted of squamous/squamoid metaplasia, epithelial hyperplasia, mucosal fibrosis and inflammation. Since changes were generally more pronounced in the anterior larynx than the posterior larynx, the following description focuses on the anterior larynx. Squamous/squamoid metaplasia of the (normally) cuboidal epithelium was present in all animals in all Folpet Technical exposure groups. Changes were greater in the anterior (versus posterior) larynx in the epithelium overlying the ventral seromucous glands and were of moderate severity in most or all animals at all exposure levels. The metaplastic epithelium was keratinized, an additional indicator of severity, in all animals at all exposure levels. Keratinization was greater in the anterior larynx (minimal - moderate versus minimal in the posterior larynx) and generally similar across exposure groups. Hyperplasia of the (normally) squamous epithelium also occurred at all exposure levels in both sexes and exhibited an exposure-level-dependent increase in incidence and severity. Minimal to slight mucosal fibrosis was observed in the anterior larynx subjacent to the epithelium. It occurred in both sexes, at all exposure levels, and exhibited an exposure-level dependent increase in severity and/or incidence. In the lung. mixed inflammatory cells were present within the mucosa in all groups, including the controls, but were increased in severity (minimal-slight in controls versus slight-moderate) in both sexes in exposed animals. Small (minimal-slight) amounts of inflammatory cell debris were found within the laryngeal lumen of 2 males and 2 females in the highest treatment group. Inflammatory changes were observed in the lungs and were limited to the highest treatment group. Minimal to slight subacute/chronic peribronchiolar inflammation was present in 4 (of 5) exposed males and all females. Perivascular mixed inflammatory cells, found in all groups including the control, were slightly increased in severity (minimal in controls versus slight) in a single male and female in the high exposure group. Pulmonary inflammation was more extensive in the unscheduled decedent and included subacute/chronic interstitial inflammation, fibrin/edema and inflammatory cell debris within airways.	

Haber's rule – k-factor calculation

The following table shows the calculation of the maximal achievable k-factor value, as k_{max} = hours exposed per day × weeks exposed × mg/m^3, for the acute and 13-week and 4-week repeated exposure studies for Captan and Folpet, respectively. For approximation purposes, only the acute studies used to set the proposed LC_{50} of

the compounds in the classification process are considered. While this exercise gives a second gross estimation on the effect of time on observed toxicity, it makes sense to use more studies for refined dosimetry modelling to account for any between-study variability observed in the acute packages.

Note: the table also includes the results from the Captan 3-week repeated exposure dose-range finding study in grey. The study reports an increase in bronchiolar associated lymphoid tissue in all animals, including controls, which possibly indicates an underlying infection. An underlying infection may have resulted in an increased sensitivity towards respiratory toxic irritation insults.

Table 6 Expected mortality according to Haber's rule. Calculation of K_{max}, K_{max}/LC_{50} ratio and expected mortality based on pro-rata extrapolation considering K_{max} and actual mortality in repeated exposure studies.

Study	Conc (mg/m³)	Cumulative exposure time[a] (h)	K_{max} (mg×h/m³) (rounded)	Ratio K_{max} / LC_{50}	Expected Mortality based on K_{max} (%)[b]	Actual Mortality (%)	
						M	F
Captan LC_{50}	670	4	2680	1.000	50[c]	--	--
Captan 15 d DRF[&]	*0.8*	*90*	*72*	*0.027*	*1*	*0*	*0*
	5.3	*90*	*477*	*0.178*	*9*	*0*	*0*
	24.8	*90*	*2232*	*0.833*	*42*	*40*	*0*
Captan 90 d	0.13	390	50.7	0.019	*1*	0	0
	0.6	390	234	0.087	*4*	0	0
	5.06	390	1973.4	0.736	*37*	0	0
	12.98	390	5062.2	1.889	*94*	50	0
Folpet LC_{50}	390	4	1560	1.000	50[c]	--	--
Folpet 28 d	5.2	120	624	0.400	*20*	0	0
	26	120	3120	2.000	*100*	0	0
	97	120	11640	7.462	*100*	20	0

[&]The results are in grey and italicized as the animals in the study appeared to have an underlying infection, which may bias the results: increased sensitivity towards respiratory irritation insults.

[a] Cumulative exposure time = total hours exposed per day × weeks exposed. 4 hours in the acute studies, 6 hours in the repeated exposure studies, 5 days/week; [b] max = 100%; [c] per LC_{50} definition

Based on the application of Haber's rule, the animals in the repeated exposure studies received higher maximal cumulative doses of the test compounds than at their respective LC_{50} value (Table 6).

The animals that died in the highest concentration group of the Captan 13-week study were exposed to about twice the dose of the LC_{50}. Thus, it is not surprising that mortality was observed, according to a pro-rata extrapolation 100% mortality would have been expected. The animals in the highest concentration group of the Folpet 28-day study were exposed towards 7.5-times the dose of the LC_{50}, but the mortality was much lower than 50%.

According to the k-factor calculation and the known acute local irritation toxic mode of action of the compounds, such low acute mortality rates are actually surprising. Hence, repeated exposure seems to be less hazardous than expected based on the received dose. This decrease in hazard could be due to tissue recovery

and/or target organ clearance between exposure periods, which would not be a significant factor in an acute exposure scenario.

Overall, Haber's rule indicates that repeated exposure to low concentrations of Captan and Folpet can result in higher cumulative exposure doses than that observed in their respective acute studies. Since the toxic mode of action is acute local irritation, the observed toxicity in the repeated exposure studies is actually less than that predicted by Haber's rule. As stated above, this may be due to the development of a certain protective or detoxifying capacity of the exposed tissue or regeneration in the repeated exposure studies. Additionally, as respiratory system surfactant in rats contains proteins with thiol-groups, a certain detoxifying capacity is expected based on the available data from studies conducted with thiols in genotoxicity assays (Arce et al., 2010) and the reaction with thiols in blood (Gordon and Williams, 1999; Gordon et al., 2001).

However, the application of Haber's rule does not take into account inhalability and, therefore, refined dosimetry has subsequently been performed using the MPPD model to further demonstrate the acute toxicity driven nature of the toxicological effects reported in the repeat exposure studies.

MPPD model

More refined dosimetry was originally performed in an unpublished report by Miller (2020) (available in the supplementary materials) using the MPPD model (Cassee et al., 1999; Miller et al., 2016). The calculations are reproduced here.

The MPPD model is a computational software program that estimates deposited doses in the respiratory tract of humans and laboratory animals based on physiology and physical properties of the inhaled particles. The MPPD model software is freely available and can be downloaded at https://www.ara.com/mppd/.

The software can be used to estimate the deposited dose and correlate this dose back to the observed toxicity. Usually, inhalation studies do not consider the inhalability of the particles available within the exposure atmosphere and, instead, rely only on nominal or actually achieved concentrations. While those are informative, in isolation they do not inform on actual inhalation risk potential.

The aim of this work is to compare the cumulative exposure in repeated exposure studies to the exposure in the acute studies. The application of Haber's rule alone indicates that the repeated exposure is similar to or higher based on nominal concentrations, and the MPPD modelling will further refine this conclusion.

For MPPD modelling, the variability between the acute studies was corrected by generating an average LC_{50} dose. This approach was considered to have a scientifically robust case based on a power-law fit (Miller et al., 2000). The LC_{50} was also derived independent of sex and study type, i.e. it considered both nose-only and whole-body exposure, which is different to the approach used for classification purposes (European Commission, 2008). However, from a regulatory perspective, this approach is conservative as it under-predicts the ratio of repeated exposure dose to LC_{50} dose.

When the X-axis is the calculated particle concentration entering the nares of the rats, the resulting LC_{50} values for Folpet and Captan are 1,080 mg/m^3 and 980 mg/m^3, respectively, which is based on the following regression fits:

$$\text{Folpet_Y} = 2.0358\ X^{0.45826} \qquad \text{(Eq. 1)}$$

$$\text{Captan_Y} = 0.25698X^{0.76522} \qquad \text{(Eq. 2)}$$

The following tables show the regional deposited doses in the respiratory system in the head (ET), the tracheobronchial airways (TB) and the alveoli for the averaged acute studies at LC_{50} (Table 7) and the repeated exposure studies (Table 8), based on MPPD calculations.

Table 7 Regional deposited doses for LC_{50}s from power law fit of mortality data

Particle	LC_{50} (mg/m^3)	Male Total Deposited Dose ($\mu g/cm^2$)			Female Total Deposited Dose ($\mu g/cm^2$)		
		ET	TB	AL	ET	TB	AL
Captan	980	2,047	130	0.52	1,965	121	0.48
Folpet	1,080	2,935	156	0.70	2,527	125	0.54
ET = head; TB = tracheobronchial airways; AL = Alveolar							

Table 8 Regional deposited doses for multiple exposure studies and dose ratios for multiple exposure studies as compared to LC_{50} dose. A gradient function (red highlight) was applied on the values with increasing ratios, which is notable when the ratios approach 1, i.e. a similar dose as at LC_{50}

Study	Conc. (mg/m^3)	Total Deposited Dose ($\mu g/cm^2$)						Study Mass/LC_{50} mass					
		Male			Female			Male			Female		
		ET	TB	AL	ET	TB	AL	ET	TB	AL	ET	TB	AL
Captan 3-week DRF&	*0.8*	*48*	*5*	*0.02*	*36*	*4*	*0.01*	0.02	0.04	0.03	0.02	0.04	0.03
	5.3	*307*	*37*	*0.13*	*229*	*31*	*0.11*	0.15	0.28	0.25	0.12	0.25	0.22
	24.8	*1,686*	*102*	*0.40*	*1,318*	*102*	*0.32*	0.82	0.78	0.77	0.67	0.85	0.67
Captan 13-week	0.13	27	3	0.02	22	2	0.01	0.01	0.02	0.04	0.01	0.02	0.03
	0.6	141	20	0.10	102	12	0.06	0.07	0.15	0.19	0.05	0.10	0.13
	5.06	1,327	182	0.71	882	122	0.51	0.65	1.4	1.38	0.45	1.01	1.06
	12.98	3,629	445	1.72	2,370	317	1.28	1.77	3.41	3.33	1.21	2.63	2.66
Folpet 4-week	5.2	512	48	0.17	327	42	0.14	0.17	0.31	0.24	0.13	0.33	0.26
	26	221	260	0.97	1,516	203	0.77	0.75	1.67	1.37	0.6	1.62	1.43
	97	8,770	907	2.98	5,582	757	2.39	2.99	5.81	4.24	2.21	6.06	4.46
ET = head; TB = tracheobronchial airways; AL = Alveolar &The results are in grey, as the animals in the study appeared to have an underlying infection, which may bias the results: increased sensitivity towards respiratory irritation insults.													

Table 8 also shows the ratio of the achieved cumulative dose in the repeated exposure studies as compared to the acute studies. Note, the lower the assumed LC_{50} for the compounds and associated alveolar dose, the higher the ratio becomes. Thus, mortality pro-rata extrapolation based on the averaged LC_{50} values is conservative as compared to the LC_{50} used for classification purposes.

The tables show that rats in the repeated exposure studies received substantially higher cumulative Captan and Folpet doses than in their respective acute studies. If there would be no detoxifying or regenerative capacity of the respiratory system, mortality could be pro-rata extrapolated, because mortality is correlated with direct local effects. Hence, doses that result in 50% mortality in acute studies (LC_{50}) would result in corresponding mortalities in the repeated exposure studies.

Table 9 explores this in more detail but shows that pro-rata extrapolation from the acute studies tremendously overpredicts mortality in the repeated exposure studies. Male mortality is much lower than expected from the cumulative dose and no female rat died due to cumulative test item exposure at levels 4-times the LC_{50} for Folpet. Hence, as repeated lower doses result in less toxicity than acute higher doses, the respiratory system seems to have both regenerative and detoxifying capacity against the test item-induced insults, which is in-line with the well-investigated mode of toxic action and can also be observed in the recovery satellite groups of the 13-week repeated exposure study with Captan.

The LC_{50} values used in this assessment are higher than the ones considered for classification purposes of acute toxicity for these compounds. Further, the associated 50% mortality occurs at lower alveolar deposited masses in the studies used for acute toxicity classification. Hence, the ratio estimated here is lower than when estimated based on the studies used for classification purposes of acute toxicity.

Table 9 Expected mortality assuming the alveolar dose drives toxicity pro-rata

Study	Concentration (mg/m^3)	Mortality (%)			
		Males		Females	
		Expected*	Actual	Expected*	Actual
Captan 3-week DRF&	*0.8*	*2*	*0*	*2*	*0*
	5.3	*13*	*0*	*11*	*0*
	24.8	*39*	*40 (!)*	*34*	*0*
Captan 13-week	0.13	2	0	2	0[1]
	0.6	10	0	7	0
	5.06	69	0	53	0[2]
	12.98	100	**50**	100	0[3]
Folpet 4-week	5.2	12	0	13	0
	26	69	0	72	0
	97	100	**20**	100	0

&The results are in grey, as the animals in the study appeared to have an underlying infection, which may bias the results: increased sensitivity towards respiratory irritation insults.
*Expected mortality = mortality at LC_{50} (50%) × dose ratio; max. 100, rounded
[1] killed because of a swelling on the head: mineralized granuloma
[2] cystitis contributed to death
[3] one female was killed due to pregnancy
NB: The expected mortality would increase if the LC_{50} and associated deposited dose for acute toxicity classification purposes would be used instead of the averaged LC_{50}

CLP criteria

The classification criteria for Europe are laid out in the CLP regulation 1272/2008 (European Commission, 2008), which amends the European regulation for chemicals, REACh (Registration, Evaluation, Authorisation and Restriction of Chemicals) (European Comission, 2006). The relevant European authority is the European Chemicals Agency (EChA) for addressing harmonised classifications and not the European Food Safety Authority (EFSA), which assesses active ingredient risk assessments relevant to pesticidal product uses. EChA maintains a guidance (EChA, 2017) which is not legal advice but helps to apply the CLP criteria.

There are five different sub-classifications with regard to STOT:

STOT-SE, specific, non-lethal target organ toxicity arising from a single exposure (SE), which is not acute toxicity, skin corrosion or irritation, eye damage or irritation, respiratory or skin sensitisation or effects associated with other defined endpoints such as carcinogenicity or reproductive toxicity. Toxic effects that contribute to the classification are morbidity or death upon a single exposure (note, this cannot be acute toxicity), functional changes, gross organ or morphological damage that may be reversible but provides clear evidence of marked organ dysfunction or necrosis.

- Category 1 or 2 based on available evidence and potency considerations, with specific guidance values of <1 mg/L and >1 and ≤5 mg/L, respectively, based on studies with 4-hour exposure.
- Category 3 considers transient target organ effects, only including narcotic effects and respiratory tract irritation and if a substance does not fall into the previous two categories. As there are no validated animal tests available to address respiratory tract irritation (RTI), this category relies primarily on human data, however, animal studies may provide useful information and may be used in weight-of-evidence evaluations according to CLP.

STOT-RE, specific target organ toxicity arising from a repeated exposure. The classification includes all significant health effects that can impair function, both reversible and irreversible, immediate and/or delayed and which is not STOT-SE or acute toxicity, skin corrosion or irritation, eye damage or irritation. Toxic effects that contribute to the classification are morbidity or death resulting from repeated or long-term exposure, due to bioaccumulation and/or overwhelming of detoxification processes. Further, functional changes, gross organ or morphological damage that may be reversible but provides clear evidence of marked organ dysfunction or necrosis.

- Category 1 or 2 also based on available evidence and potency considerations, with specific guidance values of ≤0.02 mg/L/day or >0.02 ≤ 0.2 mg/L/day, respectively, based on 90-day studies with daily 6-hour exposure. The values can be modified pro-rata, according to "Haber's rule for inhalation", to use 28-day studies for classification.

EChA (2017) further specifies (extracts below):

- *[…] Acute toxicity classification is generally assigned on the basis of evident lethality (e.g. an LD_{50}/LC_{50} value) or where the potential to cause lethality can be concluded from evident toxicity (e.g. from fixed dose procedure). STOT-SE should be considered where there is clear evidence of toxicity to a <u>specific organ</u> especially when it is observed in the absence of lethality […].*
- *[…] Acute toxicity refers to lethality and STOT-SE to non-lethal effects. However, care should be taken not to assign both classes for the same toxic effect, essentially giving a 'double classification', even where the criteria for both classes are fulfilled. In such a case the most appropriate class should be assigned. […]*
- *[…] STOT-RE should only be assigned where the observed toxicity is not covered more appropriately by another hazard class. […]*
- *[…] The purpose of STOT-RE is to identify the primary target organ(s) of toxicity […]. […]*
- *[…] Where the same target organ toxicity of similar severity is observed after single and repeated exposure to a similar dose, it may be concluded that the toxicity is essentially an acute (i.e. single exposure) effect with no accumulation or exacerbation of the toxicity with repeated exposure. In such a case classification with STOT-SE only would be appropriate. […]*

The regulatory toxicology data package demonstrates that both Captan and Folpet have irritating properties at high local concentrations in all investigated exposure (portal of entry) sites that lack a protective *stratum corneum* (e.g. the eye, respiratory tract and gastrointestinal tract), and also to skin upon repeated exposure. The irritative properties also explain their limited antibiotic and effective fungicidal activity, they are used as contact fungicides in various products.

Hence, there is no specific target organ and their inherent hazard property is clearly driven by acute irritation. Both Captan and Folpet have to be classified for acute inhalation toxicity and eye damaging irritation, which appropriately describe their hazard profiles for labelling to ensure human protection.

Histopathological changes, in the repeated exposure inhalation studies and repeated exposure studies via other routes, oral and dermal, only occur subsequent to repeated local irritation. The reason is most likely the increased cumulative exposure time, as the compounds rapidly react and degrade. The effects in the respiratory system are superficially more potent because of continuous exposure for multiple subsequent hours. Mortality, which may occur due to repeated irritation in the respiratory tract, occurs only at similar cumulative doses to those in the acute inhalation toxicity studies. Thus, the acute inhalation toxicity and acute irritation classifications for eyes are more appropriate than either STOT SE or STOT RE, hence, no additional classification is needed for hazard labelling and risk communication.

With respect to the communication of hazard prevention, a non-classification for STOT-RE1 would not affect the protectiveness against Folpet and Captan hazards. While their acute toxicity is similar, Folpet has a slightly lower minimum LC_{50}, i.e. 0.39 mg/L, as compared with Captan, 0.67 mg/L, which would result in a worst-case hazard classification for acute inhalation toxicity Categories 2 and 3, respectively. The precautionary statements for acute inhalation toxicity are more protective or at least similarly protective as the precautionary statements for STOT-RE 1 or STOT-SE 3 with respect to respiratory irritation upon single exposure, as shown in Table 10. In the case of Folpet, the precautionary prevention to wear personal protective equipment according to P284, "Wear respiratory protection" would also protect users against any potential STOT-RE 1 and STOT-SE 3 effect, as does the statement for P271 "Use only outdoors or in a well-ventilated area". Hence, a classification for STOT-RE1 is neither describing the compounds' hazards appropriately, as discussed above, nor is it relevant for users in actual exposure scenarios.

The latter is highlighted by differences in the regulatory requirements of the OECD test guidelines and CLP. Captan and Folpet were micronized in all studies to maximize respirability. However, neither are sold as micronized active substances nor used micronized in products.

This issue is recognized by the CLP guidance:

The use of highly respirable dusts and mists is ideal to fully investigate the potential inhalation hazard of the substance. However, it is acknowledged that these exposures may not necessarily reflect realistic conditions. For instance, solid materials are often micronised to a highly respirable form for testing, but in practice exposures will be to a dust of much lower respirability. […] In such situations, specific problems may arise with respect to classification and labelling, as these substances are tested in a form (i.e. specific particle size distribution) that is different from all the forms in which these substances are placed on the market and in which they can reasonably be expected to be used. A scientific concept has been developed as a basis for relating the conditions of acute inhalation tests to those occurring in real-life, in order to derive an adequate hazard classification. This concept is applicable only to substances or mixtures which are proven to cause acute toxicity through local effects and do not cause systemic toxicity (Pauluhn, 2008).

It is obvious that the communication of Captan and Folpet inhalation effects for users need to be based on the physical form their products are placed on the market, if relevant human hazard communication is intended.

Table 10 Excerpt of relevant precautionary statements regarding prevention according to CLP regulation for the discussed hazard classifications

Precautionary statements	Acute inhalation toxicity		STOT RE	STOT SE
	Category 2	Category 3	Category 1	Category 3
P260 - Do not breathe dust.	P	-	P	-
P261 - Avoid breathing dust.	-	P	-	P
P270 Do not eat, drink or smoke when using this product."	-	-	P	
P271 - Use only outdoors or in a well-ventilated area.	P	P	-	P
P284 - Wear respiratory protection.	P	-	-	-

Discussion

The observations in the repeated inhalation exposure studies are confined to the respiratory tract, confirming negligible systemic availability and specifically local effects. All observations are in-line with what one would expect from an irritant particle.

The serious eye effects demonstrate Captan and Folpet's acute irritant effects on membranes that lack a *stratum corneum*, and the aetiology of their carcinogenicity in mouse is well-established to be driven by the acute local irritant effects with no relevance for human exposure scenarios. Hence, it is evident that the effects in the respiratory tract are just another aspect of the same underlying acute local irritation.

The available acute inhalation studies demonstrate that the LC_{50} decreases with smaller particle size, which is typical for irritant aerosols (Pauluhn, 2004). The respiratory rate is affected as expected for respiratory irritation (Alarie, 1981; Castranova et al., 2002), and the more refined histopathology in the repeated exposure studies demonstrate inflammation, e.g. an influx of inflammatory cells and increased lung weights. In addition, rhinitis, laryngitis, bronchitis and alveolitis have all been diagnosed in the rat inhalation study packages for both compounds.

Fibrosis, especially in the larynx, is a typical finding subsequent to irritation and inflammation in the respiratory system of rats (Renne et al., 2009; Weber et al., 2009). Subsequent proliferation and keratinization are also typical signs of inflammation and critical for the healing process (Landén et al., 2016). The test item induced irritative effects are accordingly also repaired, which is indicated by the recovery groups in the 4-week inhalation exposure Captan study (the lack of full recovery in all animals was attributed to the short recovery period following exposure, as described in the study report, and not due to an inability to recover). Recovery was also observed in the 4-week dermal toxicity studies for Folpet, which demonstrates that the irritation depends on acute irritation insults. It needs to be highlighted that the effects in the repeated studies do not occur specifically due to repetition. As described before, the reason for differences in irritation potency between study types is most likely driven by cumulative exposure time and deposited dose.

It is generally accepted that the rat is very sensitive with respect to inhalation toxicity (Hayes, 2014; Mowat et al., 2017). Particularly, squamous metaplasia is considered to have no toxicological relevance for human health (Osimitz et al., 2007). The extreme sensitivity of the rat larynx to irritant particulates is considered to arise from anatomical, airflow, epithelial cell type and possibly clearance rates. Often degeneration of the original

epithelial cells with subsequent regeneration hyperplasia and squamous metaplasia occurs (Lewis, 1991), which was observed in the available study package. In the rat larynx, the cartilage associated with the ventral pouch is U-shaped and larynx and trachea form a relatively straight line from the nasal turbinates, which enhances the deposition of aerosols. In contrast, in humans the U-shaped pouch is absent, and the larynx is more sharply angled to the oro-nasal cavity (Kaufmann et al., 2009). Results observed in the rat larynx upon exposure towards an irritant particle are therefore of limited toxicological significance in the context of human exposure.

Observed inflammation, squamous metaplasia and keratinization are the results of subsequent acute irritation events and not adverse effects with a different aetiology. The effects are also not specific to the respiratory tract but inherent to the acute local irritation hazard. Note, similar effects were seen in the 4-week Folpet dermal toxicity study. Oedema, which caused mortality in the repeated exposure studies and was identified by pulmonary infiltrates, is the proto-typical result of acute local effects in the respiratory tract consequent to irritation. It is also notable that thiols are known to be protective against chemically induced pulmonary oedema (Lailey et al., 1991) and other lung injury (Yadav et al., 2010), which is of relevance because both Captan and Folpet are detoxified by thiols (Arce et al., 2010). This plausibly explains why Captan and Folpet are relatively less hazardous upon repeated exposure than estimated by dosimetry (relatively more cumulative burden is needed for mortality than in the acute studies): rat surfactant contains thiols which have a certain detoxifying capacity and thus reduce irritation caused by Captan and Folpet at lower concentrations, i.e. leads to less potent repeated acute local insults.

The satellite groups for the 4-week Captan study show, that the observed effects recover post exposure, which confirms this hypothesis. The effects in the respiratory tract are plausibly explained by the toxic mode of action for both compounds: release of thiophosgene and direct acute effects at the first site of contact. They are, therefore, best described by acute local hazard classifications.

Based on the mortality data of the individual studies, mortality in the repeated exposure studies occurs at higher cumulative exposures than in the acute studies. The MPPD model shows that the ratio of the cumulative alveolar mass the animals received is higher in the repeated dose studies than in the acute studies but with lower mortalities. This verifies what one can infer from the reversibility of the effects: repeated lower concentrations of both Captan and Folpet are less hazardous than an acute high concentration.

1 Conclusion

Captan and Folpet show qualitatively similar first-site-of-contact exposure toxicity, which is plausible as they both have the same toxicophore, the trichloromethyl-moiety. The effects in the acute inhalation studies, repeated exposure inhalation studies, and dermal studies, as well as the oral studies are qualitatively similar and consistent with the toxicity profile of each compound. All studies show effects that reflect both Captan and Folpet's acute irritating nature. Considering exposure duration, Haber's rule and dosimetry by MPPD-modelling, the deposited doses are quantitatively similar between the acute and repeated exposure inhalation toxicity studies. Hence, it seems that the effects in the repeated exposure inhalation toxicity studies are neither specifically targeting the respiratory tract nor toxicity with a different etiology than that of acute toxicity. Thus, a classification for STOT-RE does not appropriately reflect Captan's and Folpet's hazard profile, which is driven by local acute irritation and cumulative exposure time.

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3.13 Aspiration hazard

This hazard class not assessed in this dossier.