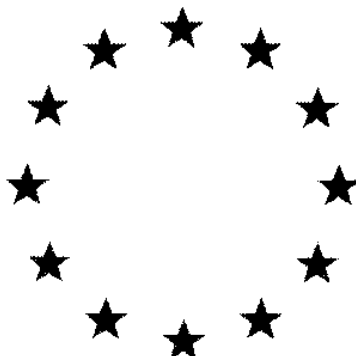


**European Commission**



**Draft (Renewal) Assessment Report prepared according to  
the Commission Regulation (EC) No 1107/2009**

**Daminozide (ISO); 4-(2,2-  
dimethylhydrazino)-4-oxobutanoic  
acid; *N*-dimethylaminosuccinamic  
acid**

**Volume 3 – B.6 (AS)**

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**B 6 TOXICOLOGY AND METABOLISM DATA****B 6.1 Absorption, distribution, metabolism, and excretion in mammals****Radiotracer metabolism study with  $^{14}\text{C}$ -ALAR in rats**

Reference	Radiotracer metabolism study with $^{14}\text{C}$ -ALAR in rats, ██████████ 1966; Report No. A.8.3.2
Guideline	The study was not conducted according to OECD guideline 417.
Deviations	-
GLP	
Acceptability	Yes
Previous evaluation	Yes, study already peer-reviewed in original DAR

**Material and method**

Rats (2/sex) were administered orally with a single dose of 1 mg of non-radioactive Alar and were housed individually in glass metabolism chambers for a 96-h observation-acclimatization period. After acclimatization, rats were administered with 1.2 mg  $^{14}\text{C}$ -Alar (purity: 98%). Faeces, urine, and  $\text{CO}_2$  were collected separately as they appeared and/or at 24-h intervals. The male animals were sacrificed at 96 h post-dose (p.d.), the two females at 192 h p.d. Upon sacrifice, the brain, heart, lungs, liver, kidneys, spleen, small intestine, large intestine, stomach, the gonads, bone were removed and blood was collected. Radioactivity was determined after homogenization of the samples, followed by evaporation and counting of the remaining solids for radioactivity.

**Results:**

Radiolabel was excreted mainly via faeces (70%). The remaining radioactivity was excreted via urine (24%) and via  $^{14}\text{CO}_2$  (2.4%). Excretion was essentially complete within 48 h, and no relevant differences were observed between males and females. At the time of sacrifice, more than 96% of the radioactivity had been eliminated. Furthermore, 1.1% of the administered radiolabel was retained in the selected organs/tissues (0.22%; excluding GIT tissue and contents) and the remaining carcass (0.83%). The highest amount of radioactivity was observed in liver (0.15% of the administered dose). No relevant differences were observed in toxicokinetics between males and females. From the data presented here, it can be delineated that the absorption of Alar is at least 28%.

**Note:** This study was not performed according to OECD guideline 417. Purities (chemical and radiochemical) were not completely presented. Furthermore, animals were pre-treated with unlabelled Alar. As a consequence, the toxicokinetic data gathered in this study may not be regarded as representative for a single oral administration. This study may be regarded as a supplementary study. Moreover, no repeated dose administration and no high dose administration were investigated. Important tissues such as muscle and fat were not examined.

**Metabolism of Daminozide (ISO); 4-(2,2-dimethylhydrazino)-4-oxobutanoic acid; N-dimethylaminosuccinamic acid ('hereafter referred to as 'daminozide')**

Reference	Metabolism of daminozide in miniature swine, [REDACTED], 1987; Report No. A.8.3.21
Guideline	The study was not conducted according to any guideline
Deviations	-
GLP	
Acceptability	Yes
Previous evaluation	Yes, study already peer-reviewed in original DAR

### Material and method

One group of male and female miniature swine (3/sex; source: [REDACTED]) was administered orally with 5 mg [<sup>14</sup>C-methyl]-daminozide/kg bw and sacrificed 96 h p.d. Another group of animals of each sex were pre-treated with daily doses of 5 mg unlabelled daminozide for 10 consecutive days followed by a radiolabelled dose and was sacrificed 48 h later. Urine and faeces (collected separately) and tissues were analysed for radioactivity by combustion analysis. Additionally, urine and faeces were extracted and analysed for metabolites with HPLC by co-elution.

### Results:

Daminozide was rapidly excreted in urine within 24 h (14%) and faeces within 48 h (59%) with no important sex differences. Pre-treatment with 10 equal daily doses did not significantly influence the faecal excretion. The urinary excretion was increased slightly for males and decreased slightly for females. From these data it can be delineated that the absorption of daminozide was at least 14%.

For single dose as well as for pre-treated animals, highest levels of radiolabel were found in liver (0.043 and 0.055g eq/kg, respectively). Residues in kidneys were 0.020 mg eq/kg for single dose animals and 0.048 mg eq/kg for pre-treated animals. Average residues in muscle, fat, gonads, bile, blood, upper, and lower colon ranged from 0.013-0.028 mg eq/kg. No significant differences were found between males and females. Except for the residue levels in kidneys, differences between single dose treated and pre-treated animals were also minimal.

In urine and faeces, daminozide was the major radiolabelled compound. At least four and two breakdown products were present in urine and faeces, respectively. 1,1-dimethylhydrazine (UDMH) and N-nitrosodimethylamine (NDMA) were identified respectively as major and minor metabolite in urine of all animals and in faeces of some animals.

**Note:** This study was actually intended to investigate residue levels in various tissues of miniature swine, but it was also examined whether it could provide any additional relevant data, useful for the evaluation of the toxicokinetics of daminozide (previous studies, not submitted here were not acceptable for the evaluation of

were not presented. Furthermore, the report on the biological part of the study is not attached. Important discrepancies were noted between study design and results with respect the question whether only a 5 mg kg/bw administration or also a higher dose administration was used. The study could therefore only be used as supplementary to other studies.

#### Metabolism of daminozide in miniature swine: Analysis for UDMH in liver tissue

Reference	Metabolism of daminozide in miniature swine: Analysis for UDMH in liver tissue, [REDACTED] 1987; Report No. A.8.3.23
Guideline	The study was not conducted according to any guideline
Deviations	-
GLP	
Acceptability	Yes
Previous evaluation	Yes, study already peer-reviewed in original DAR

#### Material and method:

In [REDACTED] (1987), the investigation of the metabolism of daminozide in miniature swine using  $^{14}\text{C}$ -labelled daminozide was described. Due to the low levels of radiolabel in the liver, formation of  $^{14}\text{C}$ -UDMH (unsymmetrical dimethylhydrazine) could not be studied. In the present study efforts are described to quantify UDMH levels in liver tissue from miniature swine. Liver tissue samples were homogenized and purified. The extract was analysed by GC/MS (selective ion monitoring).

#### Results:

UDMH was detected at a level of almost 0.001 mg eq/kg in a sample of 15 g of liver.

**Note:** This study was intended to investigate residue levels in various tissues of miniature swine, but it was also examined whether it could provide any additional relevant data, useful for the evaluation of the toxicokinetics of daminozide (previous studies, not submitted here were not acceptable for the evaluation of toxicokinetics). In this study, samples were used from [REDACTED] (1987) that was not performed according to OECD guideline 417. The present study was not performed according to OECD guideline 417 either. Furthermore, the method was described only very briefly (a report describing the complete method was not contained in the dossier). Therefore, the data found in this study should be regarded as supplementary data.

#### Metabolism and excretion of daminozide in male rats at a single oral dose level

Reference	Metabolism and excretion of daminozide in male rats at a single oral dose level, [REDACTED] 1993; Report No. 0012
Guideline	The study was not conducted according to any guideline

Deviations	-
GLP	Yes
Acceptability	Yes
Previous evaluation	Yes, study already peer-reviewed in original DAR

**Material and method:**

Rats (5 male F-344) were administered with a single oral dose of 1 mg radiolabelled daminozide/kg bw (purity: 98.5% unlabelled, 97% labelled). Urine, faeces, expired air (all collected until sacrifice at 96 h p.d.), terminal blood and plasma, liver, lungs, and the residual carcass were analysed for radiochemical content. Expired air was analysed for  $^{14}\text{CO}_2$  as well as exhaled volatile compounds. Urine collection included the urine contained in the bladder following sacrifice, which was withdrawn using a syringe. Urine and faeces were examined for the presence of unchanged test substance and UDMH (co-elution on HPLC).

**Results:**

The recovery of the administered radioactivity from all sources averaged 89% at sacrifice. An average of 87% of the dose was excreted: approximately 47% in the urine, 32% in the faeces, 7% as  $^{14}\text{CO}_2$ , and less than 1% in exhaled volatile compounds. The terminal body burden (i.e. including analysed tissues) averaged 2.5% of the administered dose. Based on these data, at least 57% of the administered dose was absorbed into the systemic circulation. Within 24 h of dosing 80% of the dose was excreted.

From liver, blood, and lungs, 0.18%, 0.10% and 0.03% of the administered dose were recovered at sacrifice, respectively. The concentrations of remaining radioactivity were 0.045, 0.020, and 0.044 mg eq/kg, respectively. From blood plasma, 0.02% of the dose was recovered (0.008 mg eq/kg). An average of 2.2% remained in the carcass (including GIT tissue and contents).

Unchanged daminozide was the predominant radiolabelled compound in the urine collected within 6 h but in urinary excretion between 6 and 12 h p.d. its hydrolysis product, 1,1-dimethylhydrazine (unsymmetrical dimethylhydrazine, UDMH), was the predominant radiolabelled compound. In the faeces excreted from 6-12 h and 12-24 h p.d., daminozide was the predominant radiolabelled compound (Table 6.1./04-1).



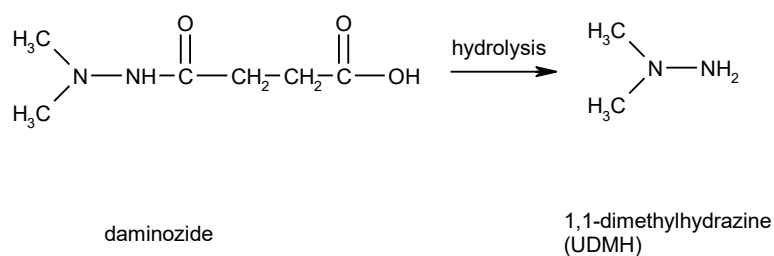
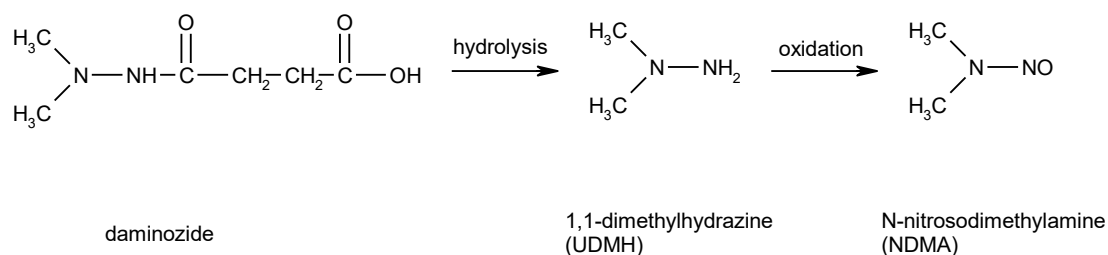
Table 6.1./04-1: Excretion of daminozide and UDMH in urine and faeces (% of the administered dose)

Urine			Faeces		
	UDMH	Daminozide		UDMH	Daminozide
0 - 6 h	0.55	6.61			
6 - 12 h	<b>8.14</b>	3.63	0 - 12 h	0.17	8.5
12 - 24 h	<b>17.4</b>	2.56	12 - 24 h	0.87	18
24 - 48 h	2.69	0.58			
Cumulative	28.7	13.4	Cumulative	1	27

**Note:** This study did not completely fulfil the requirements of OECD guideline 417 (no high and low dose administration, total recovery of the radiolabel remained far below 95%). Furthermore, very few organs and tissues were examined for radiolabel (no kidneys, muscle, fat). Therefore, this study is only suitable for the establishment of the minimal absorption of daminozide, its excretion, and the formation of UDMH, all upon single oral dose administration.

Figure 6.1-1: Proposed metabolism of daminozide

A provisional metabolism scheme is presented below. It should be used as supplementary data.



**B 6.1.1 Summary of metabolism studies**

**Absorption:** In male rats that were administered a single dose of 1 mg radiolabelled daminozide/kg bw p.o., absorption was at least 57%. Absorption of at least 28% was established in male and female rats that were administered an oral dose of 5.7 mg radiolabelled daminozide/kg bw.

In male and female miniature swine, that were administered 5 mg radiolabelled daminozide/kg bw, absorption was established to be at least 14% and was not significantly different in animals that were pre-treated with 10 daily doses of 5 mg unlabelled daminozide/kg bw.

**Excretion:** A single oral dose of 1 mg radiolabelled daminozide/kg bw to rats was excreted via urine (47%), faeces (32%) and the lungs ( $^{14}\text{CO}_2$ , 7% and some volatile compounds in expired air, <1%). Radiolabel was rapidly eliminated (nearly complete within 48 h) in rats (1.2 mg radiolabelled daminozide/kg bw) via faeces (70%), urine (24%) and the lungs (2.4%). In total, more than 96% of the administered radiolabel was excreted at time of sacrifice.

Radiolabel was rapidly excreted in miniature swine via faeces (59%; within 48 h) and urine (14% within 24 h). Pre-treatment with 10 daily 5 mg radiolabelled daminozide/kg bw doses did not importantly change the excretion data.

**Distribution:** Some organs and tissues were examined for radioactivity upon administration of a single oral dose of 1 mg radiolabelled daminozide/kg bw to male rats. Liver, blood, and lungs contained 0.18%, 0.10%, and 0.03% of the administered dose, respectively. The radiolabel levels were 0.045, 0.020, and 0.044 mg eq/kg, respectively. At 96 h p.d., in total 0.22% of the administered dose was recovered from tissues in rats, and liver in particular (0.15% of the administered dose).

In the miniature swine, radiolabel was recovered mainly from liver (0.043 mg eq/kg). Only in kidneys, importantly increased levels of radiolabel were found in pre-treated animals compared to swine that received only a single dose (0.048 compared to 0.020 mg eq/kg).

**Metabolism:** In rats that received a single oral dose of 1 mg test substance/kg bw, UDMH (unsymmetrical dimethylhydrazine) was found as an important metabolite in urine and faeces (accounting for 29% and 1% of the administered dose, respectively). Unchanged test substance accounted for 13 and 27% of the administered dose in urine and faeces, respectively.

In miniature swine, daminozide was converted to UDMH and NDMA (*N*-nitrosodimethylamine), as both compounds were reported to be found in urine and faeces. UDMH was also found in liver.

***In vitro* comparative metabolism study****[<sup>14</sup>C]-Daminozide: Comparative in vitro metabolism using mouse, rat, dog and human hepatocyte metabolism**


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Reference	<b>[<sup>14</sup>C]-Daminozide: Comparative in vitro metabolism using mouse, rat, dog and human hepatocyte metabolism, Yen-Ling Cheung, 2017</b>
Guideline	Not applicable; No OECD guideline is available for this study
Deviations	-
GLP	Yes
Acceptability	Yes
Previous evaluation	No

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**Material and method:**

[<sup>14</sup>C]-Daminozide dissolved in purified water (5 and 50 µM; purity: >98%) was incubated (at 37°C ± 1°C) with either mouse, rat, dog or human hepatocytes for 0, 0.5, 1 and 3 hours. Incubations in the absence of hepatocytes were also conducted at both [<sup>14</sup>C]-daminozide concentrations for 3 hours, to check the stability under incubation conditions. As a positive control were used samples incubated with 7-ethoxy[3-<sup>14</sup>C]coumarin (7EC) at a concentration of 50 µM for 3 hours in duplicate. The initial hepatocyte viability was determined by the trypan blue exclusion test. Incubation samples were analysed by HPLC with both off-line (5 µM [<sup>14</sup>C]-daminozide samples) and on-line (50 µM [<sup>14</sup>C]-daminozide samples) radioactive monitoring. The proportions of metabolites produced and parent [<sup>14</sup>C]-daminozide were quantified. Selected samples were analysed by LC-MS with the aim of identifying the metabolites produced.

**Results:**

[<sup>14</sup>C]-Daminozide (5 and 50 µM) essentially remained as unchanged parent compound following incubation with mouse, rat, dog and human hepatocytes for 0, 0.5, 1 and 3 hours. Only one metabolite was detected by radio-HPLC, designated M1, but at levels below the limit of quantification (<1%; in all species and in the control samples in the absence of hepatocytes). Using LC-MS/MS, only N-nitrosodimethylamine was detected and was found to be present in three of the four species tested (mouse, rat and human) and in the no-hepatocyte control sample. Because there was no material difference in the presence of N,N-dimethylhydrazine or N-nitrosodimethylamine in samples incubated with hepatocytes over the no-hepatocyte controls, the presence of these compounds may be attributed to the degradation of [<sup>14</sup>C]-daminozide rather than to any active metabolism, i.e. daminozide was not metabolised *in vitro* by hepatocytes from any of the species chosen.

Table 6.1.1-1: Parent compound: Total % of radioactivity (mean value of duplicates)

<sup>14</sup> C-Daminozide concentration  Incubation time [hours]	5µM				50µM			
	0	0.5	1	3	0	0.5	1	3
Mouse hepatocytes	96.9	97.4	97.4	97.4	91.8	95.7	91.5	93.4
Rat hepatocytes	97.2	97.2	97.1	96.9	95.8	98.7	97.0	97.0
Dog hepatocytes	97.3	97.3	97.1	97.2	97.9	97.6	96.6	96.5
Human hepatocytes	96.8	97.0	97.4	97.8	95.1	96.0	93.7	97.9

**Conclusion:**

Taken together, under the conditions of this study there were no human-specific metabolites detected. The presence of M1 (N, N-dimethylhydrazine) and N-nitrosodimethylamine in samples may be attributed to the degradation of parent [<sup>14</sup>C]-daminozide rather than to metabolism.

**RMS 2018**

We consider the conclusion regarding the presence of N,N-dimethylhydrazine and N-nitrosodimethylamine attributed by the applicant only to the degradation of parent molecule as speculative. As the incubation time of hepatocytes with Daminozide was only 3 hours, metabolic conversion of parent molecule to the N,N-dimethylhydrazine cannot be excluded.

As indicated in the study (B.Connor, J. Hart, 2012) the hydrolysis in aqueous solution from parent molecule to UDMH is characterized by maximum hydrolytic conversion between 4 - 24 hours (0.1% of parent molecule at 24h).

In addition, as indicated in toxicokinetic study (████████████████████ 1993) high UDMH urinary excretion in time intervals 6-12h (8.14% of administered dose of Daminozide) and 12-24h (17.4% of administered dose of Daminozide) suggests that UDMH may be *in vivo* product of metabolism, as it is rather unlikely that spontaneous hydrolysis *in vivo* would be that much faster than spontaneous hydrolysis in aqueous solution.

Based on above-mentioned we consider this study of limited validity regarding the presence or detection of relevant Daminozide metabolites in mammals.

Generally, as the incubation time of daminozide with tested cell culture was too short, no information on UMDH role in human metabolism can be extracted from this study.

**B 6.2 Acute toxicity****B 6.2.1 Oral****Single dose oral toxicity in rats**

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Reference	Single dose oral toxicity in rats, [REDACTED] 1994a; Report No. A.7.1.11
Guideline	The study was conducted according to OECD guideline 401
Deviations	-
GLP	Yes
Acceptability	Yes
Previous evaluation	Yes, study already peer-reviewed in original DAR

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**Material and method:**

Five male and female Wistar Albino rats (source: [REDACTED]) were dosed orally, by gavage, with Alar<sup>®</sup> technical (purity: 99.42%, stable at pH = 5, 7, 9 over 30 days) at 5000 mg/kg bw. The rats were observed 1, 2 and 4 hours post dose and once daily for 14 days for toxicity and pharmacological effects. The animals were observed twice daily for mortality. All animals were examined for gross pathology. The LD<sub>50</sub> and 95 % Confidence Limits were calculated by the method of Lichtfield&Wilcoxon JPET 96:99, 1949 or Horn H. J. Biometrics 12:311, 1956.

**Results:**

Mortality: No animals died during the exposure and 14-days post exposure period observations.

Symptoms of toxicity: Instances of soiling of the anogenital area were noted during the observation period. Bodyweight gains were normal.

Pathology: No abnormalities were observed at necropsy.

**Conclusion:**

The acute oral LD<sub>50</sub> of daminozide was found to be higher than 5000 mg/kg bw in male and female rats.

**Note:** The study is considered suitable for the overall toxicological evaluation.

**RMS 2018:** Acute oral LD<sub>50</sub> > 5000 mg/kg; classification according to the CLP (1272/2008) is not required.

**B 6.2.2 Dermal****Acute dermal toxicity in rabbits**


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Reference	Acute dermal toxicity in rabbits, [REDACTED] 1994b; Report No. A.7.1.12
Guideline	The study was conducted according to OECD guideline 402

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Deviations	-
GLP	Yes
Acceptability	Yes
Previous evaluation	Yes, study already peer-reviewed in original DAR

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**Material and method:**

Five male and five female NZW Albino rabbits (source: [REDACTED]) were dosed dermally with Alar® technical (purity: 99.42%, stable at pH = 5, 7, 9 over 30 days) at 5000 mg/kg bw. Approximately 24 hours prior to application of the test article, the dorsal area of the trunk of each animal was clipped free of hair. The prepared site was approximately 10 % of the body surface. The test article was weighed out in individual doses of 5000 mg/kg and moistened with distilled water. The test site was covered with a gauze patch, secured with non-irritating tape and gentle pressure was applied to the gauze in contact with the skin for 24 hours. Dermal responses were recorded on days 1, 7, and 14. Animals were observed for toxicity and pharmacological effects at 1, 2, and 4 hours post dose and once daily for 14 days. All animals were observed twice daily for mortality. Bodyweights were recorded pre-test, weekly, and at death or termination in the survivors. All animals were examined for gross pathology.

**Results:**

Mortality: One animal died on day 13 with no pre-death physical signs.

Symptoms of toxicity: Physical signs noted in survivors included diarrhoea, soiling of the anogenital area, emaciation and few faeces. Erythema and oedema, absent to well defined on day 1, were absent on days 7 and 14. Bodyweight gains were normal.

Pathology: No abnormalities were observed in the survivors at necropsy. The necropsy observations of the dead animal are consistent with pulmonary infection and are not considered to be related to the substance.

**Conclusion:**

The acute dermal LD<sub>50</sub> of daminozide was found to be higher than 5000 mg/kg bw for rabbits.

**Note:** The study is considered suitable for the overall toxicological evaluation.

**RMS 2018:** One animal died on day 13 - the necropsy showed a pulmonary infection, which is not relevant for application of a.i. Acute dermal LD<sub>50</sub> > 5000 mg/kg; classification according to Regulation (EC) No 1272/2008 is not required.

**B 6.2.3 Inhalation****An acute (4 hour) inhalation toxicity study of Alar Technical in the rat via nose only exposure**

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Reference	<b>An acute (4 hour) inhalation toxicity study of Alar Technical in the rat via nose only exposure, [REDACTED] 1994a; Report No. A.7.1.15</b>
Guideline	The study was conducted according to OECD guideline 403
Deviations	-
GLP	Yes
Acceptability	Yes
Previous evaluation	Yes, study already peer-reviewed in original DAR

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**Material and method:**

One group consisting of five male and five female Sprague-Dawley rats (source: [REDACTED]) was exposed to an atmosphere containing Alar® technical for four hours at a target concentration of 2.0 mg/l or at a maximum attainable concentration. The test was generated into the breathing zone of the animals as a dust (white crystalline solid, 99.42 % daminozide, storage room temperature). The test animals received an average gravimetric exposure concentration of 2.1 mg/l of Alar® technical with a range 1.2 to 3.2 mg/l. A total of 202.4 grams of test material was used during the exposure resulting in a nominal concentration of 42 mg/l. Particle size distribution measurements showed an average mass median aerodynamic diameter of 6.7 microns with an average GSD of 2.8. Approximately 3.8 percent of the dust was less than or equal to 1.0 micron in size, approximately 31 percent of the dust was less than or equal to 4.0 microns in size, and approximately 65 percent was less than or equal to 10.0 microns. All animals were held for a 14-day post-exposure observation period. A macroscopic necropsy examination was performed on all animals.

**Results:**

Mortality: No animals died during the exposure or 14-days post exposure observation period.

Symptoms of toxicity: During the 2-4 hour post-exposure period and during the 14-day observation period a few scattered signs of nasal discharge were observed. Normal bodyweight gain was noted.

Pathology: No abnormalities were observed at necropsy.

**Note:** This study is considered to be acceptable for the overall toxicological evaluation.

**Conclusion:**

The acute respiratory LC<sub>50</sub> of daminozide was found to be higher than 2.1 mg/L the highest practically achievable concentration. No classification according to Regulation (EC) No 1272/2008 as amended is required.

**RMS 2018:** Acute inhalation LC<sub>50</sub> > 2.1 mg/L; classification according to Regulation (EC) No 1272/2008 is not required.

**B 6.2.4 Skin irritation****Primary dermal irritation in albino rabbits**

Reference	Primary dermal irritation in albino rabbits, ██████████ 1994; Report No. A.7.1.13
Guideline	The study was conducted according to OECD guideline 404
Deviations	-
GLP	Yes
Acceptability	Yes
Previous evaluation	Yes, study already peer-reviewed in original DAR

**Material and method:**

Six NZW rabbits (5 males and 1 female, source: ██████████) were dosed dermally with Alar<sup>®</sup> technical (purity: 99.42%). 0.5 g was applied to one intact site and was kept in contact with the skin for 4 hours; after removal of dressing the dermal reactions were scored at 30, 60 minutes, 24, 48 and 72 hours. The skin was evaluated for ulceration and necrosis. Bodyweights were recorded.

**Results:**

Erythema and oedema, absent to slight at 30 to 60 minutes after patch removal, were absent at 24, 48, and 72 hours. There, were no abnormal physical signs noted during the study. The Modified Primary Irritation Index was 0.13.

**Table 6.2.4/01-1: Draize scores from the dermal irritation study for daminozide in albino rabbits**

Scores observed after	30-60 minutes	24 hours	48 hours	72 hours
Erythema	0, 1, 0, 0, 0, 0	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0
Oedema	0, 1, 0, 0, 0, 0	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0

**Note:** The study is considered to be suitable for the overall toxicological evaluation.



**Conclusion:** Daminozide in water was not found to be irritating at 24-48-72 hours to the rabbit skin.

**RMS 2018:** The substance did not irritate rabbit skin at 24-48-72 hours; classification according to Regulation (EC) No 1272/2008 is not required.

#### **B 6.2.5 Eye irritation**

##### **Primary eye irritation and/or corrosion in rabbits**

Reference	Primary eye irritation and/or corrosion in rabbits, [REDACTED] 1994c; Report No. A.7.1.14
Guideline	The study was conducted according to OECD guideline 405
Deviations	-
GLP	Yes
Acceptability	Yes
Previous evaluation	Yes, study already peer-reviewed in original DAR

#### **Material and method:**

Six NZW albino rabbits (4 males and 2 females) were dosed with Alar® technical (purity: 99.42%). 0.1 g of the daminozide was placed into conjunctival sac of one eye of each rabbit. After instillation, the lids were held together for approximately 1 second to insure adequate distribution. The contralateral eye served as a control. Ocular responses were recorded at 1 hour, 1, 2, 3 and 7 days. Fluorescein was used to determine corneal effects on day 1.

#### **Results:**

There was no corneal opacity noted at any observation period. Iritis, noted in 1/6 eyes, cleared by day 2. Conjunctival irritation, noted in 6/6 eyes, cleared by day 7. There were no abnormal physical signs noted during the observation period. The test article produced iritis and conjunctival irritation which cleared within 7 days.

**Table 6.2.5/01-1: Draize scores from the eye irritation study for daminozide in albino rabbits**

Scores observed after	1 hour	1 day	2 days	3 days	7 days
Cornea/opacity	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0
Iritis	0, 0, 0, 0, 0, 0	0, 1, 0, 0, 0, 0	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0
Conjunctival redness	1, 1, 1, 1, 1, 1	1, 2, 2, 2, 1, 2	1, 2, 2, 1, 2, 1	1, 2, 2, 1, 2, 1	0, 0, 0, 0, 0, 0
Conjunctival chemosis	2, 2, 2, 2, 2, 2	2, 2, 2, 2, 2, 2	2, 2, 2, 1, 2, 2	1, 2, 2, 0, 1, 0	0, 0, 0, 0, 0, 0

Scores observed after	1 hour	1 day	2 days	3 days	7 days
Conjunctival discharge	2, 2, 2 <sup>a</sup> , 2, 2, 2	2, 2, 1, 1, 2, 2	0, 2, 1, 1, 2, 2	0, 1, 0, 0, 0, 0	0, 0, 0, 0, 0, 0

<sup>a</sup> test article remaining in conjunctiva

### Conclusion:

Daminozide was found to be mildly irritating to the rabbit eye. However, the substance need not be classified according to EC classification.

**Note:** The study is considered suitable for the overall toxicological evaluation.

**RMS 2018:** Daminozide was found to be mildly irritating to the rabbit eye. The values were not sufficient to reach the trigger values for classification. All ocular abnormalities resolved on day 7.

### B 6.2.6 Skin sensitization

#### Closed patch repeated insult dermal sensitization study of Alar Technical in guinea pigs (Buehler method)

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Reference	<b>Closed patch repeated insult dermal sensitization study of Alar Technical in guinea pigs (Buehler method),</b> [REDACTED] 1994b; Report No. A.7.1.22
Guideline	The study was conducted according to OECD guideline 406
Deviations	-
GLP	Yes
Acceptability	Yes
Previous evaluation	Yes, study already peer-reviewed in original DAR

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### Material and method:

**Induction:** A Hilltop Chamber® was saturated with Alar technical (100% concentration, purity: 99.4%) moistened with 0.3 mL of 0.9% saline and applied to 10 male and female Dunkin Hartley guinea pigs (on the right side of the midline) for 6 hours. After exposure, the chamber was removed and the skin was wiped free of any excess material with distilled water and gauze. This was performed once per week, for three weeks, for a total of three exposures.

**Challenge:** Fourteen days after the last induction exposure, the challenge treatment was administered. The test or control material was administered in the same manner as in the induction phase, but at a site on the opposite side of the midline from the site used for induction. After six hours of exposure, the chamber was removed and the skin wiped free of any excess material. In order to differentiate dermal reactions produced by irritation from those produced by sensitization, five male and five female animals (previously untreated) were subjected to the same challenge procedures as the animals which received the induction exposures.

The appropriate amount of DNCB was added to 80% ethanol to produce a 0.005 g/mL (0.5% w/v) mixture or to acetone to produce a 0.003 g/mL (0.3% w/v) mixture used as a positive control during induction and challenge phase, respectively.

**Response evaluation:** Observations for mortality were made twice daily. Bodyweight was recorded pre-test and 2 days after challenge. Animals were observed prior to treatment and weekly during the study for general health; unusual observations were recorded. Dermal evaluations were made approximately 24 and 48 hours after each induction exposure as well as 24 and 48 hours after the challenge application. Dermal responses were scored.

#### **Results:**

All animals survived throughout the study and gained weight by study termination. Weekly observations were unremarkable. Based on a range-finding study, a concentration of 100% test material moistened with saline was used for induction and challenge. This maximal attainable dose was non-irritating to guinea pig skin. All 20 test animals showed no dermal response at challenge with the test material.

#### **Conclusion:**

Alar technical (daminozide technical) did not show skin sensitizing properties in a Buehler test.

**Note:** The study is considered relevant for evaluation of the sensitizing properties of daminozide.

#### **Local Lymph Node Assay in the Mouse (Individual Method)**

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Reference	<b>Local Lymph Node Assay in the Mouse (Individual Method),</b> [REDACTED] 2003; Report No. 2242/012
Guideline	The study was conducted according to OECD guideline 429 and EPA OPPTS 870.2600
Deviations	-
GLP	Yes
Acceptability	Yes
Previous evaluation	No

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#### **Material and method:**

Groups of five female CBA/CA mice (source: [REDACTED]) were subjected to topical applications of vehicle control (DMSO), positive control ( $\alpha$ -hexylcinnamaldehyde) or one of the test formulations (5, 10 or 25% in DMSO; purity: 99.7%) to the outer aspect of the auditory pinnae once daily on days 1, 2 and 3. On day 6, a 20  $\mu$ Ci dose of titrated thymidine was injected intravenously into each animal. Five hours later the auricular lymph nodes were recovered from each animal and processed through a scintillation counter. Test results are expressed in

terms of Stimulation Indices with the threshold level to be considered a positive indicator of the potential to cause skin sensitisation being 3.0. Statistical differences in DPM values between test groups and the vehicle control group were determined.

**Results:**

All animals survived treatment and there were no clinical signs indicative of a systemic effect of treatment among mice treated with any of the controls or test formulations. The vehicle and test formulation application sites remained free of irritation. Irritation was noted on the ears of the positive control group animals on days 3 and 4. A greasy appearance of the fur of the head and/or neck was noted in all test group animals on day 1 and in all positive control group animals throughout the observation period. There was no indication of treatment-related effect on bodyweight.

**Table 6.2.6/01-1: Stimulation Index from Local Lymph Node Assay in the Mouse (Individual Method)**

	Concentration of test article in applied formulation (% m/v)		
	5%	10%	25%
<b>Stimulation Index</b>	0.58	0.80	1.28

**Conclusion:**

The Local Lymph Node Assay demonstrated that daminozide technical does not have the potential to cause skin sensitization.

**RMS 2018:** Under conditions used in both studies (Buehler and LLNA) daminozide did not sensitise the skin. No classification is required according to the Regulation (EC) No 1272/2008.

**B 6.2.7 Phototoxicity**

Daminozide was shown not to absorb electromagnetic radiation within the range of 290 – 700 nm, therefore the study on phototoxicity is not required.

**B 6.2.8 Summary of acute toxicity studies**

With regard to the acute toxicity, daminozide does not need to be classified according to Regulation (EC) No 1272/2008 as amended for acute oral, dermal, respiratory toxicity, skin or eye irritation and skin sensitization. The results of the acute toxicity studies, the irritation studies, and the sensitization studies that are suitable for evaluation in the context of Annex I renewal are presented below in tabular format.

**Table 6.2-1: Acute toxicity studies**

Test substance	LD <sub>50</sub> (mg/kg bw)	Species	Route	Reference
Daminozide (>99%)	> 5000	rat	oral	Uniroyal, [REDACTED] 1994a
Daminozide (>99%)	> 5000	rabbit	dermal	Uniroyal, [REDACTED] 1994b
Daminozide (>99%)	> 2.1 mg/L (highest dose attainable)	rat	inhalation	Uniroyal, [REDACTED] 1994a

**Table 6.2-2: Skin and eye irritation studies**

Test substance	Classification	Species	Route	Reference
Daminozide (>99%)	non irritant	rabbit	dermal	Uniroyal, [REDACTED] 1994
Daminozide (>99%)	mildly irritant	rabbit	eye	Uniroyal, [REDACTED] 1994c

**Table 6.2-3: Skin sensitisation studies**

Test substance	Classification	Species	Route	Reference
Daminozide (>99%)	Non-sensitiser	guinea pig	dermal (Buehler)	Uniroyal, [REDACTED] 1994b*
Daminozide technical (99.7%)	Non-sensitiser	mice	dermal (LLNA)	Fine Agrochemicals, [REDACTED] 2003

\* supplementary study

### **B 6.3 Short-Term Toxicity**

#### **B 6.3.1 Oral 28-day studies**

No study submitted.

**B 6.3.2 Oral 90-day and 1-year studies****13 week oral (gavage) administration toxicity study in the rat**

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Reference	13 week oral (gavage) administration toxicity study in the rat, ██████████ 2005; Report No. 2242/040
Guideline	The study was conducted according to the following guidelines: OECD TG 408, US EPA OPPTS 870.
Deviations	Yes
GLP	Yes
Acceptability	Yes
Previous evaluation	No

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**RMS comment 2018:** The study has been checked for compliance. The following deviations from OECD guideline 408 (adopted on 21st September 1998) were found:

- 1) Uteri of female animals were not weighed at necropsy
- 2) The report did not include baseline values of clinical biochemistry and haematological tests

**Material and method:** Ten Wistar ██████████ rats per sex were gavaged with Daminozide Technical Grade (purity: 100.2%) at doses of 0 (control, i.e. vehicle), 40, 200, and 1000 mg/kg bw/day for 90 days. The vehicle was 0.25% w/v aqueous methyl cellulose. Dosing formulations were analysed to confirm concentration, homogeneity and stability.

All animals were observed at the beginning and the end of the working day for clinical signs. Individual bodyweights were recorded on day -7, on the first day of dosing, at weekly intervals thereafter, and before necropsy. The amount of food consumed by each cage of animals was measured once weekly from Week -1.

In addition, observations included ophthalmoscopy, functional observation battery (grip strength, posture, motor activity, gait, arousal upon opening cage, tremor, convulsion, excessive vocalisation, lacrimation, salivation, respiration, piloerection, appearance of fur, etc.), haematology (Hb, packed cell volume, mean cell volume, mean cell haemoglobin concentration, red cell distribution width, platelet count, red blood cell count, reticulocytes, total and differential white cell count, prothrombin time, and activated partial thromboplastin time), clinical chemistry (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma glutamyltransferase, Na, K, Ca, P, Cl, total protein, albumin, globulin, albumin/globulin ratio, total cholesterol, glucose, urea, total bilirubin, creatinine, triglycerides), and urinalysis (colour, turbidity, microscopy of sediment, volume, specific gravity, pH, protein, glucose, reducing substances, ketones, bilirubin, blood, urobilinogen).

At necropsy, adrenals, brain, heart, kidneys, liver, ovaries, spleen, thymus, testes, epididymides, uterus were weighed. Histopathological examination was performed on the following organs/tissues: adrenals, aorta, brain, caecum, colon, duodenum, epididymides, eyes, femur, heart, ileum, jejunum, kidneys, liver, lungs, mandibular lymph node, mammary gland, mesenteric lymph node, oesophagus, optic nerve, ovaries, pancreas, pituitary, prostate, rectum, salivary gland, sciatic nerve, seminal vesicles, skeletal muscle, skin, spinal cord [3 levels], spleen, sternum, stomach, testes, thymus, thyroid with parathyroid, trachea, urinary bladder, uterus, vagina.

## Results:

General observations: There were no decedents during the study. Clinical signs recorded were typical for this strain of rat and are considered not to be treatment-related. Thinning fur, hair loss, sores and lesions and staining were occasionally observed across all groups including controls, and are considered to be of no toxicological relevance.

Bodyweight: No consistent or dose-related effects of treatment on bodyweight/bodyweight gain or food consumption were observed.

**Table 6.3.2/01-1: Group mean bodyweight gains**

Time point (weeks)	Dose [mg/kg bw/day]								Statistics
	0		40		200		1000		
	♂	♀	♂	♀	♂	♀	♂	♀	
start – week 4	142.7	54.3	131.2	56.5	128.1	56.1	130.3	61.9	A
week 4- week 8	64.3	20.6	57.5	22.1	53.3	24.9	59.3	27.6	A, DR*♀
week 8 – week 13	32.5	10.5	36.4	8.5	30.2	6.6	25.0	6.4	A, DR* ♂+♀
Start – week 13	239.5	85.4	225.0	87.1	211.6	87.6	214.5	95.8	A

P<0.05,

DR – Significant dose response test

A=Anova

**Table 6.3.2/01-2: Group mean organ weights**

Organ weight	Dose [mg/kg bw/day]							
	0		40		200		1000	
	♂	♀	♂	♀	♂	♀	♂	♀
Adrenals (g)	0.075	0.067	0.073	0.071	0.082	0.067	0.068	0.071
Kidney (g)	1.950	1.266	1.979	1.193	1.890	1.202	1.916	1.322
Spleen (g)	0.705	0.491	0.765	0.501	0.743	0.531	0.716	0.588*
Liver (g)	8.600	5.529	9.061	5.449	8.863	5.449	8.756	5.769
Heart (g)	1.054	0.733	1.053	0.707	1.059	0.702	1.036	0.750
Brain (g)	2.001	1.839	1.962	1.836	2.017	1.825	2.014	1.865
Thyroids (g)	0.016	0.014	0.019	0.014	0.019	0.013	0.018	0.014
Thymus (g)	0.362	0.315	0.350	0.289	0.371	0.308	0.350	0.312
Testes (g)	5.461		5.555		5.542		5.568	
Ovaries (g)		0.084		0.090		0.083		0.103

\*P&lt;0.05,

**Haematology:** There were no effects of treatment on red cell or white cell lines, platelets or clotting. There were minor haematological changes noted (slight reduction of packed cell volumes in females treated with 1000 and 200 mg/kg/day and slightly higher mean cell haemoglobin in females in all treatment groups) however analysis of the data revealed that the controls showed slight variance with respect to the historical control range and the treated groups were within the range and normal. These changes therefore were considered to be of no toxicological relevance. Haemoglobin distribution width values were also slightly but significantly higher in females treated with 1000 mg/kg/day but values were within historical control range and these changes were therefore considered to be of no toxicological relevance.

**Table 6.3.2/01-3 Selected group mean haematology parameters**

Parameters	Dose [mg/kg bw/day]							
	0		40		200		1000	
	♂	♀	♂	♀	♂	♀	♂	♀
PCV (%)	52.2	51.4	53.3	49.6	52.8	49.4*	52.5	49.0*
MCV (fL)	54.9	57.7	55.4	56.1	54.8	55.6**	54.2	56.5
MCHC (g/dL)	31.1	31.3	31.0	32.3*	31.1	32.3*	31.7	32.6**

\*P&lt;0.05, \*\* P&lt;0.01,



Clinical chemistry and urinalysis: Females treated with 1000 mg/kg/day showed significantly higher calcium (outside the historical control range). Other minor changes were noted but were inconsistent or not dose-related or clearly within the normal ranges and were therefore considered not to be of toxicological significance.

Males and females treated with 1000 mg/kg/day had significantly increased specific urine gravity (outside the historical control range in males) with males also showing significantly reduced volumes. There was a slightly higher incidence of males treated with 1000 mg/kg/day with lower urine pH, darker urine and with amorphous debris. Additionally, males treated with 1000 or 200 mg/kg/day generally had a lower concentration of phosphates.

**Table 6.3.2/01-4: Group mean clinical chemistry parameters**

Parameters	Dose [mg/kg bw/day]							
	0		40		200		1000	
	♂	♀	♂	♀	♂	♀	♂	♀
AST (IU/L)	68	75	64	70	62	74	63	68
ALT (IU/L)	33	32	35	38	34	39	31	36
ALP (IU/L)	177	88	171	85	167	97	164	84
Na (mmol/l)	140	144	142	143	140	143	140	143
K (mmol/l)	5.8	4.8	5.3	4.8	4.9	4.8	5.1	4.6
Ca (mmol/l)	2.69	2.82	2.67	2.84	2.66	2.83	2.70	2.93**
P (mmol/l)	2.2	1.6	1.9	1.5	1.8**	1.4	2.1	1.7
Glucose (mmol/l)	6.6	6.1	6.0	5.4	6.2	5.4	6.4	4.8**
Urea (mmol/l)	8.7	8.6	7.3	9.1	7.1	8.6	7.1	8.7
Bilirubin (umol/l)	2.1	1.9	1.8	1.9	2.2	1.9	2.6	2.6
Creatinine (umol/l)	88	79	71	72	69	74	68	73

\*\* P<0.01,

**Table 6.3.2/01-5: Group mean urinalysis parameters**

Parameters	Dose [mg/kg bw/day]							
	0		40		200		1000	
	♂	♀	♂	♀	♂	♀	♂	♀
Volume (mL)	5.5	2.7	4.7	2.6	4.0	2.9	2.3***	2.4
Specific gravity	1.037	1.038	1.036	1.043	1.041	1.038	1.064***	1.053**
pH	7	6	7	6	6	5	5	5

\*\* P<0.01, \*\*\* P<0.001

Functional observation battery: At Week 13, there was a slight reduction in locomotor activity at a single period in males treated with 1000 mg/kg/day. This change was not consistent and was within the range of normal variability of data and is considered to be of no toxicological relevance. Overall, there were no findings from any assessment indicative of any neurotoxicological effect of the test compound.

**Pathology:** Females treated with 1000 mg/kg/day had a slightly heavier spleen, and although they had slightly heavier bodyweights, spleen weights were heavier when adjusted for bodyweight. In the absence of any histopathological correlate this finding is considered not to be of toxicological significance.

**Conclusion:**

On the basis that there were no findings of toxicological significance, the No-Observed-Adverse-Effect-Level (NOAEL) is considered to be 1000 mg/kg/day. Although there were minor changes in clinical pathology parameters and organ weights, they were considered non-adverse in the absence of corroborative histopathology, and may just reflect higher plasma solute loads.

**RMS 2018:** RMS agrees with the original NOAEL set at 1000 mg/kg bw/day (top dose). Differences in clinical biochemistry parameters (e.g. calcium concentration) or organ weights observed between the top dose and control group were only minor (although statistically significant) and not supported by accompanying macroscopic, histopathological or behavioural findings. Therefore, they are not considered to be biologically relevant.

**One Year Dietary Toxicity Study in Dogs**

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Reference	<b>One Year Dietary Toxicity Study in Dogs, ██████████ 1988a;</b> Report No. A.7.3.12
Guideline	The study was conducted according to EPA Pant 158 guideline for chronic dog studies (closely corresponds to OECD TG 452)
Deviations	Yes
GLP	Yes
Acceptability	Yes
Previous evaluation	Yes, study already peer-reviewed in original DAR

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**RMS comment 2018:** The study has been checked for compliance. The following deviations from OECD guideline 452 (adopted on 7th September 2009) were found:

- 1) The addition of the fourth test group is recommended if large intervals (6-10fold) between dosages are used
- 2) As for haematological parameters, prothrombin time and activated partial thromboplastin time were not investigated
- 3) Epididymides and uteri of animals were not weighed at necropsy
- 4) At the beginning of the study, the bodyweight variation for each sex of animals should not exceed  $\pm 20\%$  of the mean weight, however, this requirement was not met in the study

**Material and method:**

6 Beagle male and female dogs were treated orally (in diet) with daminozide (purity: 99%) at 0, 300, 3000, and 7500 ppm. A control group received basal laboratory diet on the same regimen. The dogs were observed for mortality, moribundity, signs of overt toxicity, diarrhoea, emesis, and inappetence at least twice daily throughout the study.

Detailed observations of appearance and condition, behaviour and activity, excretory functions, respiration, orifices, eyes, and palpable masses were conducted at least once each week. Individual bodyweights were determined pre-test and weekly for the first 14 weeks of study and once every 4 weeks thereafter, and at termination. Individual food consumption was measured weekly for the first 14 weeks of study, once every 4 weeks thereafter, and at termination.

Ophthalmoscopic examinations were conducted on all dogs once during the pre-test period and prior to terminal sacrifice. Physical examinations (inspection for general condition: examination of the head and neck, thorax, abdomen, external reproductive organs, skin, and extremities; heart and lung sounds evaluated by percussion and auscultation) were conducted on all dogs once during the pre-test period and at 3, 6, 9 and 12 months of study.

Clinical laboratory studies including haematology (Hb, haematocrit, mean cell volume, mean cell haemoglobin concentration, platelet count, red blood cell count, reticulocytes, total and differential white cell count), biochemistry (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, Na, K, Ca, P, Cl, total protein, albumin, globulin, albumin/globulin ratio, total cholesterol, glucose, urea, total bilirubin, creatinine, triglycerides), and urinalysis (colour, turbidity, microscopy of sediment, volume, specific gravity, pH, protein, glucose, ketones, bilirubin, blood, urobilinogen) were conducted on all animals in each group once prior to study initiation and at 6 and 12 months of study.

At necropsy, adrenals, brain, heart, kidneys, liver, ovaries, spleen, thymus, testes, pituitary, thyroids were weighed. Histopathological examination was performed on the following organs/tissues: adrenals, aorta, brain, caecum, colon, duodenum, epididymides, eyes, femur, heart, ileum, jejunum, kidneys, liver, lungs, mandibular lymph node, mammary gland, mesenteric lymph node, oesophagus, optic nerve, ovaries, pancreas, pituitary, prostate, rectum, salivary gland, sciatic nerve, seminal vesicles, skeletal muscle, skin, spinal cord [3 levels], spleen, sternum, stomach, testes, thymus, thyroid with parathyroid, trachea, urinary bladder, uterus.

## Results:

**Clinical findings:** No significant clinical findings were observed during the study. During the first 13 weeks of study, inappetence was occasionally noted in most of the female treated dogs. During study weeks 14-26, food-like emesis and/or soft stool was occasionally noted for most of the 7 500 ppm males.

**Bodyweight:** Mean bodyweight values were comparable between the control and treated animals and were not statistically different. Mean bodyweights of high dose males were approximately 4 to 6 percent below control male values throughout a majority of study weeks.

**Table 6.3.2/02-1: Group mean bodyweight (kg)**

Week of the study	Dose [mg/kg bw/day]							
	0		300		3000		7500	
	♂	♀	♂	♀	♂	♀	♂	♀
	ppm		ppm		ppm		ppm	

Week of the study	Dose [mg/kg bw/day]							
	0 ppm		300 ppm		3000 ppm		7500 ppm	
	♂	♀	♂	♀	♂	♀	♂	♀
0	12.0	8.9	11.9	8.8	12.2	9.2	11.6	8.9
5	13.1	9.6	12.3	9.8	13.6	10.0	12.6	9.7
11	13.5	10.1	13.4	10.3	13.8	10.2	13.0	9.8
18	13.6	10.3	13.3	10.5	14.3	10.4	12.7	9.8
34	14.1	10.6	13.9	10.8	14.6	11.0	13.4	10.6
52	14.6	11.3	14.4	11.1	15.3	11.1	13.7	10.9

Table 6.3.2/02-2: Group mean organ weights

Organ weight	Dose [mg/kg bw/day]							
	0 ppm		300 ppm		3000 ppm		7500 ppm	
	♂	♀	♂	♀	♂	♀	♂	♀
Adrenals (g)	1.30	1.30	1.40	1.43	1.38	1.32	1.40	1.31
Kidney (g)	59.45	47.14	60.75	45.46	60.86	45.27	61.65	43.10
Spleen (g)	121.43	80.21	137.26	61.24	115.66	80.94	97.32	70.09
Liver (g)	303.21	268.78	308.42	261.66	330.28	247.51	309.77	246.92
Heart (g)	100.49	76.07	105.03	77.43	112.04	78.90	104.02	76.52
Brain (g)	84.18	74.99	80.38	77.09	83.66	76.26	83.41	79.32
Thyroids (g)	1.43	0.92	1.21	1.05	1.25	1.15	1.38	1.06
Pituitary (mg)	83	72	79	76	76	76	89	65
Testes (g)	19.42		21.24		19.48		17.68	
Ovaries (g)		1.21		1.33		1.09		1.32

Food consumption: Mean food consumption values on g/animal/day and g/kg/day basis were comparable between the control and treated groups.

Ophthalmoscopic evaluation: No test article related ophthalmoscopic abnormalities were detected; the observations noted were representative of pathology that would be expected for this group of dogs considering age, sex, and strain.

Physical examination: Review of the physical examinations revealed no significant findings related to test article administration.

Haematology, clinical chemistry, and urinalysis: There were no test article related haematological, biochemical, and urological changes at any interval tested.

**Table 6.3.2/02-3: Selected group mean haematology parameters**

Parameters	Dose [mg/kg bw/day]							
	0 ♂	ppm ♀	300 ♂	ppm ♀	3000 ♂	ppm ♀	7500 ♂	ppm ♀
PCV (%)								
MCV (fL)	77		78		78		78	
MCHC (g/dL)	36.3		35.5		35.6		35.6	

**Table 6.3.2/02-4: Group mean clinical chemistry parameters**

Parameters	Dose [mg/kg bw/day]							
	0 ♂	ppm ♀	300 ♂	ppm ♀	3000 ♂	ppm ♀	7500 ♂	ppm ♀
AST (IU/L)	18	17	18	17	17	18	18	17
ALT (IU/L)	32	29	28	27	34	30	39	28
ALP (IU/L)	20	35	22	32	20	35	23	30
Na (mEq/l)	148	147	148	148	147	147	148	148
K (mEq/l)	4.7	4.8	4.6	4.8	4.7	4.8	4.5	5.0
Ca (mg/dL)	10.8	10.6	10.6	10.6	10.5	10.8	10.6	10.8
P (mg/dL)	4.1	4.1	3.9	3.5	3.9	3.5	3.5	4.0
Glucose (mg/dL)	106	102	102	100	102	107	104	106
Urea (mg/dL)	14.8	15.6	14.7	15.3	16.1	15.3	14.3	17.8
Bilirubin (mg/dL)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Creatinine (mg/dL)	1.0	0.8	1.0	0.9	1.0	0.9	1.0	0.9

**Table 6.3.2/02-5: Group mean urinalysis parameters**

Parameters	Dose [mg/kg bw/day]							
	0 ♂	ppm ♀	300 ♂	ppm ♀	3000 ♂	ppm ♀	7500 ♂	ppm ♀
Volume (mL)	128	137	65	114	92	77	53	193

Parameters	Dose [mg/kg bw/day]							
	0 ♂	ppm ♀	300 ♂	ppm ♀	3000 ♂	ppm ♀	7500 ♂	ppm ♀
Specific gravity	1.035	1.035	1.039	1.034	1.043	1.039	1.043	1.039
pH	8		7		7		7	

**Pathology:** There were no test article-related macroscopic changes at dietary concentrations of 300, 3,000 or 7,500 ppm. All macroscopic changes seen in these dogs were considered to be of spontaneous origin. One female in the high dose group died during the study period. Macroscopic and microscopic findings in this female suggested virus-induced acute haemorrhagic enteritis. There were no microscopic changes which were considered to be test article-related at dietary concentrations of 300, 3,000 or 7,500 ppm. All microscopic changes seen in dogs of this study were considered to be of agonal or spontaneous origin. One female in the high dose group had a renal cell adenoma in one kidney. The tumour was benign, well circumscribed, small, and not detected at necropsy or at tissue trimming but was found upon microscopic examination. In all other dogs of the high dose group, no treatment related renal changes were evident. Although it is recognized that renal cell adenoma is a rare neoplasm in young dogs, in the absence of any pre-neoplastic changes or any other test article-related changes in the high dose group, the renal cell adenoma in this female was considered to be a spontaneous tumour which occurred by chance in the high dose group.

#### Original DAR conclusion:

One female dog at 7500 mg/kg food died with acute haemorrhagic enteritis. Mean bodyweights of the high dose males were 4-6% lower than the control male values throughout a majority of the study. Based on these considerations the NOAEL is placed at 3000 mg/kg food (equal to 80.5-82.8 mg/kg bw/day).

**RMS 2018:** RMS agrees with the original NOAEL derived from this study. Setting of NOAEL at 3000 ppm (equal to 80.5 - 82.8 mg/kg bw/day) is supported by occurrence of renal cell adenoma (0.3 - 1.5 % of canine neoplasms; males are more susceptible than females; ██████████ 2012) in one female dog of the top dose group (7500 ppm equal to 199 mg/kg bw/day) as well as by higher incidence of food-like emesis and soft stool in animals of the top dose.

#### B 6.3.3 Other routes – dermal/inhalation studies

##### 28 day dermal toxicity study in rats

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Reference                      **28 day dermal toxicity study in rats, ██████████ 2012; Report No. 10519**

Guideline                      The study was conducted according to the following guidelines: OECD TG 410, EC Guideline B.9 OJEC No L 383 A/144, and EPA OPPTS 870.3200 and in compliance with GLP.

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Deviations	Yes
GLP	Yes
Acceptability	Yes
Previous evaluation	No

**RMS comment 2017:** The study has been checked for compliance. The following deviations from OECD guideline 414 (adopted on 22nd January 2001) were found:

- 1) At the start of the test, weight range 200 to 300 g is recommended for rats, which was not met in the study

#### **Material and method:**

Male and female Wistar rats (source: ), 10 rats/sex/group (one control and three test substance-dosed groups) were administered vehicle or daminozide (125, 500 or 2000 mg/kg; purity: 100%) daily by dermal application for at least 28 consecutive days (minimum of 6 hours/day) and euthanized one day following the last dose.

The weighed aliquot of daminozide was applied to pre-wetted (3 mL deionized water) gauze (3 X 4 inch Telfa pad, secured onto a dermal dosing jacket with an additional velcro strap to ensure contact with the skin) immediately prior to dose. The same procedure was performed for control animals, except only gauze moistened with 3 mL of deionized water was applied to the dose site. Each day the jackets and strap were removed and the dose site was gently wiped with water-dampened and dry gauze to remove as much test substance residue as feasible without damaging the skin.

All animals were observed twice daily for clinical signs of toxicity/mortality. Neurological examinations included evaluation of external surface areas (visual inspection and palpation for externally detectable “masses”), orifices, posture, respiration, and excretory products. During the examination, animals were also observed for piloerection, involuntary motor movements, stereotypies (e.g. excessive grooming, repetitive circling), bizarre behaviour (e.g. self-mutilation, walking backwards), vocalizations, changes in skin, fur, eyes, and mucous membranes. During Weeks 1 and 4, upon completion of the 6 h exposure and gauze pad removal, observations of each individual animal were done in the open field (standard arena).

The non-fasted bodyweight of each animal was measured on study days -1, 7, 14 and 21 and a fasted weight was measured on study Day 28. Food consumption was measured weekly.

The clinical chemistry and haematology parameters evaluated were as follows: blood cell morphology, RBC, haematocrit, haemoglobin, leukocytes, MCV, MCH, MCHC, platelets, reticulocytes, prothrombin time, activated partial thromboplastin time, ALT), albumin, A/G ratio, ALP, AST, bilirubin, blood urea nitrogen, Na, K, Ca, P, Cl, cholesterol, creatinine, creatine phosphokinase, GGT, glucose, LDH, protein, triglycerides, uric acid.

A standard necropsy was conducted on all animals. At necropsy, adrenals, brain, heart, kidneys, liver, ovaries, seminal vesicles, spleen, thymus, testes, epididymides were weighed. Histopathological examination was performed on the following organs/tissues: adrenals, aorta, brain, caecum, colon, duodenum, epididymides, eyes, femur, heart, ileum, jejunum, kidneys, liver, lungs, mandibular lymph node, mammary gland, mesenteric lymph node, oesophagus, optic nerve, ovaries, pancreas, pituitary, prostate, rectum, salivary gland, sciatic nerve, seminal vesicles, skeletal muscle, skin, spinal cord [3 levels], spleen, sternum, stomach, testes, thymus, thyroid with parathyroid, trachea, urinary bladder, uterus.

#### Results:

No effects attributable to exposure to the test substance were observed at any dose level.

#### Conclusion:

Based on the lack of adverse findings observed in this study, the 28-day dermal NOAEL in male and female rats dosed with daminozide was 2000 mg/kg/day, the highest dose level tested.

**RMS 2018:** The conclusion of this study is supported by the RMS.

#### B 6.3.4 Summary of short-term toxicity studies

In the rat 90 day gavage toxicity study (■■■■■ 2005) the NOAEL was confirmed at the limit dose of 1000 mg/kg/day. Even using oral gavage administration, there was no evidence of any neurotoxic effects. In the one year feeding study in the dog NOAEL of 3000 ppm (equal to 80.5 - 82.8 mg/kg bw/day) was established, based on occurrence of renal cell adenoma in one female as well as higher incidence of food-like emesis and soft stool in the top dose group. A new 28-day dermal toxicity study also showed no adverse effects, and the NOAEL for this study was set to the top dose at 2000 mg/kg/day (■■■■■ 2012). The results of the oral sub-chronic studies are summarised in Table 6.3.1.

**Table 6.3.1: Oral subchronic studies with their NOAELs and critical effects**

Duration	Species	Route	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Critical effects	Reference Notifier
Duration	Species	Route	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Critical effects	Reference Notifier



90-day	rat	gavage	1000 (highest dose tested)	-	No adverse effects	Fine, ██████ 2005
1 year	dog	dietary	80.5 (3000 ppm)	199 (7500 ppm)	Renal cell adenoma, food- like emesis, soft stool	Uniroyal, ██████ 1988a

#### B 6.4 Genotoxicity

##### B 6.4.1 *In vitro* studies

##### Reverse mutation in one tryptophan-requiring strain of *Escherichia coli*

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Reference	Reverse mutation in one tryptophan-requiring strain of <i>Escherichia coli</i> , <i>Williams L., 2006</i> ; Report No. 2242/50
Guideline	The study was conducted according to the following guidelines: OECD TG 471; EU Council Regulation No.440/2008, Part B, Method B13/14; Japanese MAFF Notification No. 12 Nousan 8147 GLP.
Deviations	Yes
GLP	Yes
Acceptability	Yes
Previous evaluation	No

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**RMS comment 2018:** The study has been checked for compliance. The following deviations from OECD guideline 471 (adopted on 21st July 1997) were found:

- 1) The test substance should be used with approximately half log (i.e.  $\sqrt{10}$ ) intervals between test concentration for an initial experiment, which was not met in the study
- 2) 2-aminoanthracene should not be used as the only indicator of S9 mix efficacy
- 3) Number of cells per culture was not reported in the study

#### Material and method:

Daminozide (purity: 100.2%) was assayed for mutation in tryptophan-requiring strain (WP2uvrA) of *Escherichia coli* in the absence and presence of metabolic activation by an Aroclor 1254-induced rat liver post-mitochondrial fraction (S-9), in two separate experiments. Experiment 1 (plate-incorporation) was carried out in the absence and

presence of S-9, using final concentrations of daminozide at 1.6, 8, 40, 200, 1000, and 5000 µg/plate. Experiment 2 (pre-incubation) was performed in the absence and in the presence of S-9 at daminozide concentrations 156.25, 312.5, 625, 1250, 2500, and 5000 µg/plate. Negative (vehicle) and positive controls (4-nitroquinoline-1-oxide and 2-aminoanthracene in the absence and presence of S-9, respectively) were used. The plates were examined for signs of toxicity. Colonies were counted electronically or manually where confounding factors such as split agar affected the accuracy of the automated counter.

Evaluation criteria: The test article is considered mutagenic if:

- (i) The assay was valid
- (ii) Dunnett's test gave a significant response ( $p \leq 0.01$ ) and the data set(s) showed a significant concentration correlation.
- (iii) The positive trends/responses described above were reproducible.

A test article is considered negative in this assay if none of the above criteria are met.

#### Results:

Following Experiment 1 or 2 no evidence of toxicity was observed in the absence or the presence of S-9. The test article was completely soluble in the aqueous assay system at all concentrations treated, in each of the experiment performed. Mean vehicle control counts were consistent with the normal historical control ranges, and positive controls induced large increases in revertant number which were consistent with the normal historical positive control ranges. Less than 5% of plates were lost. The assay was therefore accepted as valid.

There were no statistically significant, dose-related and reproducible increases in revertant numbers observed following treatment in the presence or absence of metabolic activation. Daminozide is therefore considered to be non-mutagenic in this assay.

**Table 6.4.1/01-1: Reverse mutation in one tryptophan-requiring strain of *Escherichia coli*:** Mean number of revertant colonies per plate; AAN = 2-aminoanthracene, NQO = 4-nitroquinoline-1-oxide;

Treatment	Dose (µg/ plate)	Revertant colonies/plate (mean ± SD)			
		Experiment 1		Experiment 2	
		-S9	+S9	-S9	+S9
Daminozide TG	0	7 ± 3	11 ± 4	13 ± 2	20 ± 4
	1.6	8 ± 2	7 ± 3		
	8	10 ± 4	13 ± 2		
	40	5 ± 1	6 ± 1		
	156.25			18 ± 6	24 ± 5
	200	6 ± 4	11 ± 3		
	312.5			16 ± 3	19 ± 6
	625			14 ± 12	20 ± 6
	1000	8 ± 4	15 ± 3		
	1250			16 ± 6	18 ± 4
	2500			11 ± 1	15 ± 7
	5000	8 ± 3	6 ± 2	15 ± 9	19 ± 6
Positive controls	NQO	1188 ± 74		986 ± 264	
	AAN		254 ± 17		99 ± 17

**Conclusion:**

Daminozide did not induce mutation of tryptophan-requiring strain of *Escherichia coli* (WP2uvrA) when tested under the conditions of this study. These conditions included treatments at concentrations up to 5000 µg/plate in the absence and in the presence S-9 metabolic activation.

**RMS 2018:** The RMS agrees that Daminozide did not exert genotoxic effect under conditions of this study.

**P7642: Assessment of its ability to induce genetic damage in *Saccharomyces Cerevisiae***

Reference	<b>P7642: Assessment of its ability to induce genetic damage in <i>Saccharomyces Cerevisiae</i>, Bootman J., Lodge D.C., 1983; Report No. Uniroyal Chemical. Report No. A.7.6.7</b>
Guideline	The study was conducted neither in compliance with GLP nor according to any guideline
Deviations	-
GLP	No

Acceptability	No
Previous evaluation	Yes, study already peer-reviewed in original DAR

#### Material and method:

Cultures of diploid, D6 strain of *Saccharomyces cerevisiae* (sensitive to cycloheximide, requiring adenine) were exposed to the test substance at the concentration of 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, and 2000 µg/mL with or without metabolic activation (S-9 mix) for 12 hours. Following incubation, each treated cell suspension was centrifuged, washed, and re-suspended in phosphate buffer. Dilutions ( $10^{-2}$  and  $10^{-4}$  in sterile saline) were prepared from each bottle, and plates of solid media were inoculated with 0.1 mL aliquots as follows: YC (liquid yeast complete) medium +  $10^{-2}/10^{-4}$  dilution; YC medium with cycloheximide +  $10^0/10^{-2}$  dilution; YM (liquid yeast minimal) medium +  $10^0$  dilution. Plates were incubated at 28° to 30°C for 5 days ( $10^{-4}$  dilution) or 10 days (all other plates), and then removed to a cold room (0° - 4°C) to await scoring. 12-0-Tetradecanoylphorbol-13-acetate (TPA) and deoxycholate were used as positive controls.

#### Results:

No significant increases were obtained in mitotic non-disjunction (aneuploidy) crossing over or mutation, either in the absence or presence of S-9 mix.

**Note:** No guideline is available for the present study. Some major shortcomings were identified: no motivation was provided for the applied dose range, based on solubility and toxicity; no information on the purity of the test substance was provided; the test was not repeated. Based on these shortcomings, this study is considered supplementary for the overall evaluation.

#### Conclusion:

The test substance did not induce an increased frequency of mitotic non-disjunction or crossing over in *Saccharomyces cerevisiae* D6 in the mitotic aneuploidy assay in the presence and the absence of a liver homogenate from Aroclor-treated rats. However, the limitations listed above make the study of limited value for the overall evaluation

#### Ames metabolic activation test to assess the potential mutagenic effect of daminozide

Reference	Ames metabolic activation test to assess the potential mutagenic effect of daminozide, Richold M., Jones E., Fenner L.A., 1984; Report No. FNA 4/84222
Guideline	The study was conducted according to OECD TG 471
Deviations	Yes

GLP	Yes
Acceptability	Yes
Previous evaluation	Yes

**RMS comment 2018:** The study has been checked for compliance. The following deviations from OECD guideline 471 (adopted on 21st July 1997) were found:

- 1) At least five strains of bacteria should be used. Even more importantly, recommended combination of *Salmonella typhimurium* strains is the following: 4 strains with GC and 1 strain with AT base pair at the primary revision site in order to detect mutagens including oxidizing and cross-linking agents. The strain with AT base pair such as TA 102 was not used in the study.
- 2) 2-aminoanthracene should not be used as the only indicator of S9 mix efficacy

#### Material and method:

Four strains of *S. typhimurium* bacteria (TA1535, TA1537, TA98 and TA100) were used in the test. In a preliminary test, four concentrations of test substance (5, 50, 500 and 5000 µg/plate; purity: 99%) were assessed for toxicity.

Five concentrations of test substance (50, 150, 500, 1500 and 5000 µg/plate) were studied in two tests. Water was used as the negative control. The positive controls were (i) with S-9 mix: 2-aminoanthracene at 2 µg/plate for all tester strains and (ii) without S-9 mix: 2-nitrofluorene at 10 µg/plate for strain TA98, 9-aminoacridine at 20 µg/plate for strain TA1537 and sodium azide at 5 µg/plate for strains TA1535 and TA100. Plates were incubated for 72 hours at 37°C. Colonies were counted using a Biotran Automatic Colony Counter, and the mean number of revertant colonies per treatment group was assessed.

#### Results:

The results of the range-finding test showed that daminozide was not toxic towards the tester strains. Therefore 5000 µg/plate was chosen as the top dose level in the mutation tests. The mean numbers of revertant colonies obtained in the first and second mutation tests are shown in 6.4.1/02-1 and 6.4.1/02-2. No substantial increases in revertant colony numbers of any of the four tester strains were observed following treatment with daminozide at any dose level, either in the presence or absence of metabolic activation.

**Table 6.4.1/02-1: Mean number (± SD) of revertant colonies obtained in the first mutation test**

	Mean ± SD revertant colony counts							
	Without metabolic activation				With metabolic activation			
Dose level (µg/plate)	Strain TA1535	Strain TA1537	Strain TA98	Strain TA100	Strain TA1535	Strain TA1537	Strain TA98	Strain TA100
5000	10±4.6	4±1.5	43±2.6	65±11.6	4±1.5	5± <sup>a</sup>	30±2.6	76±18.4
1500	8±1.5	9±2.9	40±7.9	60±5.5	7±4.2	15±4.0	25±5.8	73±9.5

<b>500</b>	9±2.0	12±4.6	43±2.6	68±12	12±4.7	27±1.2	39±3.1	90±3.1
<b>150</b>	8±3.5	10±4.0	29±2.1	65±16.5	6±3.2	20±1.5	34±3.6	97±8.5
<b>50</b>	11±1.0	16±4.0	32±5.7	78±10.0	9±3.1	14±2.6	27±1.2	95±2.3
<b>0</b>	12±5.5	10±2.3	37±4.9	71±7.9	10±1.0	20±4.5	27±3.5	85±13.1
<b>Positive controls</b>								
<b>2AA</b>	-	-	-	-	115±17.0	158±11.3	1195±125.4	702±43.7
<b>2NF</b>	-	-	475±25.7	-	-	-	-	-
<b>9AAC</b>	-	125±34.4	-	-	-	-	-	-
<b>NaN<sub>3</sub></b>	889±11.4	-	-	795±107.4	-	-	-	-

2AA: 2-aminoanthracene

9AAC: 9-aminoacridine

<sup>a</sup>: contaminated

2NF: 2-nitrofluorene

NaN<sub>3</sub>: sodium azide

Table 6.4.1/02-2: Mean number (± SD) of revertant colonies obtained in the second mutation test

	Mean ± SD revertant colony counts							
	Without metabolic activation				With metabolic activation			
<b>Dose level (µg/plate)</b>	Strain TA1535	Strain TA1537	Strain TA98	Strain TA100	Strain TA1535	Strain TA1537	Strain TA98	Strain TA100
<b>5000</b>	7±2.3	13±3.6	27±6.7	95±9.5	8±2.5	19±9.5	27±4.4	90±8.1
<b>1500</b>	8±1.0	16±1.7	22±3.6	85±11.0	10±0.6	18±2.3	20±1.5	83±28.7
<b>500</b>	7±2.1	14±4.0	27±5.7	82±12.1	11±2.6	20±1.0	18±2.1	79±18.2
<b>150</b>	10±6.2	13±2.5	30±2.5	70±4.5	8±1.5	19±1.5	22±5.5	85±16.6
<b>50</b>	5±2.0	16±3.5	27±3.5	81±16.6	4±1.2	22±1.5	23±2.9	88±14.5
<b>0</b>	10±4.0	20±0.6	26±8.5	80±3.1	14±2.5	18±3.2	21±2.5	75±13.1
<b>Positive controls</b>								
<b>2AA</b>	-	-	-	-	98±11.8	124±11.1	564±22.2	870±24.8
<b>2NF</b>	-	-	378±84.5	-	-	-	-	-
<b>9AAC</b>	-	104±28.4	-	-	-	-	-	-
<b>NaN<sub>3</sub></b>	441±38.4	-	-	411±29.1	-	-	-	-

2AA: 2-aminoanthracene

9AAC: 9-aminoacridine

2NF: 2-nitrofluorene

NaN<sub>3</sub>: sodium azide**Original DAR conclusion:**

The test substance was not mutagenic when tested in the reverse mutation assay, in the presence or absence of metabolic activation.

**RMS 2018:** The RMS agrees with the original conclusion of this study. Daminozide did not exert mutagenic effect in any of tested *Salmonella typhimurium* strains under conditions of this reverse mutation assays. However, only 4 bacteria strains were used and the strain for detection of oxidizing and cross-linking agents was not involved (see deviations from OECD TG 471 above). Therefore, this study is considered to be supplementary.

**Salmonella/mammalian-microsome plate incorporation mutagenicity assay (Ames test)**

Reference	<b>Salmonella/mammalian-microsome plate incorporation mutagenicity assay (Ames test), San R.H.C., Shelton J.B., 1991; Report No. A.7.6.18</b>
Guideline	The study was conducted according to OECD TG 471
Deviations	Yes
GLP	Yes
Acceptability	Yes
Previous evaluation	No

**RMS comment 2018:** The study has been checked for compliance. The following deviations from OECD guideline 471 (adopted on 21st July 1997) were found:

- 1) Recommended combination of *Salmonella typhimurium* strains is the following: 4 strains with GC and 1 strain with AT base pair at the primary revision site in order to detect mutagens including oxidizing and cross-linking agents. The strain with AT base pair such as TA 102 was not used in the study.
- 2) 2-aminoanthracene should not be used as the only indicator of S9 mix efficacy
- 3) The recommended maximum test concentration for non-cytotoxic substances in mutagenicity assay is 5mg/plate and not 10mg/plate
- 4) The plates should be incubated at 37 °C (37 ±2 °C in the study)

**Material and method:**

Daminozide (purity: 99.8%) was tested in Ames test for its ability to induce back mutations at selected loci of several strains of *Salmonella typhimurium* in the presence and absence of microsomal enzymes derived from Aroclor 1254-induced rat liver. The tester strains used in this study were TA98, TA100, TA1535, TA1537 and TA1538. The assay was performed in three phases: the dose-range finding study, the mutagenicity and confirmatory assays. In dose range-finding study ten dose levels (10, 33, 67, 100, 333, 667, 1000, 3333, 6667, 10000 µg/plate) of the test article were plated, one plate per dose, with an overnight culture of TA100 on selective minimal agar in both the presence and absence of microsomal enzymes. In mutagenicity and confirmatory assays, the test article was tested at five dose levels (667, 1000, 3333, 6667, 10000 µg/plate) along with appropriate vehicle (DMSO) and positive controls (2-nitrofluorene for strain TA98 and TA1538; sodium azide for strains TA1535 and TA100; ICR-191 for strain TA1537 in the absence of S-9, and 2-aminoanthracene in the presence of S-9 mix). The plates were incubated for approximately 48 hours at 37 ± 2°C.

Evaluation criteria: For a test article to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain with a minimum of two increasing concentrations of test article as specified below:

Strains TA1535, TA1537 and TA1538: data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than three times the mean vehicle control value.

Strains TA98 and TA100: data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than two times the mean vehicle control value.

#### Results:

The results of the range-finding test showed that daminozide was not toxic towards the tester strains when tested up to 10 000 µg/plate. Results of the initial and confirmatory mutation assays are presented in Table 6.4.1/03-1 and Table 6.4.1/03-2, respectively. No positive responses were observed with any of the tester strains in the presence and absence of microsomal activation. All criteria for a valid study were met.

**Table 6.4.1/03-1: Mean number ± SD of revertant colonies obtained in the initial mutation assay**

Dose level (µg/plate)	Mean revertant colony counts									
	Without metabolic activation					With metabolic activation				
	TA 98	TA 100	TA 1535	TA 1537	TA 1538	TA 98	TA 100	TA 1535	TA 1537	TA 1538
0	23±5	128±4	11±1	4±2	8±2	33±3	153±10	14±2	8±2	13±2
667	21±4	138±7	15±2	7±3	5±1	27±3	141±5	11±3	6±2	9±1
1000	26±4	160±12	13±5	5±1	6±1	27±8	146±18	14±3	6±1	8±2
3333	25±6	149±18	12±4	7±0	9±2	19±4	152±19	14±4	6±0	10±3
6667	30±12	141±7	8±0	9±3	5±1	32±4	139±18	16±3	6±5	11±2
10000	23±3	146±10	12±6	6±4	10±5	26±7	143±10	16±3	5±3	11±3
<b>Positive controls</b>										
2NF	212±50	-	-	-	321±14	-	-	-	-	-
NaN <sub>3</sub>	-	474±27	254±19	-	-	-	-	-	-	-
ICR-191	-	-	-	115±10	-	-	-	-	-	-
2AA	-	-	-	-	-	100±32	1901±115	45±9	32±4	255±19

2NF: 2-nitrofluorene

NaN<sub>3</sub>: sodium azide

2AA: 2-aminoanthracene

ICR-191: CAS 1707-45-0

**Table 6.4.1/03-2: Mean number of revertant colonies obtained in the confirmatory mutation assay**

	Mean revertant colony counts	
	Without metabolic activation	With metabolic activation



Dose level (µg/plate)	TA98	TA 100	TA 1535	TA 1537	TA 1538	TA98	TA 100	TA 1535	TA 1537	TA 1538
0	15±3	127±5	8±3	7±3	35±6	15±2	146±5	13±5	6±3	35±4
667	13±2	125±21	7±2	5±1	37±10	14±2	145±9	15±4	6±4	42±2
1000	12±2	131±4	8±4	7±1	35±11	17±1	112±17	14±1	7±1	32±10
3333	15±5	117±11	9±3	7±2	36±5	18±4	138±12	13±1	7±4	38±4
6667	9±3	118±10	10±4	4±2	32±3	20±2	131±4	9±1	8±2	34±8
10000	11±0	125±6	6±5	7±3	33±3	18±2	129±7	14±3	6±5	40±3
<b>Positive controls</b>										
2NF	161±36	-	-	-	313±23	-	-	-	-	-
NaN <sub>3</sub>	-	415±27	363±20	-	-	-	-	-	-	-
ICR-191	-	-	-	49±4	-	-	-	-	-	-
2AA	-	-	-	-	-	1389±168	1341±18	114±15	172±6	1305±63

2NF: 2-nitrofluorene

NaN<sub>3</sub>: sodium azide

2AA: 2-aminoanthracene

ICR-191: CAS 1707-45-0

#### Original DAR conclusion:

According to the authors a positive response was observed with tested strain TA 1537. However, this positive response was not dose-related and not observed in the confirmatory assay. No positive increase in revertants was observed in any of the other tester strains. Therefore the rapporteur agrees with the authors that the test substance did not induce a mutagenic effect in *Salmonella typhimurium*.

**RMS 2018:** The RMS agrees with the original conclusion of this study. Daminozide did not demonstrate mutagenic potential in any of the tested *Salmonella typhimurium* strains under conditions of this Ames test.

#### P7642: Assessment of its ability to induce primary DNA damage in *Escherichia coli*

Reference	<b>P7642: Assessment of its ability to induce primary DNA damage in <i>Escherichia coli</i>, Bootman J., Lodge D.C., May K. 1982a; Report No. Uniroyal Chemical. A.7.6.6</b>
Guideline	The study was conducted in compliance with GLP, but not according to any guideline
Deviations	-
GLP	Yes
Acceptability	No, supplementary
Previous evaluation	Yes, study already peer-reviewed in original DAR

#### Material and method:

Cultures of the WP2 strain of *Escherichia coli*, and its derivatives WP67 and CM871 were exposed to the tested compound P7642 at the concentration of 250, 1000, 2500, and 10 000 µg/mL in the presence or absence of metabolic activation (S-9 mix) for approximately 24 hours. Appropriate positive control plates were included for each test. Mitomycin C was examined in the absence of S-9 mix, while 2-aminoanthracene was tested both in the absence and presence of the activating system.

Values of percentage survival relative to the untreated cultures were used to calculate coefficients of survival ( $C_s$ ).  $C_s$  values of less than 0.1 should be considered positive, i.e. they provide evidence of selectively increased toxicity towards the repair-deficient strains.

#### Results:

P7642 caused no significant growth inhibition in any of the tester strains, either in the presence or absence of S-9 mix, at concentrations from 250 to 10 000 µg per mL of incubation medium. None of the  $C_s$  values obtained for P7672 were lower than 0.45. The majority of the values were similar to those obtained with deionised water controls.

**Note:** The present study is an indicator test of limited value for the assessment of possible genotoxic properties of daminozide. Moreover, no information was provided on the purity of the test substance. Therefore, the study is considered of limited value for the overall toxicological evaluation.

#### Original DAR conclusion:

According to the authors the test substance did not induce lethal DNA damage. However, the limitations given above make the study supplementary for evaluation

#### P7642: Investigation of mutagenic activity in the TK+/-mouse lymphoma cell mutation assay

Reference	<b>P7642: Investigation of mutagenic activity in the TK+/-mouse lymphoma cell mutation assay.</b> Bootman J., Rees R., Anderson, C. 1982b; Report No. Uniroyal Chemical. Report No. A.7.6.5
Guideline	The study was conducted in compliance with GLP and according to OECD guideline 476.
Deviations	Yes
GLP	Yes
Acceptability	Yes
Previous evaluation	Yes, study already peer-reviewed in original DAR

**RMS comment 2018:** The study has been checked for compliance. The following deviations from OECD guideline 476 (adopted on 29th July 2016) were found:

- 1) sufficient number of cells (but never less than 2 million) should be cultured during the expression period and plated for mutant selection, which was not met in the study

- 2) results should include historical negative as well as positive control data with ranges, means, standard deviations, and confidence interval

#### Material and method:

Cytotoxicity: L5178Y TK<sup>+</sup>/– mouse lymphoma cells were initially treated with ten concentrations of P7642 (1.95, 3.9, 7.81, 15.63, 31.25, 62.5, 125, 250, 500, and 1000 µg/mL). A solvent control (cells treated only with water) was also included. The cell concentration was measured after 24 hours. No significant toxicity of P7642 was seen in this experiment, so the preliminary test was recommenced using a further five P7642 concentrations of 500, 1000, 2000, 3000 and 4000 µg/mL. Each treatment was tested in the absence and presence of S-9 mix, a microsomal activating system derived from rat liver. The cell concentration was measured over two days.

Main mutation assay: L5178Y TK<sup>+</sup>/– cells were treated with P7642 at the concentration of 1500, 2000, 2333.3, 2666.7, and 3000 µg/mL in the absence or presence of S-9 mix for 4 hours. EMS and DMBA were used as positive controls. At 24 hour intervals after the initiation of treatment, the cell concentration of each culture was measured manually. Cell concentrations were adjusted, where necessary, to maintain a concentration of  $3 \times 10^5$  cells per mL, the volume of each culture remaining unchanged. This period between treatment and selection is the expression time. After the expression time of 3 days, aliquots of cells were removed from each culture to be cloned in the presence of the TFT selective agent. After one week, the colonies were manually counted using a stereomicroscope. Growth and mutation frequency data were calculated.

#### Results:

Cytotoxicity: In the presence and absence of S-9 mix there was a sharp reduction in cell growth between P7642 concentrations of 2000 and 3000 µg/mL, from over 100% growth at 2000 µg/mL to 12.5 or 13.0% growth at 3000 µg/mL. The inclusion of the activating system was therefore without effect. Dose-related decreases in pH expressed as a colour change in the medium were observed for all P7642 treated cultures.

Main mutation assay: The range of total growth of P7642 treatments was 79.4% (at 2000 µg/mL) to 16.7% (at 3000 µg/mL) in the absence of S-9 mix and 70.6% (at 1500 µg/mL) to 34.0% (3000 µg/mL) in its presence.

**Table 6.4.1/05-1 P7642: Investigation of mutagenic activity in the TK<sup>+</sup>/–mouse lymphoma cell mutation assay; Mutation frequency in L5178Y mouse lymphoma cells (mean values); EMS=Ethylmethanesulphonate, DMBA=7,12 dimethylbenzanthracene**

Treatment [ppm]	Mutation frequency per $10^5$ surviving cells	Induced mutation frequency	Total growth [%]
Metabolic activation	-S9/S9+	-S9/S9+	-S9/S9+
Control (DH <sub>2</sub> O)	5.8/5.2	0.0/0.0	100.0/100.0

<b>1500</b>	7.4/5.2	1.6/0.0	75.0/70.6
<b>2000</b>	7.2/5.9	1.4/0.7	75.4/40.1
<b>2333.3</b>	5.9/7.4	0.1/2.2	44.7/34.2
<b>2666.7</b>	8.5/6.5	2.7/1.3	31.1/47.4
<b>3000</b>	8.0/8.3	2.2/3.1	16.7/34.0
<b>EMS (300)</b>	44.1/-	38.3/-	16.4/-
<b>DMBA (5)</b>	7.4/51.3	1.6/46.1	33.3/13.4

The highest mutation frequency was 8.5 mutants per 10<sup>5</sup> survivors (at 2666.7 µg/ml) compared to 5.8 mutants per 10 survivors in the solvent control, in the absence of S-9 mix. The solvent control mutation frequencies of 5.8 and 5.2 mutants per 10<sup>5</sup> survivors was within the range of historical solvent control frequencies normally achieved at this laboratory.

**Original DAR conclusion:**

The test substance was not found to be mutagenic when tested *in vitro* for forward mutations in the TK locus of L5178Y mouse lymphoma cells, in the presence and in the absence of a liver homogenate from Aroclor-treated rats.

**RMS 2018:** Daminozide did not exert the mutagenic potential under conditions of this TK+/- mouse lymphoma cell mutation assay.

**Chromosome aberrations in Chinese Hamster Ovary (CHO) cells**

Reference	<b>Chromosome aberrations in Chinese Hamster Ovary (CHO) cells, Putman D.L., Morris M.J., 1991; Report No. A.7.6.19</b>
Guideline	The study was conducted according to OECD TG 473
Deviations	Yes
GLP	Yes
Acceptability	Yes
Previous evaluation	Yes, study already peer-reviewed in original DAR

**RMS comment 2018:** The study has been checked for compliance. The following deviations from OECD guideline 473 (adopted on 26th September 2014) were found:

- 1) The following experimental conditions should be conducted: (i) a short term treatment with the test

substance in the absence of S9 mix; (ii) a short term treatment with the test substance in the presence of S9 mix; (iii) a long term treatment with the test substance in the absence of S9 mix; The first one was not performed. In addition, during the short term treatment, the cells should be exposed to the test substance for 3-6 hours instead of 2 hours.

- 2) At least 300 well-spread metaphases should be scored per each concentration of the test substance and control, which was not met in the study
- 3) As Triethylenemelamine is not included in the list of reference substances recommended as a positive control not requiring metabolic activation, its choice should be justified

#### **Material and method:**

The clastogenic potential of daminozide (purity: 99.8%) was examined by an *in vitro* chromosomal aberration test using Chinese hamster ovary cells (CHO).

The toxicity test was performed for the purpose of selecting dose levels. CHO cells were seeded for each treatment condition at approximately  $5 \times 10^5$  cells/25 cm<sup>2</sup> flask and were incubated at  $37 \pm 1^\circ\text{C}$  for 16-24 hours. Treatment was carried out by refeeding the flasks with medium for the non-activated study or S-9 reaction mixture for the activated study, to which was added test article in solvent (0, 0.2, 0.6, 2, 6, 20, 60, 200, 600 and 2000 µg/mL) or solvent alone (DMSO). The cells were treated for six hours in the non-activated test system. Two hours after initiation of treatment, BrdU was added to each flask and incubation continued as required. At completion of the 6 hour exposure period, the cells were refed with medium containing BrdU and returned to incubator for a total of 24 hours from BrdU treatment. In the S-9 activated system, the cells were treated for two hours after which the cells were refed with medium containing BrdU and returned to the incubator for a total of 24 hours from BrdU treatment. Two hours prior to harvest by trypsinization, colcemid was added to each flask. Metaphase preparations were stained for sister chromatid differentiation using a modified fluorescence plus Giemsa technique. Slides were evaluated for the percentage of first, second and third-division metaphase cells for estimation of the test article effect on cell cycle kinetics. The mitotic index was determined for each treatment condition as the percentage of mitotic cells in a population of 500 cells scored.

For the chromosome aberration assay, CHO cells were seeded at approximately  $5 \times 10^5$  cells/25 cm<sup>2</sup> flask and incubated at  $37 \pm 1^\circ\text{C}$  for 16-24 hours. Treatment was carried out by refeeding duplicate flasks with medium for the non-activated study or S-9 reaction mixture for the activated study, to which was added test (0, 250, 500, 1000 and 2000 µg/mL) or control article (triethylenemelamine in the absence and cyclophosphamide in the presence of S-9 mix, respectively) in solvent or solvent alone. In the non-activated study, the cells were exposed for 8 hours at  $37 \pm 1^\circ\text{C}$ . Two hours prior to the scheduled cell harvest, the cells were refed with medium containing colcemid. In the S-9 activated study, the cells were exposed for 2 hours at  $37 \pm 1^\circ\text{C}$  and subsequently refed with medium and returned to the incubator for an additional 6 hours. At this time, Colcemid was added and the flasks incubated for an additional two hours. Two hours after the addition of Colcemid, metaphase cells were harvested for both the non-activated and S-9 activated studies by trypsinization. Cells were collected approximately 10 hours after initiation of treatment for both the non-activated and S-9 activated studies.

The prepared slides were stained with 5% Giemsa. Metaphase cells with  $20 \pm 2$  centromeres were examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 100 metaphase

spreads (50 per duplicate flask) were examined and scored for chromatid-type and chromosome-type aberrations. Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure were scored as a break (chromatid or chromosome). Fragments observed with an exchange figure were not scored as an aberration but instead were considered part of the incomplete exchanger. Pulverized chromosome(s), pulverized cells and severely damaged cells ( $\geq 10$  aberrations) were also recorded. Chromatid and isochromatid gaps were recorded but not included in the analysis. The XY coordinates for each cell with chromosomal aberrations were recorded using a calibrated microscope stage. The mitotic index was recorded.

#### Results:

Based upon the findings of the toxicity study, dose levels of 250, 500, 1000 and 2000  $\mu\text{g/mL}$  were selected for further study. In the absence of any observed delay in cell cycle kinetics, the harvest time was set at 10 hours for both the non-activated and S-9 activated studies in order to assure that cells were evaluated in first division metaphase after treatment.

The summary of the activity of daminozide in the induction of chromosome aberration in CHO cells when treated in the absence or presence of an exogenous source of metabolic activation is presented in Table 6.4.1/06-01.

The percentage of cells with structural aberrations in the test article-treated groups was not significantly increased above that of the solvent control ( $p > 0.05$ , Fisher's exact test) in both the presence and absence of metabolic activation. The percentage of damaged cells in the TEM group was 12% ( $p \leq 0.01$ , Fisher's exact test) and in the CP group was 13% ( $p \leq 0.01$ , Fisher's exact test), in the absence and presence of metabolic activation, respectively.

**Table 6.4.1/06-1: Summary of results of an *in vitro* chromosome aberration results**

Group	Dose ( $\mu\text{g/mL}$ )	Mitotic index (%)	Cells scored	Aberrations per cell (mean)	Cells with aberrations (%)
<b>Without metabolic activation</b>					
<b>Control</b>	Untreated	6.4	100	0.020 $\pm$ 0.141	2
	DMSO	6.5	100	0.040 $\pm$ 0.197	4
<b>Daminozide</b>	250	7.1	100	0.010 $\pm$ 0.100	1
	500	6.5	100	0.010 $\pm$ 0.100	1
	1000	6.9	100	0.000 $\pm$ 0.000	0
	2000	6.8	100	0.010 $\pm$ 0.100	1
<b>TEM</b>	0.5	2.4	100	0.250 $\pm$ 1.114	12**
<b>With metabolic activation</b>					

<b>Control</b>	Untreated	10.6	100	0.000±0.000	0
	DMSO	9.6	100	0.010±0.100	1
<b>Daminozide</b>	250	10.6	100	0.030±0.171	3
	500	10.4	100	0.000±0.000	0
	1000	10.2	100	0.030±0.171	3
	2000	11.2	100	0.000±0.000	0
<b>CP</b>	50	3.1	100	0.220± 1.040	13**

TEM: Triethylenemelamine, CP: Cyclophosphamide; \*\*:  $p \leq 0.01$  at Fisher's exact test

#### Original DAR conclusion:

Under the conditions of the assay, daminozide was found to have no potential to induce chromosomal aberrations in CHO cells.

**RMS 2018:** The RMS supports the original conclusion of this study. Under the conditions of the assay, daminozide did not induce chromosomal aberrations in CHO cells.

#### B 6.4.2 In vivo studies in somatic cells

##### In vivo micronucleus and chromosome aberration assay in mouse bone marrow cells

Reference	<b><i>In vivo</i> micronucleus and chromosome aberration assay in mouse bone marrow cells.</b> [REDACTED] 2003; Report No. AA72HH.123108.BTL
Guideline	The study was conducted according to OECD TG 474, 475
Deviations	Yes
GLP	Yes
Acceptability	Yes
Previous evaluation	No

**RMS comment:** The study has been checked for compliance. The following deviations from OECD guideline 474 (micronucleus test) and 475 (chromosomal aberrations) adopted on 29th July 2016 were found:

- 1) Intraperitoneal route of administration is not recommended.
- 2) At least 4000 immature erythrocytes per animal should be examined for evaluation of micronucleated cell incidence, which was not met in the study (2000 immature erythrocytes).

- 3) At least 200 metaphases per animal should be analysed for chromosomal aberrations (100 metaphases in the study).

**Material and method:**

Daminozide, identified as B-Nine® Technical (purity: 99.39%) in this study, was tested in the mouse micronucleus and chromosome aberrations assay. The assay was performed in two phases. The pilot toxicity phase was designed to assess toxicity of the test article and set dose levels for the definitive study. The second phase, the definitive study, was designed to evaluate the potential of the test article to increase the incidence of micronucleated polychromatic erythrocytes (MPCEs) and chromosome aberrations in bone marrow of male and female ICR mice.

In the pilot toxicity study, 5 female mice received a single intraperitoneal injection of daminozide in water at 1500 mg/kg bw; 5 male and 5 female mice were similarly treated at 1750 or 2000 mg/kg. Since differences in toxicity between the two sexes were observed, the high dose for the definitive study was set at 2000 mg/kg for male mice and at 1500 mg/kg for female mice. Mice were observed after dose administration and daily thereafter for 3 days for clinical signs of toxicity. Bodyweights were recorded before dose administration and 1 and 3 days after dose administration.

The definitive study consisted of seven groups, each containing 5 male and 5 female ICR mice. Animals in five of these groups were treated either with the controls (negative: water; positive: cyclophosphamide monohydrate at 50 mg/kg) or with daminozide at 500, 1000 and 2000 mg/kg (male mice) or 375, 750 and 1500 mg/kg (female mice) and euthanized 22-24 hours after treatment. Animals in the other two groups were treated either with the negative control or daminozide at the relevant high dose for each sex and euthanized 46-48 hours after treatment. Colchicine, used to arrest dividing cells at metaphase, was administered intraperitoneally at a dose of 2 mg/kg to all mice two to four hours prior to scheduled sacrifice time.

Bone marrow cells, collected 22-24 or 46-48 hours after treatment, were arrested in metaphase and examined microscopically for structural and numerical chromosome aberrations. A minimum of 100 metaphase spreads containing 40 (2n) centromeres were examined from each animal and scored for chromatid-type and chromosome-type aberrations. Fewer cells were scored when high numbers of aberration, i.e. >10%, were observed. Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure were scored as a break (chromatid or chromosome). Fragments observed with an exchange figure were not scored as an aberration but were considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells (>10 aberrations) were also recorded. Chromatid and isochromatid gaps were recorded but not included in the analysis. The XY coordinates for each cell with structural aberrations were recorded using a calibrated microscope stage. The mitotic index was recorded as the percentage of cells in mitosis based upon 1000 cells counted per animal. Additionally, the number of numerically damaged cells (polyploid and endoreduplicated cells) was recorded per 100 metaphase cells per animal.

For the micronucleus assay, bone marrow cells, polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs) were analysed for the presence of micronuclei. Using oil immersion (10 × 100), 2000 PCEs



per animal were scored for the presence of micronuclei. The number of MNCEs in the field of 2000 PCEs was also enumerated for each animal. The proportion of polychromatic erythrocytes to total erythrocytes (PCEs/ECs) was recorded per total of 1000 erythrocytes.

### Results:

In the pilot study: mortality was observed in 1/5 female mice at 1750 mg/kg and in 3/5 female mice at 2000 mg/kg. Clinical signs following dose administration included: lethargy and piloerection in females at doses  $\geq 1500$  mg/kg and in males at doses 1750 mg/kg. In addition, ataxia was seen in females at 1750 mg/kg and crusty eyes in males and females at 1750 mg/kg. Since differences in toxicity between the two sexes were observed, the high dose for the definitive study was set at 2000 mg/kg for male mice and at 1500 mg/kg for female mice.

In the definitive study: no mortality was observed. Clinical signs following dose administration included: piloerection in males at doses 1000 mg/kg and in females at 1500 mg/kg and lethargy in females at 1500 mg/kg and in males at 2000 mg/kg. In addition, prostration and hunched position were seen in males at 2000 mg/kg.

The percentage of damaged cells in the total population of cells scored and the number of aberrations per cell for 22-24 and 46-48 hour treatment groups are presented for male and female mice in

Table 6.4.2/01-1 and Table 6.4.2/01-2, respectively.

The mitotic index was reduced in the male treated groups (up to 16%) at 22-24 hour sampling time while no appreciable reductions were observed in female treated groups at the same time as well as in both sexes at the 46-48 sampling time. In addition, no statistically significant increase in the number of aberrant cells was observed in the treated groups relative to the negative controls regardless of sex, dose level or bone marrow sampling time ( $p > 0.05$  Fisher's exact test).

The incidence of MPCEs/10000 PCEs scored (2000 PCEs/animal) and the proportion of PCEs/ECs are summarized and presented for each treatment group by sacrifice time in Table 6.4.2/01-3. Dose-dependent reduction in the PCEs/ECs ratio was observed in the male (11% to 41%) and female (12% to 21%) 22-24 hour treated groups relative to the negative controls. Additionally, reductions of 65% and 51% were observed in the male and female 46-48 hour treated groups, respectively. These reductions demonstrate that there was bioavailability of the test article to the bone marrow target tissue. The number of MPCEs/10000 PCEs in test article treated groups was not statistically increased relative to the respective negative controls in either male or female mice, regardless of dose level or bone marrow collection time ( $p > 0.05$ , Kastenbaum-Bowman Tables).

**Table 6.4.2/01-1: Summary of *in vivo* chromosome aberration results (22-24 h post-dose)**

Group	Dose (mg/kg)	Sex	Cells scored	Mean mitotic	Cells with aberrations	Struct. Aberr.	Number of aberrations	SDC	Aberrations per cell
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					Num.	Struct.		Gap	Break	Exch.		
<b>Control (water)</b>	-	M	500	11.3	0	2	0.4	0	2	0	0	0.004±0.005
		F	500	10.1	0	1	0.2	0	1	0	0	0.002±0.004
<b>Daminozide</b>	500	M	500	10.2	0	0	0.0	0	0	0	0	0.000±0.000
	375	F	500	10.0	0	0	0.0	0	0	0	0	0.000±0.000
	1000	M	500	10.4	0	0	0.0	0	0	0	0	0.000±0.000
	750	F	500	10.3	0	2	0.4	0	2	0	0	0.004±0.005
	2000	M	500	9.5	0	2	0.4	0	2	0	0	0.004±0.005
	1500	F	500	10.6	0	0	0.0	0	0	0	0	0.000±0.000
<b>CP</b>	50	M	500	6.1	0	61*	12	0	24	0	530	1.108±0.188
	50	F	500	6.4	0	61*	12	0	42	1	470	1.026±0.228

SDC: Cells having at least 10 aberrations of any type, including pulverized chromosomes or cells.

\*: number of cells in mitosis per 1000 cells observed, expressed as a percentage (MI)

**Table 6.4.2/01-2: Summary of *in vivo* chromosome aberration results (46-48 h post-dose)**

Group	Dose (mg/kg)	Sex	Cells scored	Mean mitotic index (%) <sup>a</sup>	Cells with aberrations		Struct. Aberr. (%)	Number of aberrations			SDC	Aberrations per cell (mean)
					Num.	Struct.		Gap	Break	Exch.		
<b>Control (water)</b>	-	M	500	10.9	0	0	0.0	0	0	0	0	0.000±0.000
		F	500	10.3	0	0	0.0	0	0	0	0	0.000±0.000
<b>Daminozide</b>	2000	M	500	10.0	0	3	0.6	0	3	0	0	0.006±0.009
	1500	F	500	11.1	0	0	0.0	0	0	0	0	0.0000±0.000

SDC: Cells having at least 10 aberrations of any type, including pulverized chromosomes or cells.

\*: number of cells in mitosis per 1000 cells observed, expressed as a percentage (MI)

CP: cyclophosphamide

**Table 6.4.2/01-3: Results of an *in vivo* Micronucleus Test**

Treatment	Dose (mg/kg)	Sex	Time (h)	PCE/total erythrocytes (mean ± SD)	Micronucleated PCE <sup>a</sup> (mean ± SD)
<b>Control (water)</b>	0	M	22-24	0.509 ± 0.05	0.5 ± 0.00
	0	F		0.423 ± 0.06	0.5 ± 0.00
<b>Daminozide</b>	500	M		0.451 ± 0.03	0.3 ± 0.27
	375	F		0.446 ± 0.01	0.4 ± 0.22

	1000	M		$0.434 \pm 0.03$	$0.6 \pm 0.22$
	750	F		$0.373 \pm 0.04$	$0.8 \pm 0.27$
	2000	M		$0.302 \pm 0.04$	$1.2 \pm 0.57$
	1500	F		$0.335 \pm 0.03$	$0.9 \pm 0.42$
CP	50	M		$0.318 \pm 0.02$	$22.2 \pm 3.82^*$
	50	F		$0.339 \pm 0.03$	$23.0 \pm 4.77^*$
Control (water)	0	M	46-48	$0.510 \pm 0.02$	$0.5 \pm 0.00$
	0	F		$0.478 \pm 0.01$	$0.4 \pm 0.22$
Daminozide	2000	M		$0.176 \pm 0.03$	$1.2 \pm 0.57$
	1500	F		$0.233 \pm 0.10$	$0.5 \pm 0.35$

\*Statistically significant,  $p < 0.05$  (Kastenbaum-Bowman Tables)

<sup>a</sup>: micronucleated PCE per 1000 PCEs

PCE: polychromatic erythrocytes

CP: cyclophosphamide

#### Conclusion:

Under the conditions of the study, daminozide was concluded to be negative in the micronucleus and chromosome aberrations assay using male and female ICR mice at doses up to 2000 mg/kg (male mice) and 1500 mg/kg (female mice).

**RMS 2018:** The micronucleus and chromosome aberration assays are negative under conditions of this study.

#### Investigation of the potential for covalent binding of daminozide (ALAR) to rat liver DNA

Investigation of the potential for covalent binding of daminozide (ALAR) to rat liver DNA	
Reference	liver DNA, [REDACTED] 1986; Report No. Uniroyal Chemical. A.7.6.14
Guideline	The study was conducted neither in compliance with GLP nor according to any guideline
Deviations	-
GLP	No
Acceptability	Yes
Previous evaluation	Yes, study already peer-reviewed in original DAR

#### Material and method:

Two male Sprague-Dawley rats received labelled daminozide (37 mg/kg; 4.7 mCi/kg) in about 1 mL 0.01 N HCl by oral gavage. The precise dose given to each individual animal was determined on the basis of the weight

difference of the syringe before and after oral gavage. The treated animals were placed in glass metabolism cages where an airstream of 0.4 l/min transported the exhaled air to a trap with ethanolamine/methanol to collect expired CO<sub>2</sub>.

24 hours after the administration, the animals were bled, the livers were excised, and chromatin was prepared. Subsequently, the DNA was isolated and purified; and nucleotides were analysed. The chromatin protein was isolated and its amount determined. DNA was also isolated from an untreated animal held together with the treated ones. The respective total count (upon comparison with historical controls) was used to show that the work-up of the DNA samples was performed without external contamination with radiolabels.

### Results:

**DNA Radioactivity:** Radioactivity was clearly detectable in all DNA samples isolated from the daminozide-treated animals. Between the first and the second purification of the DNA the specific radioactivity of the DNA from the daminozide treated rats remained constant so that it was concluded that non-covalently bound contaminants had been completely removed. Under the preliminary assumption that all radioactivity measured on the DNA was due to covalently bound metabolites of the test compounds, the specific activities were converted to the units of the Covalent Binding Index, CBI = ( $\mu\text{mol chemical bound per mol nucleotide/mmole chemical applied per kg bodyweight}$ ). An apparent CBI value of about 9 resulted for daminozide. Radioactivity on the DNA isolated from an animal that has been treated with a radiolabelled substance is not necessarily due to covalent interactions of the test compound with DNA, but could be derived from three additional sources: (i) Non-covalent interaction of the test compound with DNA. This is unlikely because the specific activity remained constant upon repetitive purification; (ii) Contamination of DNA with protein. The interaction of daminozide or one of its radiolabelled metabolites with chromatin protein resulted in specific activities which were only about 4 times higher in protein than of DNA so that the protein contamination of the DNA cannot have contributed substantially to the DNA radioactivity; (iii) Biosynthetic incorporation of radioactivity into DNA and protein. This is a more likely reason for a part of the observed DNA radioactivity if the compound or impurities administered are degraded to small molecules able to enter the pool of nucleic acid (or protein) precursors. Such is obviously the case with compounds that are degraded to yield <sup>14</sup>CO<sub>2</sub>. On average, 1.3% of the radioactivity dose administered was exhaled within 24 hours in the form of <sup>14</sup>CO<sub>2</sub> after the administration of [<sup>14</sup>C]daminozide. Analysis of the nucleotides for radioactivity will now show to what extent DNA synthesis contributed to the total DNA radioactivity.

**HPLC analysis of DNA constituents:** The recovery of DNA was 93% containing 99% of the total radioactivity. Six percent of the radioactivity eluted after the natural nucleotides without optical density in the region where the methylated nucleotides are known to elute. For an identification of the alkylation products, DNA was depurinated and the resulting purines were separated by HPLC. The recovery of radioactivity was 100% where of 42% co-chromatographed with guanine and adenine and 52% eluted together with the apurinic acid. 6% of the radioactivity co-chromatographed with added 7-methylguanine. These results indicate that the radioactivity on the DNA after oral administration of the [<sup>14</sup>C] labelled daminozide was mostly due to biosynthetic incorporation of metabolic breakdown products of [<sup>14</sup>C] labelled compounds into DNA. Only about 6% of the radioactivity was due to methylation of DNA.

**Note:** For the present study, no guideline was available. Because the purity of the test substance was not provided, the conclusion regards only the tested substance, and not daminozide in general.

#### Conclusion:

Covalent binding to DNA of target cells was studied *in vivo* by analysing the DNA isolated from liver of 2 male rats which had been administered radiolabelled daminozide. About 6% of the DNA radioactivity co-chromatographed with 7-methylguanine. The results indicated that the radioactivity associated with the DNA, as determined 24 hours after oral administration of radiolabelled daminozide, was mostly due to biosynthetic incorporation of radiolabelled nucleotide precursors into DNA and that methylation of liver DNA by daminozide contributed little to the overall DNA radioactivity. The extent of this DNA damage, expressed in units of CBI, was in the order of 0.5 (6% of the apparent value 9) for daminozide. According to the authors, nitrosodimethylamine exhibits a CBI of 6000 under similar conditions and the authors consider daminozide as negative in the present study.

**RMS 2018:** RMS supports the conclusion of study authors. Daminozide did not exert the potential to damage DNA via covalent binding to a relevant extent.

#### B 6.4.3 In vivo studies in germ cells

##### Dominant lethal assay of Alar in the male mouse

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Reference	<b>Dominant lethal assay of Alar in the male mouse,</b> [REDACTED] 1973; Report No. A.7.6.1 Uniroyal Chemical.
Guideline	The study was conducted prior to OECD guideline 478. The study was not performed in compliance with GLP.
Deviations	-
GLP	No
Acceptability	Yes
Previous evaluation	No

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#### Material and method:

Four groups of 20 male mice were treated with Alar (purity: unknown) at 0, 10, 300, and 1000 mg/kg food (equivalent to 0, 1.5, 45, and 150 mg/kg bw/day) for 5 consecutive days. Mating period lasted 1 week (4 matings; 1f/1m). Male animals were observed for signs of toxicity, bodyweight and food consumption. Females were examined to determine total implantations, viable embryos, and early/late deaths.

In a dose-range finding study, male mice (n = 10) were exposed to dietary levels of 10 000, 25 000, 50 000 and 75 000 mg/kg food for 10 consecutive days.

#### Results:

Treatment at all dose groups, except 10 000 mg/kg food, was associated with diarrhoea and retarded weight gain; both responses were dose related. No treatment-related effects on mating performance, pregnancy rate, embryonic deaths and implantation loss were observed.

#### Conclusion:

The test substance does not induce dominant lethal effects in germ cells of male mice, under the conditions of the present study

**Note:** Since no information was provided on the purity of the test substance, the conclusion regards only the tested substance, and not daminozide in general

#### B 6.4.4 Summary of genotoxic studies

Daminozide did not induce gene mutations in Ames tests with bacteria (*Escherichia coli*, *Salmonella typhimurium*) either in the presence or absence of metabolic activation. The test substance was also negative in a chromosome aberration study with CHO cells and a TK+/- mouse lymphoma cell mutation assay. A combined *in vivo* micronucleus study and a chromosome aberration study in mouse bone marrow cells conducted with the technical material were submitted in 2003. According to their results the test substance did not induce an increase in micronuclei or chromosome aberrations in male or female mice.

Based on the negative results of *in vitro* and new *in vivo* studies, daminozide is considered to have no genotoxic properties.

The genotoxicity studies which are considered acceptable or supplementary for the overall toxicological evaluation of daminozide are summarised in Table 6.4-01 and 6.4-02.

**Table 6.4-01: *In vitro* genotoxicity studies**

Type of study		Result		Reference / Notifier	Batch information
Indicator cells	Endpoint	Without activation	With activation		
<i>E. coli</i> (WP2uvrA)	point mutations	-	-	Williams (2006), Fine	Batch number 2003-10-01, 100.2% daminozide
<i>S. Cerevisiae</i>	mitotic aneuploidy	- <sup>1</sup>	- <sup>1</sup>	Bootman and Lodge (1983), Uniroyal	batch P7642, UDMH/NDMA content undetermined , 99.0% daminozide
<i>S. typhimurium</i>	point mutations	-	-	Sans and Shelton (1991), Uniroyal	batch number 806M006, with a daminozide content of 99.78- 99.96%, UDMH content of 25 mg/kg and a NDMA content of 0 ppm (1 ppm as LOD)
<i>E. coli</i>	differential killing	- <sup>2</sup>	- <sup>2</sup>	Bootman et al. (1982a), Uniroyal	batch P7642, UDMH/NDMA content undetermined , 99.0% daminozide
mouse lymphoma cells	thymidine kinase activity	-	-	Bootman et al. (1982b),	batch P7642, UDMH/NDMA content undetermined , 99.0%

Type of study		Result		Reference / Notifier	Batch information
Indicator cells	Endpoint	Without activation	With activation		
				Uniroyal	daminozide
Chinese Hamster Ovary cells	chromosomal aberrations	-	-	Putman and Morris (1991), Uniroyal	batch number 806M006, with a daminozide content of 99.78-99.96%, UDMH content of 25 mg/kg and a NDMA content of 0 ppm (1 ppm as LOD)
<i>S. typhimurium</i>	point mutations	<sup>-1</sup>	<sup>-1</sup>	Richold et al., (1984), Fine	batch no. 83/0668. According to the certificate of analysis, this batch contains 99% w/w daminozide, 7 ppm UDMH and <20ppb NDMA (LOD at that time).

<sup>1</sup> The study is considered supplementary due to some shortcomings in study design.

<sup>2</sup> The study is considered supplementary because the indicator test is of limited value for assessment of possible genotoxic effects of daminozide.

**Table 6.4-02: *In vivo* genotoxicity studies**

Type of study		Result	Reference / Notifier	Batch information
Species	Endpoint			
Mouse (ICR)	Micronuclei (bone marrow)	-	██████████ (2003), ██████████	B-Nine <sup>®</sup> Technical (daminozide 99.39% pure)
Mouse (ICR)	Chromosome aberration in bone marrow cells	-	██████████ (2003), ██████████	B-Nine <sup>®</sup> Technical (daminozide 99.39% pure)
rats	DNA methylation in liver	<sup>-1</sup>	██████████ (1986), Uniroyal	
mice, males	dominant lethality	<sup>-1</sup>	██████████ (1973), Uniroyal	

<sup>1</sup> Since no information is provided on the purity of the test substance, only conclusions can be drawn for the tested substance and not for daminozide in general.

## **B 6.5 Long-term toxicity and carcinogenicity**

### **B 6.5.1 Chronic toxicity and carcinogenicity**

#### **Two year dietary toxicity and oncogenicity study in rats**

Reference	Two year dietary toxicity and oncogenicity study in rats, ██████████ 1988b; Report No. A.7.3.9
Guideline	The study was conducted according to OPPTS Guideline 83-2, OECD TG 453

Deviations	Yes
GLP	Yes
Acceptability	Yes
Previous evaluation	Yes, study already peer-reviewed in original DAR

**RMS comment 2018:** The study has been checked for compliance. The following deviations from OECD guideline 453 (adopted on 7th September 2009) were found:

- 1) As for haematological parameters, prothrombin time and activated partial thromboplastin time were not investigated
- 2) Epididymides, uterus, and thyroid should be weighed at necropsy after the chronic toxicity phase

#### Material and method:

Groups of 60 male and 60 female [REDACTED] (source: [REDACTED]) were offered Alar® Technical (daminozide; purity: 99%) in the diet at concentrations of 0 (control), 100, 500, 5000 or 10000 ppm.

All animals were observed twice daily to ensure they were in good health. In addition, each animal was given a detailed physical examination at weekly intervals (appearance and condition, behaviour and activity, excretory function, respiration, orifices, eyes and palpable masses). Investigations also included ophthalmoscopy (prior to study, after 12 and 24 months), haematology (leukocyte, erythrocyte count, haemoglobin, haematocrit, MCV, MCH, MCHC, platelets, reticulocytes, differential leucocyte count), biochemistry (Na, P, K, Cl, Ca, AST, ALT, CPK, total bilirubin, urea nitrogen, creatinine, albumin, globulin, total protein, cholesterol, glucose), and urinalysis (after 6, 12, 18 and 24 months). After 12 months, satellite groups of 10 males and 10 females were used for interim sacrifice and the remaining survivors sacrificed after 24 months of treatment. Individual bodyweights and food consumption were recorded pre-initiation, once weekly for weeks 1-16 and every 4 weeks thereafter. Terminal investigations included gross necropsy, organ weights, and histopathology. All animals were examined for evidence of neoplasia.

At necropsy, adrenals, brain, heart, kidneys, liver, ovaries, testes, epididymides were weighed. Histopathological examination was performed on the following organs/tissues: adrenals, aorta, brain, caecum, colon, duodenum, stomach, epididymides, eyes, femur, heart, ileum, jejunum, kidneys, liver, lungs, mediastinal lymph node, mammary gland, mesenteric lymph node, oesophagus, optic nerve, ovaries, pancreas, pituitary, prostate, rectum, salivary gland, sciatic nerve, seminal vesicles, skeletal muscle, skin, spinal cord [3 levels], spleen, sternum, stomach, testes, thymus, thyroid with parathyroid, trachea, urinary bladder, uterus.

#### Results:

During the study, 41, 40, 31, 35 and 28 rats died/were sacrificed *in extremis* in the control, 100, 500, 5000 and 10000 ppm dosage groups, respectively. Mortality was slightly higher for males than females, but overall extreme sacrifices and deaths were not treatment-related. There were no treatment-related differences in the mean weekly



bodyweights observed during the study. Although statistically significant differences were present, they were very slight and/or did not follow a dose-related pattern. Therefore, the differences were not considered to be biologically meaningful. The survival of animals per dose group is given in table 6.5.1/01-1.

**Table 6.5.1/01-1 Survival data: number of survived rats (% survival)**

Time point (weeks)	Dose [mg/kg bw/day]									
	0		100		500		5000		10000	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
total number	60	60	60	60	60	60	60	60	60	60
week 52	59	60	59	59	60	60	60	60	59	60
rats after 1st kill	49	50	49	49	50	50	50	50	49	50
week 80	48	46	47	45	50	48	47	48	48	47
week 92	40	43	39	42	44	44	39	45	45	42
week 104	26/50 (52 %)	36/50 (72 %)	26/50 (52 %)	37/50 (74 %)	33/50 (66 %)	39/50 (78 %)	27/50 (54 %)	40/50 (80 %)	38/50 (76%)	38/50 (76%)

No test article related haematological and biochemical changes, in any group, at any of the intervals evaluated, were observed. A small number of statistically significant differences were present in the absolute organ weights and the ratio of organ weight to brain or bodyweight. In the males, the ratio of the kidney to brain weight was significantly decreased in the 100 ppm group ( $p < 0.05$ ). The absolute weight of the kidneys of the 5000 ppm females was significantly decreased ( $p < 0.05$ ) and the kidney/brain weight ratio of the 5000 ppm females was decreased ( $p < 0.05$ ). These changes could not be correlated with microscopic findings and were not believed to be related to the administration of the test article.

**Table 6.5.1/01-2 Absolute and relative organ weights**

organ	Dose [mg/kg bw/day]										
	weeks	0		100		500		5,000		10,000	
		ppm		ppm		ppm		ppm		ppm	
		♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Testis weight absolute (g)	52	3.35		3.51*		3.44		3.42		3.35	
	104	5.90		5.76		4.80		6.53		5.85	

<b>Kidney weight absolute (g)</b>	52	3.27	1.92	3.15	1.90	3.21	1.86	3.19	1.98	3.16	1.90
	104	3.77	2.34	3.55	2.36	3.66	2.31	3.69	2.22*	3.81	2.26
<b>Kidney/Brain Weight, %*10-2</b>	52	1.73	1.12	1.64	1.09	1.67	1.08	1.67	1.16	1.69	1.12
	104	1.96	1.33	1.81*	1.33	1.96	1.33	1.89	1.25*	1.95	1.29
<b>Heart/Bodyweight, %*10</b>	52	2.80	3.44	2.75	3.33	2.81	3.37	2.78	3.41	2.75	3.54
	104	3.37	3.45	3.42	3.48	3.39	3.47	3.15	3.59	3.35	3.72*

\* ( $p \leq 0.05$ ) significantly different from controls

Neoplastic lesions were few and they occurred sporadically in control and treated groups. The only neoplasm seen in the high dose group was an adamantinoma in a male. Although this tumour is rare, it is occasionally seen in untreated rats. Oral tumours of this type often manifest macroscopically as swelling or deformities, even in early stages, and can be easily detected during ante-mortem or post-mortem examination. Since no significant macroscopic oral pathology was seen in treated groups, the occurrence of this tumour in one high dose animal was considered an incidental finding.

Bile duct hyperplasia in the liver appeared to occur more often in the treated females than in the control females, but was not more prominent in the control males compared to the treated males. This finding is illustrated in Table 6.5/01-3. No other indications of hepatic toxicity were evident in either sex.

Ovarian atrophy and ovarian cysts also appeared to be slightly more common in treated compared to control females. This increased incidence may have been test article related. Incidence of ovarian cysts is illustrated in Table 6.5/01-3.

Chronic progressive nephropathy is a common lesion in old rats and is more prominent in the males than the females. In this study, the microscopic lesion of chronic progressive nephropathy often correlated with the macroscopic observation of granular surface of the kidneys. A brown pigment was also a common finding in the renal tubules of both male and female rats in all dose groups. Haemolytic anaemia has been reported to be associated with mononuclear cell leukaemia.

Pituitary adenomas were prominent and correlated to macroscopic enlargement or the macroscopic presence of coloured foci. The macroscopically enlarged spleens and the granular livers often correlated microscopically with the diagnosis of mononuclear cell leukaemia. Mononuclear cell leukaemia is common in the Fischer rat and has also been referred to as Fischer rat leukaemia and large granular lymphocyte lymphoma/leukaemia.

The macroscopically-observed testicular foci correlated with the diagnosis of interstitial cell tumour. Testicular interstitial cell tumours occurred in a very high percentage of males in all dose groups. The interstitial cell tumours

were designated as either benign or malignant based upon their morphological appearance. Some of the interstitial cell tumours were quite large; however, none of them metastasized distant from the testes. Testicular atrophy was also a common finding in the male rats and appeared to be a sequela to pressure of the interstitial tumours.

All of the above lesions occurred across dose levels and appeared to be spontaneous in origin and consistent with what would normally be expected in a two year chronic study in Fischer rats.

**Table 6.5/01-3 104 week oral toxicity study in rats; Incidence of microscopic observations – 12 months to termination.**

		Dose [mg/kg bw/day]									
		0 ♂	ppm ♀	100 ♂	ppm ♀	500 ♂	ppm ♀	5000 ♂	ppm ♀	10 000 ♂	ppm ♀
<b>Adrenal, Cortex</b>											
Haemolymphoreticular neoplasm	104 weeks	8/49	2/50	7/26	1/12	1/19	2/14	6/28	4/11	3/49	5/50
<b>Adrenal, Medulla</b>											
Haemolymphoreticular neoplasm	104 weeks	2/49	2/50	5/26	3/12	2/19	2/14	5/28	3/11	4/49	6/50
hyperplasia	104 weeks	7/49	1/50	7/26	0/12	3/19	3/14	7/28	3/11	13/49	3/50
Pheochromocytoma, benign	104 weeks	8/49	1/50	1/26	0/12	2/19	0/14	3/28	0/11	8/49	3/50
Pheochromocytoma, malignant	104 weeks	1/49	2/50	2/26	1/12	3/19	0/14	5/28	1/11	4/49	2/50
<b>Bone marrow, Femur</b>											
Haemolymphoreticular neoplasm	104 weeks	20/49	4/50	7/26	2/12	4/19	3/15	11/24	5/11	11/49	8/50
<b>Brain</b>											
Haemolymphoreticular neoplasm	104 weeks	3/49	1/50	2/26	0/14	1/19	0/13	1/24	0/11	1/49	1/50
<b>Cecum</b>											
Haemolymphoreticular neoplasm	104 weeks	1/49		0/26		0/19		0/24		0/49	
Metastatic tumor	104 weeks	0/49		0/26		0/19		0/24		1/49	
<b>Colon</b>											
Cyst, severe	104 weeks		0/50		0/12		0/13		0/11		1/50

Metastatic tumour	104 weeks	0/49		0/26		0/19		0/24		1/49	
<b>Duodenum</b>											
Haemolymph o-reticular neoplasm	104 weeks	1/49	0/50	0/26	0/12	0/19	0/13	0/24	1/11	0/49	0/50
Metastatic tumour	104 weeks	0/49		0/26		0/19		0/24		1/49	
<b>Epididymis</b>											
mesothelioma, benign	52 weeks	0/11		0/1		1/1		0/0		0/11	
	104 weeks										
relative aspermia, mild	52 weeks	0/11		0/1		0/1		0/0		1/11	
Haemolymph oreticular neoplasm	104 weeks	1/49		0/26		0/19		1/24		0/49	
Metastatic tumour	104 weeks	0/49		2/28		1/18		0/24		1/49	
<b>Eye</b>											
Cataract	52 weeks	0/11	0/10	0/9	0/9	0/5	1/9	0/8	0/6	1/11	0/10
	104 weeks	0/49	2/50	4/45	1/41	8/40	7/42	2/42	3/40	0/49	0/50
Keratitis	52 weeks	4/11	4/10	4/9	2/8	0/5	2/9	3/8	2/6	3/11	2/10
	104 weeks	6/49	5/50	4/45	4/41	8/40	8/42	3/42	5/40	7/49	4/50
Retinopathy, mild	52 weeks	1/1	0/10	0/9	1/8	0/5	1/9	0/8	0/6	0/11	0/10
<b>Heart</b>											
Haemolymph oreticular neoplasm	52 weeks	1/11		0/1		0/0		0/0		0/11	
	104 weeks	3/49	1/50	0/26	1/14	1/18	3/13	5/24	2/11	1/49	4/50
<b>Haemolymphoreticular system</b>											
Mononuclear cell leukemia	52 weeks	1/11		0/1		0/0		0/0		0/11	
	104 weeks	18/23	10/12	18/20	7/9	10/14	11/12	15/18	10/13	16/18	11/11
Granulocytic leukemia	104 weeks	0/23		0/20		0/14		0/18		1/18	
Malignant lymphoma	104 weeks	2/18	1/12	2/20	1/9	2/14	1/12	3/18	3/13	1/18	0/11
Hystocytic sarcoma	104 weeks	3/18	1/12	0/20	1/9	2/14	0/12	0/18	0/13	0/18	0/11
<b>Ileum</b>											

Haemolymphoreticular neoplasm	104 weeks	2/49		0/26		0/18		0/24		0/49	
Metastatic tumour	104 weeks	0/49		0/26		0/18		0/24		1/49	
<b>Jejunum</b>											
adenocarcinoma	104 weeks	0/49		0/26		2/19		0/24		0/49	
<b>Kidney</b>											
Haemolymphoreticular neoplasm	104 weeks	5/49	2/50	3/49	1/49	2/50	3/50	5/50	3/50	1/49	2/50
Metastatic tumour	104 weeks	0/49	0/50	0/49	1/49	0/50	0/50	0/50	0/50	1/49	0/50
Renal cell adenoma	104 weeks	0/49		0/49		0/50		0/50		1/49	
Transitional cell carcinoma	104 weeks	0/49		1/49		0/50		0/50		0/49	
<b>Liver</b>											
Bile duct hyperplasia, mild	52 weeks	4/11	1/10	0/1	0/1	0/0	0/0	0/0	0/1	4/11	2/10
	104 weeks	36/49	5/50	33/49	16/49	38/50	13/50	39/50	16/50	41/49	14/50
Haemolymphoreticular neoplasm	52 weeks	1/11		0/1		0/0		0/0		0/11	
	104 weeks	22/49	12/50	20/49	9/49	13/50	12/50	17/50	13/50	17/49	11/50
Hepatocellular adenoma	104 weeks	4/49	1/50	2/49	0/49	0/50	1/50	2/50	0/50	1/49	1/50
Hepatocellular carcinoma	104 weeks	0/49		1/49		0/50		0/50		0/50	
<b>Lung</b>											
Haemolymphoreticular neoplasm	52 weeks	1/11		0/1		0/0		0/0		0/11	
	104 weeks	12/49	3/50	8/49	3/49	6/50	4/50	10/50	5/50	4/49	8/50
Alveolar bronchiolar adenoma	104 weeks	1/49	0/50	0/49	0/49	0/50	0/50	0/50	1/50	0/49	0/50
Metastatic tumour	104 weeks	2/49	1/50	0/49	1/49	1/50	1/50	0/50	1/50	0/49	0/50
<b>Lymph Node: Mediastinal</b>											
Haemorrhage	52 weeks	5/11	8/10	1/1	0/1	0/0	0/0	0/0	0/0	4/11	7/10
Haemolymphoreticular neoplasm	104 weeks	9/4	3/50	4/26	3/12	3/19	2/12	5/24	4/11	4/49	6/49

<b>Lymph Node: Mesenteric</b>											
Haemolymphoreticular neoplasm	104 weeks	6/49	3/50	7/27	4/12	3/18	2/13	7/23	6/12	5/49	9/50
<b>Lymph Node: Submandibular</b>											
Haemolymphoreticular neoplasm	104 weeks	10/49	2/49	5/26	3/12	1/18	3/13	5/24	5/12	2/49	7/49
<b>Mammary gland</b>											
Fibroadenoma	52 weeks	0/11	0/10	0/1	1/2	1/1	0/0	0/0	0/0	0/11	0/10
	104 weeks	1/2	3/50	0/2	2/14	1/3	0/15	0/1	2/16	3/4	6/50
adenocarcinoma	104 weeks	0/2	1/50	0/2	0/14	0/3	2/15	0/1	3/16	1/4	0/50
<b>Pancreas</b>											
Haemolymphoreticular neoplasm	104 weeks	2/49	2/50	1/26	1/13	0/18	2/13	1/23	3/11	1/48	2/50
Islet cell adenoma	104 weeks	2/49	0/50	1/26	1/13	1/18	0/13	1/23	0/11	6/48	1/50
Islet cell carcinoma	104 weeks	1/49		0/26		0/18		0/23		1/48	
Metastatic tumour	104 weeks	0/49	1/50	0/26	1/13	1/18	0/13	0/23	0/11	1/48	0/50
<b>Pituitary</b>											
cyst	104 weeks	3/49	14/50	1/30	1/24	3/27	4/31	6/27	4/24	5/48	10/50
Haemolymphoreticular neoplasm	104 weeks	4/49	2/50	2/30	1/24	1/27	1/31	2/27	0/24	1/48	1/50
adenoma	104 weeks	24/49	21/50	12/30	18/24	17/27	26/31	12/27	19/24	27/48	27/50
<b>Testis</b>											
Interstitial cell hyperplasia, mild	52 weeks	2/11		0/1		0/1		0/0		3/11	
	104 weeks	9/49		10/49		4/49		5/50		4/49	
Interstitial cell tumour, benign	52 weeks	1/11		0/1		0/1		0/0		0/11	
	104 weeks	13/49		8/49		12/49		9/50		3/49	

Interstitial cell tumour, malignant	104 weeks	32/49		38/49		33/49		34/50		42/49	
Mesothelioma, benign	52 weeks	0/11		0/1		1/1		0/0		0/11	
	104 weeks	0/49		2/49		1/49		0/50		1/49	
<b>Ovary</b>											
Haemolymphoreticular neoplasm	52 weeks										
	104 weeks		2/50		1/49		2/50		3/50		2/50
cyst	52 weeks		1/10		3/4		5/5		1/1		1/10
	104 weeks		2/50		5/49		6/50		7/50		8/50
atrophy	52 weeks										
	104 weeks		2/50		5/49		9/50		5/50		10/50
<b>Thyroid</b>											
Hyperplasia, mild	52 weeks	0/11		0/1		0/1		0/0		1/11	
Parafollicular cell adenoma	104 weeks	6/49	1/50	1/26	1/12	1/19	0/14	0/25	1/12	10/49	4/50
Parafollicular cell carcinoma	104 weeks	4/49	2/50	0/26	0/12	1/19	0/14	2/25	1/12	0/49	2/50
Parafollicular cell hyperplasia	104 weeks	7/49	7/50	3/26	2/12	1/19	1/14	3/25	2/12	16/49	8/50

**Table 6.5/01-4: Summary of findings in the 2 year rat chronic toxicity and carcinogenicity study with daminozide.**

	Dose [mg/kg bw/day]									
	0 ppm		100 ppm		500 ppm		5000 ppm		10000 ppm	
	DOS	SAC	DOS	SAC	DOS	SAC	DOS	SAC	DOS	SAC
<b>Males</b>	(25)	(24)	(26)	(23)	(18)	(32)	(24)	(26)	(12)	(37)
Bile duct hyperplasia	17	19	17	16	11	27	18	21	10	31
<b>Females</b>	(15)	(35)	(12)	(37)	(13)	(37)	(11)	(39)	(15)	(35)
Bile duct hyperplasia	1	4	3	13	2	11	6	10	2	12

Ovarian atrophy	2	0	4	1	6	3	2	3	7	3
Ovarian cysts	2	0	0	5	2	4	2	5	2	6

() = Total number examined

DOS = Died on study

SAC = Scheduled sacrifice

**Table 6.5/01-5 Tumour analysis**

<b>Tumour analysis - males (ppm)</b>					
	<b>Dose [mg/kg bw/day]</b>				
	<b>0</b>	<b>100</b>	<b>500</b>	<b>5000</b>	<b>10 000</b>
<b>ADRENAL - Pheochromocytoma, benign</b>					
overall rates	8/60 (13.3%)	1/27 (3.7%)	2/19 (10.5%)	3/28 (10.7%)	8/60 (13.3%)
terminal rates	6/25 (24.0%)	1/3 (33.3%)	0/2 (0.0%)	0/4 (0.0%)	4/37 (10.8%)
<b>ADRENAL - Pheochromocytoma, malignant</b>					
overall rates	1/60 (1.7%)	2/27 (7.4%)	3/19 (15.8%)*	5/28 (17.9%)*	4/60 (6.7%)
terminal rates	0/25 (0.0%)	0/3 (0.0%)	2/2 (100%)	4/4 (100%)	4/37 (10.8%)
<b>HAEMOLYMPHORETICULAR SYSTEM – Mononuclear cell leukaemia</b>					
overall rates	19/60 (31.7%)	18/60 (30.0%)	10/60 (16.7%)*	15/60 (25.0%)	16/60 (26.7%)
terminal rates	9/25 (36.0%)	7/26 (26.9%)	6/33 (18.2%)	4/26 (15.4%)	11/37 (29.7%)
<b>MAMMARY GLAND - Fibroadenoma</b>					
overall rates	1/60 (1.7%)	0/60 (0.0%)	2/60 (3.3%)	0/60 (0.0%)	3/60 (5.0%)
terminal rates	1/25 (4.0%)	0/26 (0.0%)	1/33 (3.0%)	0/26 (0.0%)	3/37 (8.1%)
<b>PANCREAS – Islet cell adenoma</b>					
overall rates	2/60 (3.3%)	1/27 (3.7%)	1/18 (5.6%)	1/23 (4.3%)	6/60 (10.0%)
terminal rates	1/25 (4.0%)	1/3 (33.3%)	0/1 (0.0%)	0/0	5/37 (13.5%)
<b>PITUITARY - Adenoma</b>					
overall rates	25/60 (41.7%)	12/31 (38.7%)	17/27 (63.0%)	12/27 (44.4%)	27/59 (45.8%)
terminal rates	11/25 (44.0%)	4/7 (57.1%)	9/10 (90.0%)	3/3 (100%)	19/36 (52.8%)
<b>TESTIS – Interstitial cell tumour, benign</b>					
overall rates	14/60 (23.3%)	8/50 (16.0%)	12/50 (24.0%)	9/50 (18.0%)	3/60 (5.0%)**
terminal rates	1/25 (4.0%)	1/26 (3.8%)	7/32 (21.9%)	2/26 (7.7%)	1/37 (2.7%)
<b>TESTIS – Interstitial cell tumour, malignant</b>					
overall rates	32/60 (53.3%)	38/50 (76.0%)*	33/50 (66.0%)	34/50 (68.0%)	42/60 (70.0%)*
terminal rates	24/25 (96.0%)	25/26 (92.6%)	24/32 (75.0%)	24/26 (92.3%)	36/37 (97.3%)
<b>THYROID – Parafofollicular cell adenoma</b>					
overall rates	6/60 (10.0%)	1/27 (3.7%)	1/19 (5.3%)	0/25 (0.0%)	10/60 (16.7%)
terminal rates	5/25 (20.0%)	0/3 (0.0%)	0/2 (0.0%)	0/2 (0.0%)	8/37 (21.6%)



Tumour analysis - females (ppm)					
	0	100	500	5000	10 000
ADRENAL – Pheochromocytoma, benign					
overall rates	1/60 (1.7%)	0/13 (0.0%)	0/14 (0.0%)	0/11 (0.0%)	3/59 (5.1%)
terminal rates	0/36 (0.0%)	0/0	0/1 (0.0%)	0/0	2/36 (5.6%)
ADRENAL – Pheochromocytoma, malignant					
overall rates	2/60 (3.3%)	1/13 (7.7%)	0/14 (0.0%)	1/11 (9.1%)	2/59 (3.4%)
terminal rates	1/36 (2.8%)	0/0	0/1 (0.0%)	0/0	2/36 (5.6%)
HAEMOLYMPHORETICULAR SYSTEM – Mononuclear cell leukemia					
overall rates	10/60 (16.7%)	7/60 (11.7%)	11/60 (18.3%)	10/60 (16.7%)	11/60 (18.3%)
terminal rates	4/36 (11.1%)	2/37 (5.4%)	6/37 (16.2%)	3/39 (7.7%)	5/36 (13.9%)
MAMMARY GLAND - Fibroadenoma					
overall rates	3/60 (5.0%)	3/60 (5.0%)	0/60 (0.0%)	2/60 (3.3%)	6/60 (10.0%)
terminal rates	3/36 (8.3%)	2/37 (5.4%)	0/37 (0.0%)	2/39 (5.1%)	4/36 (11.1%)
PITUITARY - Adenoma					
overall rates	22/59 (37.3%)	18/25** (72.0%)	27/32*** (84.4%)	19/25*** (76.0%)	27/58 (46.6%)
terminal rates	16/36 (44.4%)	12/12 (100%)	18/18 (100%)	13/13 (100%)	20/36 (55.6%)
THYROID – Parafollicular cell adenoma					
overall rates	1/60 (1.7%)	1/13 (7.7%)	0/14 (0.0%)	1/12 (8.3%)	4/60 (6.7%)
terminal rates	1/36 (2.8%)	0/0	0/1 (0.0%)	0/1 (0.0%)	3/36 (8.3%)
THYROID – Parafollicular cell carcinoma					
overall rates	2/60 (3.3%)	1/13 (7.7%)	0/14 (0.0%)	1/12 (8.3%)	4/60 (6.7%)
terminal rates	1/36 (2.8%)	0/0	0/1 (0.0%)	0/1 (0.0%)	3/36 (8.3%)
UTERUS - Polyp					
overall rates	9/60 (15.0%)	5/21 (23.8%)	8/23 (34.8%)*	8/23 (34.8%)*	8/60 (13.3%)
terminal rates	7/36 (19.4%)	4/8 (50.0%)	7/10 (70.0%)	8/12 (66.7%)	7/36 (19.4%)

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

#### Original DAR Conclusion:

- No evidence of toxicity was seen after 2 years oral exposure of rats to daminozide (purity 99%) up to the high dose of 10,000 mg/kg food (500 mg/kg bw/day).
- No evidence of oncogenic potential was seen in this study.
- The NOAEL in this study is  $\geq 10,000$  mg/kg food ( $\geq 500$  mg/kg bw/day).

**RMS 2018:** We do not agree with the original conclusion that Daminozide did not reveal any carcinogenic potential. It seems that higher occurrence of pituitary adenomas at females was induced by the treatment. This effect was mainly observed at doses 100, 500, 5000 ppm, while at the highest dose the incidence was only slightly higher compared to control (46.6 vs. 37%). However, the incidence at the top dose was still higher compared to the

normal incidence, which is considered to be 42% (Hayes, 2014)

Despite the fact that lower number of animals at mid-dose groups was examined and absence of dose response, we consider the occurrence of pituitary adenomas as related to the treatment. The NOAEL for carcinogenicity cannot be derived as the effect was observed already at the lowest dose of 100ppm. However, a provisional NOAEL of 100ppm which corresponds to 5mg/kg/bw day is derived.

The AOEL<sub>long-term</sub> of Daminozide is based on the lowest dose of 100ppm corresponding to 5 mg/kg bw/day with additional safety factor of 2.

#### **Two year dietary oncogenicity study in mice**

Reference	Two year dietary oncogenicity study in mice, ██████████ 1988c; Report No. A.7.3.8
Guideline	The study was conducted according to OPPTS Guideline 83-2, OECD TG 451
Deviations	-
GLP	Yes
Acceptability	Yes
Previous evaluation	Yes, study already peer-reviewed in original DAR

**RMS comment 2018:** The study has been checked for compliance. No deviations from OECD guideline 451 (adopted on 7<sup>th</sup> September 2009) were found.

#### **Material and method:**

Groups of 50 male and 50 female CD-1 mice (██████████) were given Alar<sup>®</sup> Technical (daminozide; purity: 99%) in the diet at concentrations of 0 (controls), 300, 3000, 6000 and 10000 ppm for 24 months. The mice were observed for moribundity and mortality three times each day, Monday-Friday, and twice a day on weekends and holidays. The mice were observed for signs of overt toxicity at the times of the moribundity/mortality checks. Detailed observations of appearance and condition, behaviour and activity, excretory functions, respiration, orifices, eyes and palpable masses were conducted at least once weekly. Individual bodyweights and food consumption were recorded prior to study initiation, weekly for weeks 1-16 and every 4 weeks thereafter. Haematological evaluations (leukocyte, erythrocyte count, haemoglobin, haematocrit, MCV, MCH, MCHC, platelets, reticulocytes, differential leucocyte count) were conducted on samples collected from 10 selected animals /group at 12, 18, and 24 months of study.

Histopathological examination was performed on the following organs/tissues: adrenals, aorta, brain, caecum, colon, duodenum, stomach, epididymides, eyes, femur, heart, ileum, jejunum, kidneys, gallbladder, liver, lungs with bronchi, mediastinal lymph node, mammary gland, mesenteric lymph node, oesophagus, optic nerve, ovaries,

pancreas, pituitary, prostate, rectum, salivary gland, sciatic nerve, seminal vesicles, skeletal muscle, skin, spinal cord [3 levels], spleen, sternum, stomach, testes, thymus, thyroid with parathyroid, trachea, urinary bladder, uterus.

### Results:

Clinical signs seen in moribund animals generally included hunched posture, sluggishness, pale appearance, laboured/rapid respiration, swollen abdomen and/or thin appearance. The incidences and causes of morbidity and mortality in controls and treated animals were generally similar and consistent with the usual pattern of causes of demise in mice of this strain. During the study, 56, 57, 51, 62 and 66 mice died/were sacrificed *in extremis* in the control, 300 ppm, 3000 ppm, 6000 ppm and 10000 ppm dosage groups, respectively. The incidence of male mortality in the 6000 and 10000 ppm dosage groups (33 and 35 non-surviving mice, respectively) was slightly elevated relative to that in the control group (29 non-surviving mice). Female mortality was comparable in the control and treated groups. The death of one female in the 10000 ppm group during week 88 was accidental. Deaths/sacrifices *in extremis* were observed during weeks 20-105, with the majority of the mortality in the control and treated groups occurring during the second year (week 53-105). No test article-related differences in group mean bodyweights and food consumption were detected at any interval.

**Table 6.5/02-1 Survival data: number of survived rats (% survival)**

	Dose [mg/kg bw/day]									
	0 ppm		300 ppm		3000 ppm		6000 ppm		10000 ppm	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
total number	50	50	50	50	50	50	50	50	50	50
week 52	48	46	49	49	50	49	49	47	47	50
week 80	37	38	38	40	36	45	35	38	34	45
week 92	31	31	30	33	31	37	27	31	24	38
End of the study	22/50 (44%)	24/50 (48%)	25/50 (50%)	20/50 (40%)	25/50 (50%)	25/50 (50%)	17/50 (34%)	22/50 (44%)	18/50 (36%)	19/50 (38%)

Haematology: Females in the 3000, 6000 and 10000 ppm groups had decreased mean platelet counts at the 24 month intervals. Although this parameter is highly variable in rodents, the pattern of occurrence was indicative of a test article-related effect. In males, the data were too variable to detect a similar effect. Males in the 6000 and 10000 ppm groups displayed decreased (statistically significant) red cell parameters at the 18 month interval, however, not at 24 months. Females in the 10000 ppm group had decreased haematocrit values at 18 months and decreased erythrocytes at 24 months. All mean values, except the erythrocytes in the 10000 ppm females, were within the range of historical control values for mice of this age in this laboratory. It is unknown whether this

apparent anaemia was an artefact of animal variation, due to a direct effect of the test article, or was a secondary manifestation of underlying disease processes. Males in the 3000 ppm group had a statistically significant increase in mean leukocyte counts at the 24 month interval. This increase was not seen in males at higher levels, or in females, and more than likely was attributed to normal animal variation. Accordingly, there were no apparent test article related effects in the 300 ppm animals.

**Table 6.5/02-2 Haematological changes**

	Dose [mg/kg bw/day]														
	0 ppm			300 ppm			3000 ppm			6000 ppm			10000 ppm		
month	12	18	24	12	18	24	12	18	24	12	18	24	12	18	24
<b>Leukocytes, x10<sup>3</sup>/cmm</b>															
Males	6.9 (10)	5.2 (10)	5.7 (10)	7.7 (10)	5.7 (10)	<b>9.7*</b> (10)	7.9 (10)	7.6 (10)	7.0 (10)	7.3 (10)	7.9 (9)	7.9 (10)	5.3 (10)	6.9 (10)	6.2 (10)
Females	4.4 (10)	5.0 (10)	6.9 (10)	4.4 (10)	5.4 (10)	7.2 (10)	5.5 (10)	5.6 (10)	10.6 (10)	5.1 (10)	4.8 (10)	6.1 (10)	5.0 (10)	5.8 (10)	5.2 (10)
<b>Erythrocytes, x 10<sup>6</sup>/cmm</b>															
Males	7.8 (10)	7.6 (10)	7.3 (10)	7.6 (10)	7.4 (10)	7.6 (10)	7.9 (10)	7.6 (10)	7.0 (10)	7.6 (10)	7.1 (9)	7.1 (10)	7.4 (10)	<b>6.7*</b> *(10)	7.0 (10)
Females	7.49 (10)	7.19 (10)	7.07 (10)	7.71 (10)	6.70 (10)	6.67 (10)	7.52 (10)	7.18 (10)	7.03 (10)	7.48 (10)	6.91 (10)	6.51 (10)	7.60 (10)	6.63 (10)	<b>5.83**</b> (10)
<b>Haematocrit, %</b>															
Males	39.4 (10)	39.2 (10)	39.0 (10)	38.4 (10)	37.8 (10)	40.5 (10)	39.5 (10)	38.4 (10)	37.9 (10)	38.8 (10)	<b>36.2</b> **(9)	36.9 (10)	37.8 (10)	<b>34.3</b> *(10)	37.2 (10)
Females	39.2 (10)	38.2 (10)	38.1 (10)	40.6 (10)	35.0 (10)	37.5 (10)	39.1 (10)	37.5 (10)	38.8 (10)	38.5 (10)	36.3 (10)	36.5 (10)	38.8 (10)	<b>34.7</b> *(10)	33.2 (10)
<b>Haemoglobin, g/dl</b>															

	Dose [mg/kg bw/day]														
	0 ppm			300 ppm			3000 ppm			6000 ppm			10000 ppm		
Males	14.7 (10)	14.4 (10)	13.7 (10)	14.4 (10)	13.7 (10)	14.4 (10)	14.8 (10)	14.3 (10)	13.4 (10)	14.4 (10)	<b>13.3</b> ** (9)	13.2 (10)	13.9 (10)	<b>12.8</b> *(10)	13.2 (10)
Females	14.6 (10)	14.0 (10)	13.3 (10)	14.9 (10)	12.9 (10)	13.2 (10)	14.5 (10)	13.8 (10)	13.7 (10)	14.4 (10)	13.5 (10)	12.8 (10)	14.4 (10)	12.9 (10)	11.8 (10)
<b>Platelets, x10<sup>3</sup>/cmm</b>															
Males	903 (9)	1223 (10)	1169 (10)	994 (8)	1152 (9)	1084 (9)	886 (10)	1000 (10)	1131 (10)	791 (10)	1096 (9)	856 (9)	973 (10)	1003 (10)	919 (10)
Females	850 (9)	778 (10)	877 (10)	878 (8)	773 (10)	756 (10)	807 (10)	794 (10)	<b>594*</b> *(10)	<b>732*</b> (10)	694 (10)	<b>576*</b> *(10)	774 (10)	646 (10)	<b>415**</b> (10)

\*p<0.05, \*\*p<0.01, () number of animals.

Nodules and masses: An increased incidence in nodules/masses in the lungs was present in the treated mice of both sexes as compared to the controls and may have been related to the administration of the test article.

A variety of both neoplastic and non-neoplastic lesions were evident in both sexes of all dose groups. Multifocal inflammation of the liver and brown pigment in liver was common in both the males and females. Both appeared to be slightly increased in the treated males compared to control males, while the females did not appear to be affected. This increase in males may have been a test article effect.

The inflammation in the liver was predominantly multifocal and chronic in nature. The macroscopic enlarged spleens correlated microscopically with an increase in extramedullary haematopoiesis and macroscopically enlarged spleens and lymph nodes correlated with haemolymphoreticular neoplasms. Haemolymphoreticular neoplasms included a variety of malignant lymphomas and histiocytic sarcomas. They are a common spontaneous finding in many mouse strains and the incidence did not appear to be increased with the administration of the test article.

Neoplasms: The macroscopic masses or nodules in the livers correlated microscopically with either hepatocellular neoplasms or vascular neoplasms (haemangiomas and haemangiosarcomas). Neither of these tumours appeared to be increased with the administration of the test article. Haemangiomas are benign neoplasms and haemangiosarcomas are malignant neoplasms originating from endothelial cells lining blood vascular spaces. Since blood vessels with endothelial linings are common to all organs and tissues, haemangiomas and haemangiosarcomas can theoretically arise from any organ or tissue in the body. In reality they are found in a number of organs including: liver; spleen; skin/subcutis; ovary; uterus; bone marrow; and lymph nodes.

Haemangiosarcomas may occasionally occur in more than one organ of the same animal (multicentric origin) or the primary neoplasm may metastasize to a secondary organ. It is sometimes difficult to distinguish between multicentric origin and metastatic neoplasms. If either multicentric origin or metastatic haemangiosarcomas are present, the neoplasm is designated as being present in only a single organ so that only a single neoplasm will be listed for that animal. Other involved organs are noted with special notations in the individual animal microscopic report (IADR). Also, because both haemangiomas and haemangiosarcomas are not unique to a single organ or tissue, they are usually listed as present for the animal and not for a specific tissue. Although the incidence of haemangiomas and haemangiosarcomas did not appear to be increased by the administration of the test article, information is provided because of the possible relationship of this particular tumour type to the administration of the test article.

Although a dose response was not evident, the incidence of pulmonary tumours did appear to be increased in the treated mice when compared to controls. Lung tumours occur spontaneously in many strains of mice and the incidence varies between strains with a higher incidence in the males compared to the females. The neoplasms appear to arise either from the alveolar cells lining the pulmonary alveoli or from Clara cells found normally within bronchioles. Since these two types of lung neoplasms cannot be easily distinguished at the light microscopic level, they are usually designated as alveolar/bronchiolar adenomas or carcinomas. The incidence varies quite markedly between different studies conducted in the same strain of mice in the same laboratory.

**Table 6.5/02-3 Tumour analysis;** \*p<0.05, \*\*p<0.01, Fisher exact test

<b>Tumour analysis - males (ppm)</b>					
	<b>Dose [mg/kg bw/day]</b>				
	<b>0</b>	<b>300</b>	<b>3000</b>	<b>6000</b>	<b>10 000</b>
<b>LIVER - hepatocellular adenoma</b>					
overall rates	4/50 (8.0%)	4/50 (8.0%)	3/50 (6.0%)	4/50 (8.0%)	5/50 (10.0%)
terminal rates	2/21 (9.5%)	4/24 (16.7%)	3/24 (12.5%)	3/17 (17.6%)	2/15 (13.3%)
<b>LIVER - haemangioma</b>					
overall rates	2/50 (4.0%)	0/50 (0.0%)	2/50 (4.0%)	1/50 (2.0%)	2/50 (4.0%)
terminal rates	0/21 (0.0%)	0/24 (0.0%)	1/24 (4.2%)	0/17 (0.0%)	0/15 (0.0%)
<b>LIVER - haemangiosarcoma</b>					
overall rates	3/50 (6.0%)	1/50 (2.0%)	0/50 (0.0%)	2/50 (4.0%)	7/50 (14.0%)
terminal rates	0/21 (0.0%)	0/24 (0.0%)	0/24 (0.0%)	0/17 (0.0%)	0/15 (0.0%)
<b>LIVER - hepatocellular carcinoma</b>					
overall rates	4/50 (8.0%)	9/50 (18.0%)	7/50 (14.0%)	5/50 (10.0%)	2/50 (4.0%)

terminal rates	2/21 (9.5%)	3/24 (12.5%)	4/24 (16.7%)	2/17 (11.8%)	1/15 (6.7%)
LUNG - alveolar bronchiolar adenoma					
overall rates	20/50 (40.0%)	26/50 (52.0%)	28/50 (56.0%)	31/50 (62.0%)*	27/50 (54.0%)
terminal rates	9/21 (42.9%)	13/24 (54.2%)	18/24 (75.0%)	13/17 (76.5%)	8/15 (53.3%)
LUNG - alveolar bronchiolar carcinoma					
overall rates	5/50 (10.0%)	2/50 (4.0%)	5/50 (10.0%)	7/50 (14.0%)	6/50 (12.0%)
terminal rates	2/21 (9.5%)	1/24 (4.2%)	4/24 (16.7%)	3/17 (17.6%)	0/15 (0.0%)
LUNG - alveolar bronchiolar adenoma/carcinoma					
overall rates	25/50 (50.0%)	28/50 (56.0%)	33/50 (66.0%)	38/50 (76.0%)**	33/50 (66.0%)
terminal rates	11/21 (52.4%)	14/24 (58.3%)	22/24 (91.7%)	16/17 (94.1%)	8/15 (53.3%)
LIVER - haemangioma/haemangiosarcoma					
overall rates	5/50 (10.0%)	1/50 (2.0%)	2/50 (4.0%)	3/50 (6.0%)	9/50 (18.0%)
terminal rates	0/21 (0.0%)	0/24 (0.0%)	1/24 (4.2%)	0/17 (0.0%)	0/15 (0.0%)
<b>Tumour analysis - females (ppm)</b>					
	<b>0</b>	<b>300</b>	<b>3000</b>	<b>6000</b>	<b>10 000</b>
LIVER - hepatocellular adenoma					
overall rates	2/50 (4.0%)	0/50 (0.0%)	1/50 (2.0%)	2/50 (4.0%)	3/50 (6.0%)
terminal rates	1/23 (4.3%)	0/19 (0.0%)	0/26 (0.0%)	1/22 (4.5%)	2/20 (10.0%)
LIVER - haemangiosarcoma					
overall rates	1/50 (2.0%)	1/50 (2.0%)	1/50 (2.0%)	0/50 (0.0%)	3/50 (6.0%)
terminal rates	1/23 (4.3%)	0/19 (0.0%)	0/26 (0.0%)	0/22 (0.0%)	1/20 (5.0%)
LUNG - alveolar bronchiolar adenoma					
overall rates	20/50 (40.0%)	26/50 (52.0%)	27/50 (54.0%)	28/50 (56.0%)	26/50 (52.0%)
terminal rates	12/23 (52.2%)	12/19 (63.2%)	16/26 (61.5%)	17/22 (77.3%)	13/20 (65.0%)
LUNG - alveolar bronchiolar carcinoma					
overall rates	0/50 (0.0%)	3/50 (6.0%)	2/50 (4.0%)	2/50 (4.0%)	4/50 (8.0%)
terminal rates	0/23 (0.0%)	0/19 (0.0%)	0/26 (0.0%)	0/22 (0.0%)	0/20 (0.0%)

UTERUS - haemangiosarcoma					
overall rates	1/50 (2.0%)	0/42 (0.0%)	0/36 (0.0%)	0/41 (0.0%)	4/50 (8.0%)
terminal rates	0/23 (0.0%)	0/11 (0.0%)	0/12 (0.0%)	0/13 (0.0%)	1/20 (8.0%)
LUNG - alveolar bronchiolar adenoma/carcinoma					
overall rates	20/50 (40.0%)	29/50 (58.0%)	29/50 (58.0%)	30/50 (60.0%)*	30/50 (60.0%)*
terminal rates	12/23 (52.0%)	12/19 (63.2%)	16/26 (61.5%)	17/22 (77.3%)	13/20 (65.0%)
LIVER - haemangioma/haemangiosarcoma					
overall rates	3/50 (6.0%)	1/50 (2.0%)	2/50 (4.0%)	1/50 (2.0%)	4/50 (8.0%)
terminal rates	2/23 (8.7%)	0/19 (0.0%)	1/26 (3.8%)	0/22 (0.0%)	2/20 (10.0%)

**Table 6.5/02-4 Incidence of macroscopic masses/nodules in the lungs**

	0 ppm		300 ppm		3000 ppm		6000 ppm		10000 ppm	
	DOS	SAC	DOS	SAC	DOS	SAC	DOS	SAC	DOS	SAC
<b>Males</b>	(29)	(21)	(26)	(24)	(26)	(24)	(33)	(17)	(35)	(15)
	5	6	6	10	4	15	12	14	13	6
<b>Females</b>	(27)	(23)	(31)	(19)	(25)	(25)	(29)	(21)	(31)	(19)
	3	4	10	7	4	8	7	3	4	7

() = Total number examined

DOS = Died on study

SAC = Scheduled sacrifice

**Table 6.5/02-5: Incidence of inflammation/brown pigmentation of the liver**

INCIDENCE OF INFLAMMATION OF THE LIVER		0 ppm		300 ppm		3000 ppm		6000 ppm		10000 ppm	
		DOS	SAC	DOS	SAC	DOS	SAC	DOS	SAC	DOS	SAC
	<b>Males</b>	(29)	(21)	(26)	(24)	(26)	(24)	(33)	(17)	(35)	(15)
		0	3	2	6	1	8	7	5	5	5
	<b>Females</b>	(27)	(23)	(31)	(19)	(25)	(25)	(29)	(21)	(31)	(19)
		2	17	2	10	3	9	2	5	9	10
BROWN PIGMENT		0 ppm		300 ppm		3000 ppm		6000 ppm		10000 ppm	
		DOS	SAC	DOS	SAC	DOS	SAC	DOS	SAC	DOS	SAC



	<b>Males</b>	(29)	(21)	(26)	(24)	(26)	(24)	(33)	(17)	(35)	(15)
		1	1	1	3	2	2	8	4	11	3
	<b>Females</b>	(27)	(23)	(31)	(19)	(25)	(25)	(29)	(21)	(31)	(19)
		3	2	3	2	6	5	1	4	5	3

() = Total number examined

DOS = Died on study

SAC = Scheduled sacrifice

**Table 6.5/02-6: Incidence of vascular neoplasms**

	0 ppm		300 ppm		3000 ppm		6000 ppm		10000 ppm	
	DOS	SAC	DOS	SAC	DOS	SAC	DOS	SAC	DOS	SAC
<b>Males</b>	(29)	(21)	(26)	(24)	(26)	(24)	(33)	(17)	(35)	(15)
Haemangiomas	3	0	0	0	1	1	5	0	2	0
Haemangiosarcomas	4	0	1	0	1	0	2	0	8	0
Total vascular neoplasms	7	0	1	0	2	1	7	0	10	0
<b>Females</b>	(27)	(23)	(31)	(19)	(25)	(25)	(29)	(21)	(31)	(19)
Haemangiomas	2	1	1	1	0	4	2	1	1	1
Haemangiosarcomas	2	2	1	0	2	0	0	0	6	2
Total vascular neoplasms	4	3	2	1	2	4	2	1	7	3

() = Total number examined

DOS = Died on study

SAC = Scheduled sacrifice

**Table 6.5/02-7: Incidence of pulmonary neoplasms**

	0 ppm		300 ppm		3000 ppm		6000 ppm		10000 ppm	
	DOS	SAC	DOS	SAC	DOS	SAC	DOS	SAC	DOS	SAC
<b>Males</b>	(29)	(21)	(26)	(24)	(26)	(24)	(33)	(17)	(35)	(15)
Alveolar/bronchiolar adenomas	11	9	13	13	10	18	18	13	19	8
Alveolar/bronchiolar carcinomas	3	2	1	1	1	4	4	3	6	0
Total pulmonary neoplasms	14	11	14	14	11	22	22	16	25	8
<b>Females</b>	(27)	(23)	(31)	(19)	(25)	(25)	(29)	(21)	(31)	(19)
Alveolar/bronchiolar adenomas	8	12	14	12	12	15	12	16	13	13
Alveolar/bronchiolar carcinomas	0	0	3	0	2	0	2	0	4	0
Total pulmonary neoplasms	8	12	17	12	14	15	14	16	17	13

( ) = Total number examined

DOS = Died on study

SAC = Scheduled sacrifice

#### Original DAR conclusion:

- Inflammation and brown pigment in the livers of male mice were more prevalent in the two highest dose groups than in the controls.
- Females in the 3 highest dose groups had statistical significant decreased platelet counts. In males, a slight decrease in the same parameter was observed. According to the author, the pattern of occurrence of decreased platelet counts was indicative of a treatment-related effect, which is agreed upon by the reviewer.
- Female mice exposed to 10,000 mg/kg food showed a statistical significant decrease in erythrocyte counts.
- It was concluded that there was no treatment-related oncogenic effects in this study.
- No treatment-related effects were observed at 300 mg/kg food (45 mg/kg bw/day).

**RMS 2018:** We do not agree with the original conclusion that there were no treatment-related oncogenic effects. The NOAEL for carcinogenicity cannot be derived from this study because the incidence of alveolar/bronchiolar adenomas as well as alveolar/bronchiolar adenomas combined with carcinomas was increased in each treated group in both sexes (at the maximum by 26% in males at 6000 ppm) when compared to the control. Although the clear dose-response relationship was not observed, the rise in the incidence of pulmonary neoplasms is regarded by RMS as the treatment related (oncogenic) effect of the test substance.

### B 6.5.2 Summary of long-term toxicity and carcinogenicity

Long term dietary studies with daminozide were conducted in rats and mice. In the two year rat study, the increased incidence of pituitary adenomas was observed at females from the lowest dose. Therefore NOAEL was not established. In the second study in mice an increased incidence of pulmonary neoplasms (adenomas+carcinomas) was considered as related to the treatment. As this effect was evident from the lowest dose, NOAEL was not established from the second study either. We consider the observed effects from both chronic studies as treatment-related, indicating oncogenic potential of Daminozide. Thus, the original conclusions that these effects are not related to the treatment were revised and changed by the RMS. Based on the results of the chronic toxicity/carcinogenicity studies and according to the CLP criteria, a classification as **Carc. 1B** should be considered warranted for Daminozide.

The results of the oral long-term studies with daminozide are summarised in Table 5.5/01.

**Table 6.5-1: Dietary carcinogenicity studies conducted in rats and mice with daminozide**

Duration	Species	Route	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Critical effects	Reference Notifier
2 years	rat	oral	-	-	Pituitary adenomas, Bile duct hyperplasia	Uniroyal, [REDACTED] 1988b
2 years	mouse	oral	-	-	Pulmonary neoplasms (Carcinomas + adenomas)	Uniroyal, [REDACTED] 1988c

### B 6.6 Reproductive toxicity

#### B 6.6.1 Generation studies

##### Two-generation reproduction study with Alar in rats (one litter per generation)

Reference	<b>Two-generation reproduction study with Alar in rats (one litter per generation),</b> [REDACTED] 1987; Report No. A.7.6.15
Guideline	The study was conducted according to US EPA Guideline 83-4, OECD TG 416 and in compliance with GLP. No individual data were submitted by the notifier. However, the study is considered acceptable for overall evaluation.
Deviations	Yes
GLP	Yes
Acceptability	Yes

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Previous evaluation	Yes, study already peer-reviewed in original DAR
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**RMS comment 2018:** The study has been checked for compliance. The following deviations from OECD guideline 416 (adopted on 22nd January 2001) were found:

- 1) In general, the addition of the fourth test group is recommended if large intervals (6-10fold) between dosages are used. In dietary studies the dose interval should not exceed 3fold.
- 2) In F1 weanlings selected for mating, the age at vaginal opening or preputial separation should be determined
- 3) Sperm parameters, i.e. total cauda epididymal sperm number, percent progressively motile sperm, percent morphologically normal sperm, and percent of sperm with each identified abnormality were not evaluated
- 4) The following organs: uterus, ovaries, testes, prostate, seminal vesicles with coagulating glands, brain, liver, kidneys, spleen, pituitary, thyroid and adrenal glands of all P and F1 parental animals were not weighed at the time of termination
- 5) Brain, spleen, and thymus of randomly selected F1 and F2 pups of each sex and litter were not weighed
- 6) Quantitative evaluation of primordial follicles of F1 females was not performed

#### **Material and method:**

Groups of 25 male and 25 female rats (F0 parents; albino [REDACTED]) were fed diets containing 0, 100, 1000, or 10000 ppm of daminozide (mentioned as ALAR in this study; purity: 99%) for 10 weeks. The F0 parents were mated to produce an F1 litter. Groups of 25 male and 25 female F1 animals were continued on their parents' diet for 10 weeks post-weaning, and the breeding program was repeated to produce F2 animals. The study was terminated with the F2 weanlings.

All animals were observed twice daily for mortality and once daily for any overt changes in appearance or indications of toxicity. Bodyweights for males were recorded weekly throughout the study. Females' bodyweights were recorded weekly during pre-mating phases, on days 0, 7, 14 and 20 of gestation, and on days 0, 7, 14 and 21 of lactation. Food consumptions were recorded weekly during pre-mating phases for both males and females.

In each generation, breeding was initiated by randomly selecting one male and one female from the same treatment group. The presence of a copulatory plug or sperm was considered evidence of positive mating, and that day was considered day 0 of gestation. The first day, an entire litter was observed, was considered day 0 of lactation for that litter. Litters were examined as soon as possible after delivery. Litters weights and the number of live pups were recorded. Intact dead pups were examined macroscopically. Four days after birth the number and sex of pups and litter weights were recorded. Pups were culled from the litters at random to achieve a maximum litter size of eight, with four males and four females for each litter, if possible. After this, the pups were weighed by litter and individually examined for external abnormalities on days 7, 14 and 21 of lactation.

Pups were allowed to nurse for 21 days before weaning. Animals were selected at random from the available litters so that each litter was represented during the continuation of the study. The following indices were calculated and reported for all groups in each generation: mating, female fertility, male fertility, gestation, viability, weaning, and

sex ratio. All F0 and F1 adult animals (including infertile males and females) were subjected to a gross necropsy after the litters had been weaned. Epididymides, vagina, any gross lesions, ovaries, testes, prostate, uterus, and seminal vesicles were preserved in formalin. These tissues were sectioned and stained with haematoxylin and eosin and evaluated microscopically from all F0 and F1 high-dose and control animals.

### Results:

**F0 animals:** There were no treatment-related observations. Survival was 100% for all groups of F0 males, except for the 10000 ppm group, where it was 96%. For F0 females, survival was 100% in the 0 and 10000 ppm groups and 96% for the 100 and 1000 ppm groups. These deaths were not related to treatment. No differences in bodyweights and bodyweight gains were noted in either F0 males or F0 females during any phase of the study. No differences in food consumption were noted in F0 males. Food consumption for F0 females was significantly higher than that of controls during weeks 1-9 for the 10000 ppm group and significantly lower during week 10 for the 1000 ppm group.

No differences were noted for male fertility, mating, female fertility, gestation, days required to mate and length of gestation in any of the F0 groups. No females aborted or had physical or behavioural abnormalities during gestation or lactation. No differences were noted for the number of pups born alive, litter size, pup survival, pup weights, pup weight gains or sex ratio. No pups had external abnormalities.

**Table 6.6.1/01-1: Two-generation reproduction study with Alar in rats; Group mean bodyweight gain in F0 animals [g]**

Dose [ppm] Week of study	Males				Females			
	0	100	1000	10000	0	100	1000	10000
1	48.3	50.1	52.0	52.1	20.2	20.8	21.3	22.9
2	38.4	38.3	40.4	37.6	14.9	13.5	15.6	17.7
4	30.6	30.3	32.6	32.0	12.6	13.6	13.3	11.4
6	22.7	24.2	24.9	21.2	9.0	9.3	9.7	9.0
8	18.8	18.5	18.6	15.8	6.3	9.8	4.9	6.4
10	11.7	14.5	13.3	14.3	4.1	1.9	1.5	1.7
12	12.2	9.7	9.7	15.1	-	-	-	-
14	8.3	12.8	11.0	7.5	-	-	-	-
	Overall bodyweight gain (week 15-Start)				Overall bodyweight gain (week 10-Start)			
	301.8	306.5	314.3	302.5	101.1	94	96.8	98.8

Table 6.6.1/01-2 Bodyweights (g) in F0 animals

<b>Dose (ppm) Week</b>	<b>0</b>	<b>100</b>	<b>1000</b>	<b>10000</b>
<b>Males</b>				
<b>0</b>	263.2	265.3	264.3	264.7
<b>1</b>	311.5	315.4	316.2	316.8
<b>2</b>	349.9	353.7	356.7	354.4
<b>3</b>	385.4	388.9	391.6	388.4
<b>4</b>	416.0	419.2	424.2	420.4
<b>5</b>	432.3	436.3	443.4	437.0
<b>6</b>	455.0	460.5	468.3	458.2
<b>7</b>	474.3	479.8	487.5	475.5
<b>8</b>	493.1	498.3	506.0	491.3
<b>9</b>	512.6	516.1	524.2	506.5
<b>10</b>	524.3	530.6	537.5	520.7
<b>11</b>	528.3	536.3	542.7	521.8
<b>12</b>	540.5	546.0	552.5	536.9
<b>13</b>	546.0	550.4	560.2	540.6
<b>14</b>	554.2	563.2	571.2	548.1
<b>15</b>	565.0	571.8	578.6	567.2
<b>Females</b>				
<b>0</b>	164.4	160.4	164.3	167.1
<b>1</b>	184.6	181.2	185.6	190.0
<b>2</b>	199.5	194.7	201.2	204.7
<b>3</b>	213.3	205.6	213.6	218.0
<b>4</b>	226.0	219.2	226.9	229.5
<b>5</b>	232.0	224.4	232.0	235.4
<b>6</b>	241.0	233.7	241.7	244.3

7	247.2	236.4	246.9	251.4
8	253.4	246.3	251.8	257.9
9	261.4	252.5	259.6	264.2
10	265.5	254.4	261.1	265.9

Table 6.6.1/01-3 Food consumption (g) in males

Week \ Dose (ppm)	F0 animals				F1 animals			
	0	100	1000	10000	0	100	1000	10000
1	181.7	189.3	187.3	188.8	158.7	153.5	158.7	156.0
2	182.8	192.6	190.6	191.8	185.8	177.9	183.3	183.1
3	184.1	191.8	192.1	195.2	201.1	195.3	196.0	196.2
4	187.0	193.0	195.6	199.6	207.8	201.9	201.6	204.9
5	186.6	190.6	199.4	200.0	212.4	204.6	207.6	211.6
6	186.3	194.6	202.8*	200.8	215.7	202.3	211.5	211.1
7	194.8	201.2	208.8	203.5	215.0	207.6	211.8	210.1
8	191.0	198.4	205.8	198.4	217.2	208.9	211.3	216.3
9	196.6	198.0	206.7	196.3	211.4	200.6	202.1	204.1
10	195.0	199.1	206.8	210.4	211.9	202.4	205.9	204.6

\* Statistically significant ( $p < 0.05$ )

Table 6.6.1/01-4 Food consumption (g) in F0 and F1 females

Week \ Dose(ppm)	F0 animals				F1 animals			
	0	100	1000	10000	0	100	1000	10000
1	123.2	121.3	126.9	131.0*	128.9	130.9	133.6	137.7
2	129.5	122.7	133.5	144.9*	136.8	136.3	140.6	151.2*

3	129.6	126.9	133.0	145.2*	144.3	145.6	153.7	158.1*
4	139.6	140.1	142.0	163.2*	139.6	141.7	150.5	159.0*
5	130.9	130.8	137.6	154.5*	147.5	141.2	151.6	162.8*
6	134.1	134.9	141.0	155.5*	147.6	147.0	163.0*	164.3*
7	140.5	134.4	138.5	157.9*	146.8	148.1	160.9*	159.1*
8	136.6	137.4	135.8	152.0*	150.4	153.6	164.4	161.9
9	133.0	132.9	133.6	149.7*	144.8	140.9	149.2	154.3
10	132.2	125.6	123.8*	132.4	150.0	154.6	153.3	156.6

\* Statistically significant ( $p < 0.05$ )

F1 animals: There were no treatment-related observations for F1 males and females. Survival was 100% for all groups of F1 males and females. During lactation, bodyweights of the 100 ppm female group were significantly lower than those of the controls on days 14 and 21.

**Table 6.6.1/01-5 Bodyweights (g) in F1 males**

<b>Dose (ppm) Week</b>	<b>0</b>	<b>100</b>	<b>1000</b>	<b>10000</b>
<b>0</b>	113.7	113.3	118.9	108.8
<b>1</b>	174.9	173.6	182.2	168.6
<b>2</b>	233.5	231.2	239.6	223.9
<b>3</b>	295.6	291.0	298.4	281.6
<b>4</b>	340.8	334.3	341.9	327.1
<b>5</b>	380.0	370.6	380.1	362.2
<b>6</b>	414.4	399.7	413.2	393.4
<b>7</b>	440.3	424.4	437.8	419.2
<b>8</b>	459.0	443.5	455.5	439.0
<b>9</b>	486.0	467.6	480.8	462.0
<b>10</b>	506.4	483.2	497.0	474.3



<b>11</b>	524.1	494.7	513.0	486.1*
<b>12</b>	528.0	503.2	519.1	491.4*
<b>13</b>	539.3	517.0	532.2	502.9
<b>14</b>	555.1	531.3	543.0	513.2
<b>15</b>	564.4	538.5	549.9	521.2*
<b>16</b>	579.8	554.2	563.1	534.4*
<b>17</b>	588.8	563.5	574.1	539.7*
<b>18</b>	602.6	573.0	584.1	550.2*
<b>19</b>	607.8	579.0	588.4	557.2*

\* Statistically significant ( $p < 0.05$ )

There were no differences in food consumption for males. Food consumption for females was significantly higher than that of controls during weeks 2 through 7 for the 10000 ppm group and during weeks 6 and 7 for the 1000 ppm group.

**Table 6.6.1/01-6 Bodyweights (g) in F1 females**

<b>Dose (ppm) Week</b>	<b>0</b>	<b>100</b>	<b>1000</b>	<b>10000</b>
<b>0</b>	99.9	98.2	105.0	97.8
<b>1</b>	138.8	137.8	141.9	132.9
<b>2</b>	166.7	163.3	168.4	163.8
<b>3</b>	192.7	186.7	192.0	188.9
<b>4</b>	210.5	202.5	208.6	208.2
<b>5</b>	227.1	216.5	224.9	224.6
<b>6</b>	240.9	229.5	237.9	237.9
<b>7</b>	250.4	239.5	250.1	248.4
<b>8</b>	256.2	244.9	255.4	252.8
<b>9</b>	269.3	257.9	266.6	267.3
<b>10</b>	275.2	264.0	272.6	271.3
<b>11</b>	279.0	267.7	275.9	276.0
<b>12</b>	300.7	282.1	294.2	290.7

Lactation (days post partum)				
<b>0</b>	309	292	311	299
<b>4</b>	306	289	308	306
<b>7</b>	314	302	316	315
<b>14</b>	331	313*	329.3	327.0
<b>21</b>	316	301*	318	313

\* Statistically significant ( $p < 0.05$ )

No differences were noted for male fertility, mating, female fertility, gestation, days required to mate and length of gestation in any of the F1 groups. No females aborted or had physical or behavioural abnormalities during gestation or lactation. No differences were noted for the number of pups born alive, litter size, pup survival or sex ratio. There were significant differences in pup bodyweights on day 7 and weight gains on days 4 through 7 for pups from dams treated with 100 ppm. However, the differences were not consistent or toxicologically meaningful.

**Table 6.6.1/01-7: Two-generation reproduction study with Alar in rats; Group mean bodyweight gain in F1 animals [g];**

Dose [ppm] Week of study	Males				Females			
	0	100	1000	10000	0	100	1000	10000
<b>1</b>	61.2	60.3	63.3	59.9	38.8	39.6	36.9	35.0
<b>2</b>	58.5	57.6	57.4	55.3	27.9	25.5	26.5	30.9
<b>4</b>	45.2	43.3	43.5	45.4	17.8	15.8	16.5	19.3
<b>6</b>	34.4	29.1	33.1	31.1	13.8	13.0	13.0	13.3
<b>8</b>	18.8	19.1	17.7	19.8	5.9	5.4	5.3	4.4
<b>10</b>	20.5	15.6	16.2	12.3*	5.9	6.1	6.1	4.0
<b>12</b>	3.8	8.4	6.2	5.3	21.7	14.3*	18.4	14.7*
<b>14</b>	15.8	14.3	10.8	10.3	-	-	-	-
<b>16</b>	15.4	15.7	13.2	13.2	-	-	-	-
<b>18</b>	13.8	9.5*	10.0*	10.6	-	-	-	-
<b>19</b>	5.2	6.0	4.3	7.0	-	-	-	-
	Overall bodyweight gain (week 19-Start)				Overall bodyweight gain (week 12-Start)			
	494.1	465.7	469.5	448.4	200.8	183.9	189.2	192.9

\* $p \leq 0.05$ ;

**Table 6.6.1/01-8: Two-generation reproduction study with Alar in rats; Group mean bodyweight gain in F0 and F1 females during gestation [g]**

Dose [ppm] Day of gestation	F0 females				F1 females			
	0	100	1000	10000	0	100	1000	10000
0-7	25.1	27.4	28.7	30.7	30.1	23.4*	27.1	28.2
7-14	27.8	23.5	22.2	22.0	26.7	26.0	26.2	25.0
14-20	56.6	59.2	54.2	58.0	60.7	55.9	62.9	54.5
0-20	109.6	110.1	105.1	110.8	117.4	105.3	116.3	107.6

\*p≤0.05

**Table 6.6.1/01-9: Two-generation reproduction study with Alar in rats; Group mean bodyweight gain in F0 and F1 females during lactation [g]**

Dose [ppm] Day of lactation	F0 females				F1 females			
	0	100	1000	10000	0	100	1000	10000
0-4	5.6	11.8	13.8	7.4	-2.7	-2.7	-3.9	5.6
4-7	5.9	4.8	3.6	7.7	8.1	12.25	8.52	9.6
7-14	22.3	24.8	23.5	28.2	16.6	11.4	13.2	11.6
14-21	-21.1	-28.3	-29.4	-33.0	-14.91	-12.5	-11.0	-13.74
0-21	12.7	13.0	11.6	10.4	7.0	11.4	7.1	13.2

**Table 6.6.1/01-10 Mean pup weights (g)**

Dose levels	day 1	day 4	day 7	day 14	day 21
F <sub>1</sub> 0 ppm	6.4	10.3	16.7	32.1	51.5
100 ppm	6.3	10.1	16.7	32.4	51.3
1000 ppm	6.2	10.3	17.0	33.2	52.4
10000 ppm	6.4	10.1	16.2	31.6	49.1
F <sub>2</sub> 0 ppm	6.4	10.7	17.6	33.7	54.2
100 ppm	6.3	10.2	16.4*	32.3	52.9
1000 ppm	6.5	11.0	17.8	34.3	55.9
10000 ppm	6.5	11.0	17.6	33.19	54.1

\* Statistically significant (p < 0.05)

**Table 6.6.1/01-11: Two generation reproduction toxicity study in rat; Group mating data and group mean litter data in P and F1 generation**

Parameter \ Dose[ppm]	P generation				F1 generation			
	0	100	1000	10000	0	100	1000	10000
<b>Fertility index in males [%]</b>	100	92	96	92	92	96	100	92
<b>Fertility index in females [%]</b>	100	100	100	100	92	96	100	100
<b>Mating [%]</b>	100	92	96	92	92	96	100	92
<b>Gestation [%]</b>	100	100	100	100	100	100	100	100
<b>Viability</b>	97	98	96	99	98	97	99	98
<b>Weaning [%]</b>	100	100	99	99	99	100	99	100
<b>No. of pups born alive/No. of pups born dead</b>	316/0	305/1	309/4	309/4	297/5	310/6	329/0	297/6
<b>No. of pups born (Mean no. / female)</b>	316 (12.2)	306 (13.3)	313 (13.0)	313 (13.6)	302 (13.1)	316 (13.2)	329 (13.2)	303 (13.2)

**Original DAR Conclusion:**

- Changes in food consumption and bodyweight were observed in the parental animals of the highest dose group. Therefore, the NOAEL for parental toxicity is established at 50 mg/kg bw/day.
- Since no developmental effects were observed, the NOAEL for developmental toxicity is established at  $\geq 500$  mg/kg bw/day.
- Since no effects on fertility were observed, the NOAEL for reproductive effects is established at  $\geq 500$  mg/kg bw/day.

**RMS 2018:** The original conclusion of this study is partly supported by the RMS. The RMS agrees with original NOAELs derived from this study. The NOAEL for maternal toxicity is established at 1000 ppm (50 mg/kg bw/day) based on the significant decrease in bodyweight in F1 males of the top dose group. In parental animals, the effect on bodyweight and bodyweight gain was not revealed. The NOAEL for developmental and reproductive toxicity is set at  $\geq 10000$  ppm (500 mg/kg bw/day).

**Oral (gavage) rat two-generation reproductive toxicity study**

Reference	<b>Oral (gavage) rat two-generation reproductive toxicity study, [REDACTED]</b> 1994; Report No. JSA/2/94
Guideline	The study was conducted according to OECD TG 416
Deviations	Yes
GLP	Yes
Acceptability	Yes
Previous evaluation	Yes, study already peer-reviewed in original DAR

**RMS comment 2018:** The study has been checked for compliance. The following deviations from OECD guideline 416 (adopted on 22nd January 2001) were found:

- 1) In general, the addition of the fourth test group is recommended if large intervals (6-10fold) between dosages are used. In dietary studies the dose interval should not exceed 3fold.
- 2) Sperm parameters, i.e. total cauda epididymal sperm number, percent progressively motile sperm, percent morphologically normal sperm, and percent of sperm with each identified abnormality were not evaluated
- 3) Quantitative evaluation of primordial follicles of F1 females was not performed
- 4) The following organs: uterus, prostate, epididymides, seminal vesicles with coagulating glands, spleen, thyroid and adrenal glands, and target organs (i.e. lungs) of all P and F1 parental animals were not weighed at the time of termination
- 5) Brain, spleen, and thymus of randomly selected F1 and F2 pups of each sex and litter were not weighed

#### Material and method:

Four groups of 30 male and 30 female rats of the Sprague Dawley derived OFA(SD)IOPS-Caw strain (source: [REDACTED]) were dosed for ten weeks and were then paired (1M: 1F) for mating. Dosing continued for the males during mating and for a further three weeks until day before necropsy, and for the females during mating, pregnancy and lactation. The treated animals (groups 2, 3 and 4, respectively) were dosed orally by gavage with solutions of daminozide (purity >99%) at concentrations of 60, 360 or 1200 mg/kg/day. The control group (group 1) received the vehicle (purified water) alone. Pregnant females were allowed to litter and rear their offspring (F1 generation) to weaning on day 21 post-partum.

Twenty-five male and twenty-five female F1 pups were selected from each group for rearing in the second generation. These animals were dosed with solutions of daminozide at the same concentrations as their parents from day 25 post-partum. At approximately 17 weeks of age the animals were paired (1M:1F) within dose groups for mating. Dosing continued for the males during mating and for a further three weeks until the day before necropsy, and for the females during mating, pregnancy and lactation. Pregnant females were allowed to litter and to rear their offspring (F2 generation) to weaning on day 21 post-partum.

Animals were observed twice daily for morbidity or mortality and once daily for changes in clinical condition. Individual bodyweights and food consumption of F0 animals were recorded at weekly intervals throughout the study commencing on day -7. Bodyweights for the selected F1 generation were recorded daily from day 24 post-partum until the group sizes were reduced to 25, and then at weekly intervals. Additionally, for pregnant females individual bodyweights were recorded on days 0, 4, 7, 11, 14 and 20 of pregnancy and, for lactating females on days 1, 4, 7, 14 and 21 post-partum. Food consumption was not recorded during late lactation (days 14 to 21 post-partum) as the pups also start eating the diet.

Vaginal smears were taken daily until sperm was found in the smear; the stage of the oestrus cycle or the presence of sperm was recorded. The day on which sperm were observed was designated day 0 of pregnancy.

The following indices were calculated and reported for all groups in each generation: copulation, fertility, gestation, lactation, viability, live birth, sex ratio, cumulative survival. The litter size and pup sexes were recorded as soon as possible after birth and daily thereafter until day 21 post-partum. Clinical signs were recorded daily and any abnormalities recorded. Individual pup bodyweights were recorded as soon as possible after birth and on days 2, 4, 7, 14 and 21 post-partum.

Histopathological examination was performed on the following organs/tissues of F0 and F1 animals: brain, kidneys, liver, lungs, pituitary, ovaries, vagina, uterus, prostate, testes, epididymides, seminal vesicles, coagulating gland. The same organs of pups were fixed and stored.

## Results:

In the F0 and F1 generation in group 4 (1200 mg/kg/day) loose faeces, perianal fur staining, and excess salivation were observed. No signs were noted in the animals of lower dose levels. Water consumption of the F0 generation males measured over days 86 to 91 of the dosing period was markedly increased in group 4 (1200 mg/kg/day) compared with the controls and the difference achieved statistical difference. Water consumption of the males in groups 2 and 3 (60 and 360 mg/kg/day) was similar to that of the controls.

**Table 6.6.1/02-1 Group mean water consumption (g/rat/day) in F0 males**

<b>Group</b> <b>Days</b>	<b>1</b> <b>(control)</b>	<b>2</b> <b>(60mg/kg/day)</b>	<b>3</b> <b>(360mg/kg/day)</b>	<b>4</b> <b>(1200mg/kg/day)</b>
<b>86-91</b>	<b>51.5</b>	<b>46.9</b>	<b>50.7</b>	<b>658.4**</b>

\*\* Statistically significant ( $p < 0.01$ ).

No treatment-related differences in organ weights, histopathology, number, sex, bodyweights, and development of the pups were observed.

The clinical observations made for the pups in all groups in both generations during lactation were of a type and frequency that are generally observed in developing pups of this strain of rat. A low incidence of pups died or was missing (presumed cannibalised). Only a low incidence of pups showed major abnormalities considered to be congenital in origin. At necropsy of the F1 generation pups that died, one control exhibited exencephaly and two in group 4 (1200 mg/kg/day) showed a shortened gut (possibly associated with maternal cannibalisation of an

umbilical hernia). In the F2 generation, one pup in group 2 (60 mg/kg/day) exhibited hydrocephaly and one control and one in group 3 (360 mg/kg/day) showed a shortened gut. Amongst the weaned pups that were subject to necropsy, one F1 generation pup in group 3 showed asymmetrically sized testes and in the F2 generation, one control and two in group 3 had diaphragmatic hernias. As these abnormalities were diverse in nature and occurred at a low incidence, they were considered to be unrelated to parental treatment with daminozide. Other abnormalities were common findings in rat pups during development.

F0 animals: There were no consistent treatment-related effects on the group mean bodyweights or bodyweight gains for either the males or the females.

Group 4 (1200 mg/kg/day) male bodyweight gain between weeks 6 and 7 did show a statistically significant reduction in comparison with the controls. However, in view of the isolated nature of this observation and, since with this exception, the male bodyweight gains observed throughout the treatment period in this group were essentially similar to those of the controls, this difference could not be related to treatment.

**Table 6.6.1/02-2 Group mean bodyweight gains (g) in F0 males**

<b>Group</b>  <b>weeks</b>	<b>1</b> <b>(control)</b>	<b>2</b> <b>(60mg/kg/day)</b>	<b>3</b> <b>(360mg/kg/day)</b>	<b>4</b> <b>(1200mg/kg/day)</b>
to 1	74	73	72	74
1 to 2	57	54	57	55
2 to 3	47	45	49	47
3 to 4	36	35	40	34
4 to 5	35	34	36	34
5 to 6	27	27	25	26
6 to 7	20	18	19	15*
7 to 8	22	22	20	19
8 to 9	19	19	23	21
9 to 10	13	14	12	12
10 to 11	14	13	12	12
11 to 12	12	9	11	7 (29)
12 to 13	10	12	13	16 (29)
13 to 14	13	11	10	14
14 to 15	13	13	15	14
15 to 16	15	16	13	14
16 to 17	12	9	12	11

\* Statistically significant ( $p < 0.05$ )

Number of animals in each group was 30,

( )=Number of animals, were different from original

**Table 6.6.1/02-3 Food consumption (g/rat/day) in F0 males**

<b>Dose</b> <b>Week</b>	<b>0</b> <b>(mg/kg/day)</b>	<b>60</b> <b>(mg/kg/day)</b>	<b>360</b> <b>(mg/kg/day)</b>	<b>1200</b> <b>(mg/kg/day)</b>
1	30.0	30.1	29.5	30.4
2	31.7	31.2	31.0	30.9
3	32.9	32.2	33.4	33.3
4	32.9	32.4	33.6	33.5
5	33.9	33.2	33.7	34.0
6	32.8	32.3	33.1	33.1
7	33.1	32.5	33.2	32.8
8	34.8	34.1	34.1	34.3
9	33.8	33.5	33.7	32.9
10	32.5	32.1	32.1	30.5
11	-	-	-	-
12	32.5	31.5	32.0	32.7
13	34.3	33.7	33.7	34.4
14	34.3	34.3	34.3	35.0
15	33.6	32.9	33.4	33.7

Statistical reductions in female bodyweight gain were observed in groups 2 and 4 between weeks 1 and 2, and for group 2 between weeks 6 and 7. In view of the absence of any dose-relationship, these differences were considered to be unrelated to treatment with daminozide.

During lactation, females in group 4 exhibited a significant reduction in bodyweight gain between day 1 and 4, and an increase in bodyweight gain between day 7 and 14 in comparison with the controls. As there was no consistent effect upon female bodyweight gain this was considered unlikely to be related to daminozide treatment. There was no significant effect of treatment on the food consumption.

**Table 6.6.1/02-4 Group mean bodyweight gains (g) in F0 females**

<b>Group</b> <b>weeks</b>	<b>1</b> <b>(control)</b>	<b>2</b> <b>(60mg/kg/day)</b>	<b>3</b> <b>(360mg/kg/day)</b>	<b>4</b> <b>(1200mg/kg/day)</b>
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to 1	44	44	44	44
1 to 2	33	<b>27**</b>	29	<b>25**</b>
2 to 3	28	25	28	26
3 to 4	20	19	20	21
4 to 5	19	18	18	18
5 to 6	13	12	13	12
6 to 7	10	<b>5*</b>	7	6
7 to 8	8	9	8	9
8 to 9	9	9	7	8
9 to 10	4	4	6	6
10 to 11	8	9	10	6
<b>Days of pregnancy</b>				
0 to 4	19	17	19	16
4 to 7	9	10	12	10
7 to 11	18	16	16	17
11 to 14	17	16	20	16
14 to 20	85	91	91	87
<b>Lactation (days post partum)</b>				
1 to 4	22	22	21	<b>17*</b>
4 to 7	8	8	9	5
7 to 14	18	18	21	<b>26*</b>
14 to 21	-22	-20	-26	-26

\* Statistically significant ( $p < 0.05$ )

\*\* Statistically significant ( $p < 0.01$ )

Table 6.6.1/02-5 Food consumption (g/rat/day) in F0 females

<b>Dose</b> <b>Week</b>	<b>0</b> <b>(mg/kg/day)</b>	<b>60</b> <b>(mg/kg/day)</b>	<b>360</b> <b>(mg/kg/day)</b>	<b>1200</b> <b>(mg/kg/day)</b>
1	23.6	22.3	22.3	21.5
2	25.4	24.6	24.6	23.9
3	26.2	25.6	25.6	25.5
4	26.7	25.6	26.4	26.5
5	26.4	25.1	24.9	24.7
6	25.7	24.4	24.6	25.3
7	25.5	24.8	24.4	24.9
8	27.4	26.7	27.1	26.2
9	25.0	24.1	24.2	23.6
10	24.3	23.9	24.0	23.4
<b>Days of pregnancy</b>				
0 to 7	26.5	24.9	25.6	24.6
7 to 14	29.5	27.9	28.1	27.1
14 to 20	33.1	32.2	33.0	31.9
<b>Days post partum</b>				
1 to 4	43.5	47.7	44.0	43.5
4 to 7	51.9	50.8	53.8	51.6
7 to 14	67.7	68.2	68.4	68.1

There was no effect of treatment with daminozide in any group in either generation on the oestrous cycle pattern, the pre-coital interval, the number of males and females mating or their fertility.

F1 animals: There were no consistent effects of treatment on the male bodyweights or bodyweight gains. Inter-group differences in female bodyweights were observed at the start of the F1 generation dosing and by week 4 of age, these differences from the control group in mean bodyweight were significant ( $p < 0.05 - 0.01$ ) in both groups 2 and 4. The differences from the control group remained statistically significant throughout most of the F1 generation in group 2 and occasionally in group 4.

Table 6.6.1/02-6 Bodyweights (g) in F1 females

<b>Dose</b> <b>Week</b>	<b>0</b> <b>(mg/kg/day)</b>	<b>60</b> <b>(mg/kg/day)</b>	<b>360</b> <b>(mg/kg/day)</b>	<b>1200</b> <b>(mg/kg/day)</b>
<b>4</b>	116	106**	112	109*
<b>5</b>	154	141**	147	145*
<b>6</b>	179	166**	172	170
<b>7</b>	215	199**	205	206
<b>8</b>	248	229**	236	235
<b>9</b>	270	249**	257	257
<b>10</b>	290	269*	278	276
<b>11</b>	302	283	291	287
<b>12</b>	312	294	300	296
<b>13</b>	322	300*	306	303
<b>14</b>	330	310*	314	308*
<b>Gestation (day of pregnancy)</b>				
<b>0</b>	331	304**	313	310*
<b>4</b>	351	321**	331	328*
<b>7</b>	363	331**	344	340*
<b>11</b>	382	348**	362	358*
<b>14</b>	400	364**	378	377
<b>20</b>	479	439**	456	464
<b>Lactation (day post partum)</b>				
<b>1</b>	364	325**	352	365
<b>4</b>	388	353**	372	384
<b>7</b>	398	364**	386	396
<b>14</b>	410	375**	395	402
<b>21</b>	392	357**	369*	381

\* Statistically significant ( $p < 0.05$ )\*\* Statistically significant ( $p < 0.01$ )

However, the bodyweight gains were generally similar amongst all groups and, as there was no dose-relationship, these differences in bodyweight were considered to be fortuitous and unrelated to treatment.

**Table 6.6.1/02-7 Bodyweights gain (g) in F1 females**

<b>Dose Week</b>	<b>0 (mg/kg/day)</b>	<b>60 (mg/kg/day)</b>	<b>1000 (mg/kg/day)</b>	<b>10000 (mg/kg/day)</b>
<b>To 4</b>	36	29*	31	33
<b>4 to 5</b>	38	35	35	36
<b>5 to 6</b>	25	24	25	25
<b>6 to 7</b>	36	34	33	35
<b>7 to 8</b>	33	30	31	30
<b>8 to 9</b>	21	20	21	22
<b>9 to 10</b>	20	19	21	19
<b>10 to 11</b>	12	15	12	12
<b>11 to 12</b>	10	10	9	9
<b>12 to 13</b>	10	6	6	7
<b>13 to 14</b>	8	10	8	5
<b>Days of pregnancy</b>				
<b>0 to 4</b>	20	17	18	18
<b>4 to 7</b>	12	11	13	12
<b>7 to 11</b>	20	17	18	18
<b>11 to 14</b>	18	16	16	19
<b>14 to 20</b>	79	75	79	87
<b>Days post partum</b>				
<b>1 to 4</b>	24	27	20	19
<b>4 to 7</b>	9	11	14	12
<b>7 to 14</b>	12	11	9	6
<b>14 to 21</b>	-17	-18	-26	-21

There were no consistent effects of treatment on the mean male and female food consumption. The food consumption in the treated groups was generally similar to that of the control group. A lower mean food consumption was observed for group 2 (60 mg/kg/day) during pregnancy. However, as there were no similar reductions in groups 3 and 4 (360 and 1200 mg/kg/day), the lower food consumption in group 2 was considered to be unrelated to treatment with daminozide.

**Table 6.6.1/02-8 Food consumption (g/rat/day) in F1 females**

<b>Dose</b> <b>Week</b>	<b>0</b> <b>(mg/kg/day)</b>	<b>60</b> <b>(mg/kg/day)</b>	<b>1000</b> <b>(mg/kg/day)</b>	<b>10000</b> <b>(mg/kg/day)</b>
<b>5</b>	22.0	20.6	21.1	20.7
<b>6</b>	23.9	22.7	23.0	23.4
<b>7</b>	25.0	24.0	24.1	22.9
<b>8</b>	25.9	24.3	24.3	24.2
<b>9</b>	26.6	24.2	25.3	24.8
<b>10</b>	25.2	24.1	24.6	24.0
<b>11</b>	25.3	24.2	24.0	23.6
<b>12</b>	24.0	23.2	22.8	22.7
<b>13</b>	23.5	22.3	24.1	22.5
<b>Days of pregnancy</b>				
<b>0 to 7</b>	26.5	23.7**	24.7	24.8
<b>7 to 14</b>	29.0	26.0**	27.3	27.7
<b>14 to 20</b>	31.7	28.6*	30.4	32.0
<b>Days post partum</b>				
<b>1 to 7</b>	42.5	43.4	42.8	45.4
<b>7 to 14</b>	64.6	66.5	64.1	65.1

\* Statistically significant ( $p < 0.05$ )

\*\* Statistically significant ( $p < 0.01$ )

Eight of the mated females (five in group 2 and one in each of the control group and groups 3 and 4) were found not to be pregnant. Although the incidence of non-pregnant females in group 2 was unusually high, in the absence

of similar findings in groups 3 and 4, this was considered to be co-incidental and unrelated to treatment with daminozide.

**Original DAR/applicant conclusion:**

- The NOAEL for parental toxicity is established at 360 mg/kg bw/day based on the clinical signs observed in the F0 and F1 animals and the increased water consumption in the F1 males.
- The decrease in bodyweight of the F1 pups was only observed at 14 and 21 days post-partum. Moreover, this decrease in bodyweight was not statistically significant and was not observed in the F2 pups. Therefore, the NOAEL for developmental toxicity is established at  $\geq 1200$  mg/kg bw/day.
- Since no effects on reproductive performance were observed the NOAEL for reproductive effects is  $\geq 1200$  mg/kg bw/day.

**RMS 2018:** The RMS agrees with original NOAELs derived from this study. The NOAEL for the developmental and reproductive toxicity is set at 1200 mg/kg bw/day (top dose). The NOAEL for the parental toxicity is proposed to be established at 360 mg/kg bw/day based on loose faeces, perianal fur staining, and excess post-dose salivation observed in both F0 and F1 animals of the top dose.

**B 6.6.2 Teratogenicity studies**

**Teratologic assessment of maleic hydrazide and daminozide, and formulations of ethoxyquin, thiabendazole and Haled in rats**

Reference	<b>Teratologic assessment of maleic hydrazide and daminozide, and formulations of ethoxyquin, thiabendazole and Haled in rats, Khera K.S., Whalen C., Trivett G., Angers G., 1979; Report Environ J., Sci. Health; 1979 Vol. B14 (6), pp. 563-577</b>
Guideline	The study was not conducted according to any guideline or in compliance with GLP.
Deviations	-
GLP	No
Acceptability	No, supplementary
Previous evaluation	Yes, study already peer-reviewed in original DAR

**Material and method:**

Groups of twenty mated female rats were given daily oral doses of daminozide (purity >99%) of 0, 300, 600, and 1000 mg/kg bw/day, suspended in distilled water, on days 6-15 of gestation, by gavage. On day 22 of gestation, all rats were killed and subjected caesarean section.

**Results:**

According to the authors no signs of toxicity were observed and pregnancy rates, numbers of corpora lutea, implantation and resorption rates, foetal deaths, sex ratio, foetal weights, number of live foetuses, the incidence of foetal anomalies and skeletal malformations were not significantly different from control values. The NOAELs for maternal and developmental toxicity is established at 1000 mg/kg bw/day.

**RMS Note:** This study is considered supplementary for the overall evaluation since not all observations were reported and no individual data were presented.

#### **Daminozide oral (gavage) rat developmental toxicity (teratogenicity)**

Reference	<b>Daminozide oral (gavage) rat developmental toxicity (teratogenicity),</b> [REDACTED] 1993; Report No. JSA/3-4/92
Guideline	The study was conducted according to OECD TG 414
Deviations	Yes
GLP	Yes
Acceptability	Yes
Previous evaluation	Yes, study already peer-reviewed in original DAR

**RMS comment 2018:** The study has been checked for compliance. The following deviations from OECD guideline 414 (adopted on 22nd January 2001) were found:

- 1) The relative humidity in the animal room was 48 – 74%, however, it should not exceed 70 %

#### **Material and method:**

This study was designed to investigate the effects of the test article (daminozide) on the embryonic and foetal development of the rat when administered during the period of organogenesis.

Groups of twenty-five timed-mated female Sprague-Dawley derived rats of the OFA(SD)IOPS-Caw strain ([REDACTED]) were dosed once daily by the oral route, between days 6 and 15 of pregnancy, with solutions of daminozide (purity: >99%) at dose levels of 0 (vehicle control), 150, 750 or 1500 mg/kg/day (groups 1 to 4, respectively). Day 0 of pregnancy was the day of observation of a sperm positive smear.

The females were observed twice daily for morbidity or mortality and once daily for changes in clinical condition. Bodyweights were recorded on days 0, 6 to 15, inclusive, and 20 of pregnancy. Food consumption was recorded daily during pregnancy and the consumption over days 0 to 6, 6 to 9, 9 to 12, 12 to 15, 15 to 18 and 18 to 20 of pregnancy was reported.

The females were killed on day 20 of pregnancy and necropsy was performed. Non-gravid uteri and unilaterally implanted uteri were stained to determine if any very early implantation sites were present. The following data were recorded: weight of the gravid uterus, number of corpora lutea, number (and distribution) of implantation

sites. The implantations were classified as early resorptions, late resorptions, dead fetuses or live fetuses and were numbered separately for the right and left horns.

The live fetuses were removed from *utero*, examined for external abnormalities, weighed and then sexed. One half of the fetuses were examined for visceral abnormalities. The bones in each fetus were identified for normality with respect to shape, size and the extent of ossification. Serial sections of the head, heart, kidneys, and inspection of all major organs as well as blood vessels were performed. Structural congenital abnormalities that impair or potentially impair the survival or fitness of the fetus were regarded as major abnormalities. Other defects were classified as minor abnormalities. Commonly observed variations in the degree of ossification from that expected of a day 20 gestation fetus, additional ribs at the thoraco-lumbar border, common variations in the extent of renal pelvic cavitation and ureter dilation and thin areas in the midline of the secondary palate were recorded as variants.

### Results:

Observations: There were no treatment-related changes in clinical condition. One female in each group showed alopecia, two females in the 750 mg/kg/day group showed periorbital fur staining and one female in the same group was observed with noisy respiration one day; after biting through the dosing catheter. All these findings were considered to be incidental and unrelated to treatment.

Bodyweight and food consumption: There was a dose-related retardation of bodyweight gain at the onset of dosing. The difference from the controls in bodyweight gain between days 6 and 9 of pregnancy was significant ( $p < 0.001$ ) in the groups treated at 750 and 1500 mg/kg/day. There was also a significant ( $p < 0.05$ ) difference in bodyweight gain of the group treated at 1500 mg/kg/day in comparison with the control group during the entire dosing period as a consequence of the initial effect. These differences were considered to be an effect of treatment.

**Table 6.6.2/02-1 Maternal bodyweights gain (g)**

<b>Dose</b> <b>Days of pregnancy</b>	<b>0 (mg/kg/day)</b>	<b>150 (mg/kg/day)</b>	<b>750 (mg/kg/day)</b>	<b>1500 (mg/kg/day)</b>
0 to 6	29.0	30.8	30.1	29.9
6 to 7	6.5	4.9	2.0***	2.1***
6 to 8	10.3	9.1	7.1**	5.8***
6 to 9	17.2	15.8	11.9***	11.1***
9 to 12	20.7	20.5	18.3	17.1
12 to 15	25.5	29.2	29.3	27.6
15 to 20	83.3	83.2	84.6	87.8
6 to 15	63.3	65.5	59.5	55.8*



<b>No. of animals in group</b>	23	25	24	25
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\* Statistically significant ( $p < 0.05$ )

\*\* Statistically significant ( $p < 0.01$ )

\*\*\* Statistically significant ( $p < 0.001$ )

There was a dose-related reduction in food consumption in the groups treated at 750 and 1500 mg/kg/day at the onset of dosing. This was considered to be an effect of treatment. The difference from the control group between days 6 and 12 of pregnancy was significant ( $p < 0.05$ ) in the group treated at 1500 mg/kg/day, but did not achieve statistical significance at 750 mg/kg/day. After day 12 of pregnancy, mean food consumption in these two treated groups was similar to or slightly greater than in the control group. Mean food consumption in the group treated at 150 mg/kg/day was similar to the control group throughout pregnancy.

**Table 6.6.2/02-2 Food consumption (g/rat/day)**

<b>Dose</b> <b>Days of pregnancy</b>	<b>0 (mg/kg/day)</b>	<b>150 (mg/kg/day)</b>	<b>750 (mg/kg/day)</b>	<b>1500 (mg/kg/day)</b>
0 to 6	24.8	25.4	25.0	24.8
6 to 9	27.6	27.8	26.0	25.4*
9 to 12	29.5	30.2	28.2	27.5*
12 to 15	31.8	32.2	31.8	31.6
15 to 18	32.4	33.8	33.8	33.2
18 to 20	30.8	31.8	31.4	32.2
<b>No. of animals in group</b>	23	25	24	25

\* Statistically significant ( $p < 0.05$ )

Pregnancy data: All females were pregnant. However, for one control female and for one female treated at 750 mg/kg/day, all the implantations were dead. The presence of implantations was confirmed by uterine staining. There were 23, 25, 24 and 25 litters of live foetuses in the control group and groups treated at 150, 750 and 1500 mg/kg/day, respectively. Mean numbers of corpora lutea and implantations, and pre-implantation losses, were within expected ranges in all groups. The mean numbers of live foetuses were slightly greater, and post-implantation losses were slightly lower, in the treated groups than in the control group. However, these differences were not adverse effects of treatment, since the treated values were superior to those of the control group.

Foetal data: Mean foetal weights were marginally lower than in the control group in the groups treated at 750 and 1500 mg/kg/day. Since the differences did not achieve statistical significance and since the litter sizes in these two

treatment groups were slightly larger, this was considered not to be an effect of treatment. Mean foetal weight in the group treated at 150 mg/kg/day was marginally greater than in the control group.

**Table 6.6.2/02-3Pregnancy and foetal data**

<b>Dose</b> <b>Parameter</b>	<b>0 (mg/kg/day)</b>	<b>150 (mg/kg/day)</b>	<b>750 (mg/kg/day)</b>	<b>1500 (mg/kg/day)</b>
No. of corpora lutea	16.5	17.3	17.6	17.9
No. of implantation	15.2	15.6	15.9	16.5
No. of live foetus	14.0	14.8	14.8	15.5
Pre- /Postimplantation loss (%)	9.1 / 11.3	9.4 / 4.5	8.7 / 7.5	6.9 / 6.3
Sex ratio	49 : 51	52 : 48	46 : 54	49 : 51
No. of pregnant	24	25	25	25
Foetal weight (g)	4.09	4.13	3.97	3.97

Foetal abnormalities:

*Major abnormalities*

There was a low incidence of foetuses with major abnormalities. Only two foetuses were observed with major abnormalities (one with cleft palate and one with umbilical hernia) and both were in the group treated at the lowest dosage (150 mg/kg/day). Since no major abnormalities were observed at the higher dose levels, the abnormalities at the low dose level were considered to be spontaneous and unrelated to treatment.

**Table 6.6.2/02-4 Foetal examination data**

<b>Dose</b> <b>Parameter</b>	<b>0 (mg/kg/day)</b>	<b>150 (mg/kg/day)</b>	<b>750 (mg/kg/day)</b>	<b>1500 (mg/kg/day)</b>
<b>External and visceral examination</b>				
No. of foetuses (litters)	337 (23)	371 (25)	369 (24)	387 (25)
No. with minor abnormalities only/ <b>Mean %</b>	1 (1) / <b>0.3</b>	3 (3) / <b>0.9</b>	4 (4) / <b>1.0</b>	5 (4) / <b>1.3</b>

No. with major abnormalities/ <b>Mean %</b>	0 (0) / <b>0.0</b>	2 (6) / <b>0.6</b>	0 (0) / <b>0.0</b>	0 (0) / <b>0.0</b>
<b>Skeletal examination</b>				
No. of foetuses (litters)	169 (23)	185 (25)	185 (24)	194 (25)
No. with minor abnormalities only/ <b>Mean %</b>	4 (4) / <b>2.4</b>	13 (10) / <b>7.4</b>	13 (11)* / <b>6.6</b>	7 (6) / <b>3.5</b>
No. with major abnormalities/ <b>Mean %</b>	0 (0) / <b>0.0</b>	1 (1) / <b>0.7</b>	0 (0) / <b>0.0</b>	0 (0) / <b>0.0</b>
<b>Combined examination</b>				
No. with any major abnormalities/ <b>Mean, %</b>	0 (0) / <b>0.0</b>	2 (2) / <b>0.6</b>	0 (0) / <b>0.0</b>	0 (0) / <b>0.0</b>

\* Statistically significant ( $p < 0.05$ )

#### *Minor abnormalities*

The overall incidence of foetuses with minor external/visceral abnormalities was also low, but incidences were slightly higher in treated groups than in the control group. However, since the control group was unusually low, since differences were not statistically significant and since the treated group values were all at the lower end of the background range, this was considered fortuitous and unrelated to treatment. All but two of the minor abnormalities were absence of the innominate artery. This is one of the most common minor abnormalities of this strain of rat.

For skeletal examination, the incidences of foetuses with minor abnormalities were similar to the control group in the group treated at 1500 mg/kg/day and slightly higher in the groups treated at 150 and 750 mg/kg/day. The difference at 750 mg/kg/day achieved statistical significance. All the treated group values were within background range and the lack of a dose response precluded any association with treatment. There was no treatment-related increase in the incidence of any particular type of minor skeletal abnormal observed.

#### *Variants*

Three types of external/visceral variant were observed in this study. For increased renal pelvic cavitation and dilated ureter, the incidences in the treated groups were similar to or lower than in the control group and all values were within background range. The other type of external/visceral variant observed was an underdeveloped (thin) area in the mid-line of the secondary palate. This finding had not previously been observed for this strain of rat. It was classified as a variant as there was no structural abnormality of the palate but merely an area of transparency which was concluded to be a slightly lower condensation of mesenchyme in the region of palatal fusion. A concurrent developmental toxicity study of the same strain of rat showed similar findings in the foetuses, but a

slightly higher incidence. In concurrent littering studies, the same finding was not observed in the pups on day 4 post-partum or at weaning, which indicated that the finding was probably only a transient developmental delay. The slightly higher incidences in the group treated at 1500 mg/kg/day in comparison with the control group in this study was considered to be fortuitous and to be of no biological significance.

As usual for findings that occur at high frequencies, there was intergroup variation in the incidences of skeletal variants. Despite this variation, there was no dose-related increase in the incidences of any particular type of skeletal variant and all values were within background range; apart from the incidence of fetuses with one or more cervical centra ossified. This was slightly higher in the groups treated at 150 and 750 mg/kg/day than in the control group. However, this indicated that the fetuses in these groups were slightly better ossified and, since the incidence of this finding in the group treated at 1500 mg/kg/day was similar to the control group, was considered to be fortuitous.

**Table 6.6.2/02-5 Examination of foetus**

<b>Dose</b>	<b>0 (mg/kg/day)</b>	<b>150 (mg/kg/day)</b>	<b>750 (mg/kg/day)</b>	<b>1500 (mg/kg/day)</b>
<b>Findings</b>				
<b>External and visceral examination</b>				
Cleft palate ( <b>major</b> )	0 (0.0)	1 (0.3)	0 (0.0)	0 (0.0)
Palate secondary: undeveloped areas in midline ( <b>variant</b> )	2 (0.6)	4 (1.1)	2 (0.5)	8 (2.0)
Innominate artery: absent. Right common carotid & right subclavian arteries arising directly from aortic arch ( <b>minor</b> )	0 (0.0)	3 (0.9)	4 (1.0)	4 (1.0)
Umbilical hernia ( <b>major</b> )	0 (0.0)	1 (0.3)	0 (0.0)	0 (0.0)
Abdominal haemorrhage ( <b>minor</b> )	1 (0.3)	0 (0.0)	0 (0.0)	1 (0.3)
Kidneys-uni- or bilateral: increased pelvic cavitation ( <b>variant</b> )	32 (10.1)	<b>13 (3.3)**</b>	42 (11.0)	27 (7.0)
Ureter-uni- or bilateral: dilated ( <b>variant</b> )	52 (15.4)	<b>27 (7.4)*</b>	58 (15.1)	57 (14.8)
<b>Skeletal examination: Skull</b>				
Cleft palate ( <b>major</b> )	0 (0.0)	1 (0.7)	0 (0.0)	0 (0.0)

Hyoid: not ossified (variant)	2 (1.0)	1 (0.7)	5 (2.3)	4 (1.9)
Occipital: retarded ossification (variant)	1 (0.5)	5 (3.1)	5 (2.6)	3 (1.3)
Interparietal: retarded ossification (variant)	8 (4.7)	15 (8.6)	16 (8.2)	17 (8.7)
Parietals: retarded ossification (variant)	2 (1.1)	3 (1.9)	10 (4.8)	3 (1.6)
Temporals: retarded ossification (variant)	0 (0.0)	1 (0.7)	1 (0.5)	1 (0.7)

( )-mean, %

\* Statistically significant ( $p < 0.05$ )

\*\* Statistically significant ( $p < 0.01$ )

#### Original DAR/applicant conclusions

- The NOAEL for maternal toxicity was established at 150 mg/kg bw/day, based on the decreased bodyweight and food consumption in the two highest dose groups.
- Since the litter sizes in the two highest dose groups were slightly larger than in the control group, the decreased foetal bodyweight was not considered an effect of treatment. Therefore, the NOAEL for developmental toxicity is established at  $\geq 1500$  mg/kg bw/day.
- No treatment-related teratogenic effects were observed.

**RMS 2018:** The RMS agrees with the setting of maternal toxicity at 150 mg/kg bw/day based on the reduced bodyweight gain in 750 and 1500 mg/kg bw/day dose groups. The NOAEL for developmental toxicity is supported to be established at  $\geq 1500$  mg/kg bw/day. No treatment-related teratogenic effects were observed.

#### Oral dosage-range developmental toxicity study of daminozide technical in rabbits

Reference	<b>Oral dosage-range developmental toxicity study of daminozide technical in rabbits, [REDACTED], 2006a; Report No. TZE00003</b>
Guideline	The study was conducted according to OECD TG 414 and OPPTS 870.3700
Deviations	-
GLP	Yes
Acceptability	Supplementary
Previous evaluation	No

**Material and method:**

In a preliminary study designed to investigate doses for subsequent developmental toxicity testing, three groups of five presumed pregnant rabbits (New Zealand White [Hra: (NZW) SPF]; source: [REDACTED]) were administered daily doses of 300, 500 and 700 mg/kg/day daminozide technical (purity: 100%) during days 7-28 of presumed gestation.

Five virgin female rabbits were added to extend the study and administered 1000 mg/kg/day daminozide technical daily for 14 days (sacrificed on day 15).

Checks for viability were made at least twice daily, clinical observations were recorded at least once during the pre-dosage period and daily during dosage and post-dosage periods. Bodyweights and feed consumption were recorded daily during dosage and post-dosage periods. All surviving rabbits were sacrificed on day 29 of presumed gestation and a gross necropsy of the thoracic, abdominal and pelvic viscera performed. The number and distribution of corpora lutea, implantation sites and uterine contents were examined. Foetuses were weighed and examined for gross external alterations and sex.

Mortality: One 500 mg/kg/day animal was sacrificed due to its moribund condition and one 700 mg/kg/day animal was found dead. The sacrifice of the 500 mg/kg/day animal was not considered to be related to the test material because the animal had evidence of trauma produced by the dosing procedure. The death of the 700 mg/kg/day animal was considered to be unexplained. All five animals in study extension survived 14 days of consecutive dosage.

Clinical and necropsy observations: There were minor clinical observations that appear to be related to the test material administration in the 500 and 700 mg/kg/day groups. Clinical observations in both groups included soft or liquid faeces. Additional observations included scant faeces and perivaginal substance in the 500 mg/kg/day group and ungroomed coat in the 700 mg/kg/day group. No gross necropsy findings related to the test material were observed. One 500 mg/kg/day animal had a mottled tan and black gallbladder and two black areas on the pancreas. These observations were not considered related to the test material because the incidence was not dosage-dependent.

Soft or liquid faeces and ungroomed coat occurred in all animals of study extension. No other adverse clinical or any necropsy observations occurred.

Bodyweight and food consumption: Bodyweights, bodyweight gains, and food consumption were comparable among the three dosage groups. No toxicologically important dosage-dependent differences occurred during the dosage period.

In study extension, an average bodyweight loss of 220 g occurred during the dosage period for the five animals. The average weight loss during the first week of dosage was greater than the second week (170 g vs 60 g). Reduction in food consumption of approximately 50% was observed.

**Table 6.6.2/03-1 Bodyweight changes (kg) and food consumption (g/day) in high dose group (1000 mg/kg/day): mean±SD**

Days of study	Bodyweight changes	Food consumption
1 to 8	-0.17 ± 0.14	81.8 ± 40.4
8 to 15	-0.06 ± 0.13	69.9 ± 50.5
1 to 15	-0.22 ± 0.13	75.8 ± 40.8

Caesarean-sectioning and litter observations: Pregnancy occurred in five rabbits in all dosage groups. Caesarean-sectioning observations were based on 100%, 60% and 80% pregnant rabbits with one or more live foetuses in the 300, 500 and 700 mg/kg/day dosage groups, respectively (one animal in the 500 mg/kg/day group had a litter that consisted of all resorptions). The litter averages for corpora lutea, implantations, litter sizes, live foetuses, early and late resorptions, foetal bodyweights, percent resorbed conceptuses and percent live male foetuses were comparable in the 300 and 700 mg/kg/day groups. There was an unexplained decrease in the litter averages for corpora lutea, implantations, litter size, live foetuses and percent live male foetuses in the 500 mg/kg/day group.

**Table 6.6.2/03-2 Caesarean-sectioning and litter observations**

<b>Dose</b>	<b>300 (mg/kg/day)</b>	<b>500 (mg/kg/day)</b>	<b>700 (mg/kg/day)</b>
<b>Findings</b>			
Pregnant	5 (100%)	5 (100%)	5 (100%)
Found dead	0 (0 %)	0 (0%)	1 (20%)
Moribund sacrificed	0 (0%)	1 (20%)	0 (0%)
No. of caesarean-sectioned animal	5	4	4
Corpora lutea	10.0 ± 1.9	6.5 ± 1.9	10.5 ± 1.0
Implantation	9.6 ± 1.3	6.0 ± 1.2	9.0 ± 1.4
Litter size	9.2 ± 1.8	4.2 ± 3.0	8.8 ± 1.7
Resorption	0.4 ± 0.5	1.8 ± 3.5	0.2 ± 0.5
Does with any resorption	2 (40%)	1 (25%)	1 (25%)
Does with all conceptuses resorbed	0 (0%)	1 (25%)	0 (0%)
Does with viable	5 (100%)	4 (100%)	4 (100%)

foetuses			
Live foetuses	46	17	35
Live male foetuses	22	10	14
Live foetal bodyweight (g)	39.64 ± 4.25	47.77 ± 2.72	41.33 ± 2.58

There was one foetus with flexion in the fore and/or hindlimbs in the 700 mg/kg/day group and one incidence of gastroschisis in the 300 mg/kg/day group. These findings were not considered to be treatment-related because the gastroschisis was not dose-related and the limb flexion is a common finding in this strain of rabbit.

**Table 6.6.2/03-3 Foetal gross external alterations**

<b>Dose</b>	<b>300(mg/kg/day)</b>	<b>500 (mg/kg/day)</b>	<b>700 (mg/kg/day)</b>
<b>Findings</b>			
Litter evaluated	5	3	4
Foetuses evaluated	46	17	35
Fore and hindlimbs: Flexed			
Litter incidence	0 (0%)	0 (0%)	1 (25%)
Foetal incidence	0 (0%)	0 (0%)	1 (2.8 %)
Body: Gastroschisis			
Litter incidence	1 (20%)	0 (0%)	0 (0%)
Foetal incidence	1 (2.2%)	0 (0%)	0 (0%)

#### **Conclusion:**

Based on the results from the main study, 700 mg/kg/day daminozide technical did not produce enough toxicity to be considered a high dose level for a developmental toxicity study in rabbits. Therefore, dose levels greater than 700 mg/kg/day would need to be evaluated to determine a maximum tolerated dose for a full developmental toxicity study.

Based on the results of the study extension, 1000 mg/kg/day was considered a maximum tolerated dose. This dose was not lethal but did produce adverse clinical observations, reductions in bodyweight and a reduction in food consumption of approximately 50%.

Based on the results of the main study and study extension, dosages of 0, 250, 500 and 1000 mg/kg/day were recommended for the full developmental toxicity study in rabbits.



**RMS 2018:** The purpose of this preliminary study was the setting of dose levels for the complete developmental study. Thus, this study represents the supplementary material.

#### **Oral Developmental Toxicity Study of Daminozide Technical in Rabbits**

Reference	<b>Oral Developmental Toxicity Study of Daminozide Technical in Rabbits,</b> ██████████ 2006b; Report No. TZE00002
Guideline	The study was conducted according to OECD TG 414 and OPPTS 870.3700.
Deviations	Yes
GLP	Yes
Acceptability	Yes
Previous evaluation	No

**RMS comment 2018:** The study has been checked for compliance. The following deviations from OECD guideline 414 (adopted on 22nd January 2001) were found:

- 1) At the end of the study only 15 and 8 pregnant females were alive in the 500 and 1000 mg/kg group, respectively. However, each test group should contain approximately 20 pregnant females at necropsy, groups with fewer than 16 animals may be inappropriate. Maternal mortality should not exceed 10 percent, which was not met in the study.

#### **Material and method:**

One hundred timed-mated female Hra: (NZW)SPF rabbits (source: C ██████████ ) were randomly assigned to four dosage groups (Groups I to IV) with 25 animals per group. The test material (99.5%) or vehicle was administered orally (via stomach tube) once daily on days 6 through 28 of presumed gestation (DG 6 through 28) at doses of 0, 250, 500 and 1000 mg/kg/day to rabbits in Groups I to IV, respectively. The dosage volume was 10 mL/kg. All rabbits were examined for clinical observations, abortions, premature deliveries and deaths before dosage, 60 ± 10 minutes after dosage administration and once daily during the post-dosage period. Bodyweights were recorded on DG 0 and daily during the dosage and post-dosage periods. Food consumption values were recorded daily. All surviving rabbits were sacrificed on DG 29, Caesarean-sectioned and a gross necropsy of the thoracic, abdominal and pelvic viscera was performed. Foetuses were weighed and examined for sex, gross external, soft tissue and skeletal alterations.

#### **Results:**

**Observations:** Seven and eight animals died in the 500 and 1000 mg/kg/day dosage groups, respectively, and the early sacrifice of two and six animals in these groups. Each of these deaths were considered to be test-material related (with the exception of one death per group which was considered to be the result of intubation accidents) because the deaths were preceded by adverse clinical observations and/or reductions in bodyweight gain and food

consumption. Two animals in the 1000 mg/kg group aborted and these were also considered to be test material related. One animal in each of the 0 and 250 mg/kg/day groups were sacrificed due to severe reductions in food consumption and resorption of litters; these deaths were not considered to be test material related. Two animals in the 1000 mg/kg/day group had totally resorbed litters.

The number of animals with soft or liquid faeces and ungroomed coat was increased or significantly increased in the 500 and 1000 mg/kg/day groups. These observations, along with the following clinical observations, were considered to be test material related and generally occurred in the animals that did not survive to scheduled sacrifice. In both of these groups, the number of animals with hyperactivity, perinasal substance, hyperpnoea, convulsions (clonic or tonic extension), tremors, red perioral substance, impaired righting reflex, gasping, ungroomed coat and no faeces in the cage pan were increased or significantly increased. In the 1000 mg/kg/day group, the number of animals with scant faeces, mucoid faeces, decreased motor activity, dehydration, dyspnoea, ptosis, blue or light blue colouring around the mouth and cold to touch was significantly increased. Twitches and mydriasis also occurred in the 1000 mg/kg/day group.

All other clinical observations e.g. sparse hair coat, scabs and localised alopecia, were considered to be unrelated to the test material. Red substance in the cage pan occurred in one control, two 250 mg/kg/day and two 1000 mg/kg/day animals and was a sign of impending abortion or delivery.

**Table 6.6.2/04-1 Clinical observation (No. of animal with observation)**

<b>Dose</b> <b>Findings</b>	<b>0</b> <b>(mg/kg/day)</b>	<b>250</b> <b>(mg/kg/day)</b>	<b>500</b> <b>(mg/kg/day)</b>	<b>1000</b> <b>(mg/kg/day)</b>
Mortality	1	1	9*	14**
<i>Found dead</i>	0	0	7**	8**
<i>Moribund sacrificed</i>	1	1	2	6
Aborted and sacrificed	0	0	0	2
Soft and liquid feces	9	9	20*	25**
Ungroomed coat	4	5	14	24**
Scant feces	3	5	5	16**
Mucoid feces	0	1	2	12**
Decreased motor activity	0	0	1	7**
Hyperactivity	0	0	3*	5**
Dehydration	1	0	0	6**

Dyspnea	0	0	1	4**
Ptosis	0	0	0	4**
Perinasal substance	0	0	4**	3**
Hyperpnea	0	0	4**	3**
Convulsion	0	0	3	3
Tremors	0	0	1	3
Blue mouth	0	0	0	3**
Cold to touch	0	0	0	3**
Perioral substance	0	0	1	2
Red substance in cage pan	1	2	0	2
Twitches	0	0	0	2
Mydryasis	0	0	0	2
Impaired righting reflex	0	0	2	1
Gasping	0	0	1	1

\* Statistically significant ( $p < 0.05$ )

\*\* Statistically significant ( $p < 0.01$ )

Bodyweight and food consumption: Bodyweights, uterine weights and corrected bodyweights (bodyweight on day 29 of presumed gestation minus the uterine weight) did not differ significantly among the groups. Bodyweight gains for the entire dosage period were 21% reduced in the 1000 mg/kg/day group compared to the controls. Within this interval, average bodyweight losses occurred for the intervals DG 6 to 9, 9 to 12, 15 to 19 and 19 to 24. Bodyweight gains increased by 29% in the 250 and 500 mg/kg/day groups compared to the controls. Within this interval, significant increases in bodyweight gains occurred for the interval DG 24 to 29. Bodyweight gains were also significantly increased in these two groups for the entire study period.

Absolute and relative food consumption values were reduced in the 1000 mg/kg/day groups for the entire dosage period compared to the controls. Within this interval, significant reductions in absolute and relative feed consumption values occurred for DG 6 to 9, 9 to 12, 12 to 15, 15 to 19 and 19 to 24. Absolute and relative feed consumption values were significantly increased for DG 24 to 29 in the 250 and 500 mg/kg/day groups.

**Table 6.6.2/04-2 Maternal bodyweight changes (kg)**

Dose	0 (mg/kg/day)	250	500 (mg/kg/day)	1000 (mg/kg/day)

<b>Days</b> \		<b>(mg/kg/day)</b>		
0 to 6	0.13	0.16	0.15	0.09
6 to 9	0.00	0.03	0.03	-0.06
9 to 12	0.03	0.02	-0.02	-0.06
12 to 15	0.10	0.06	0.03	0.01
15 to 19	0.07	0.06	0.08	-0.02*
19 to 24	0.10	0.11	0.09	-0.00**
24 to 29	0.00	0.10*	0.11*	0.09
6 to 29	0.34	0.44	0.44	0.27
0 to 29	0.47	0.61*	0.61*	0.41

\* Statistically significant ( $p < 0.05$ )

\*\* Statistically significant ( $p < 0.01$ )

**Table 6.6.2/04-3 Maternal feed consumption (g/kg/day)**

<b>Dose</b> \	<b>0 (mg/kg/day)</b>	<b>250 (mg/kg/day)</b>	<b>500 (mg/kg/day)</b>	<b>1000 (mg/kg/day)</b>
<b>Days</b>				
6 to 9	45.4	45.4	43.2	30.0**
9 to 12	42.4	41.4	40.7	23.3**
12 to 15	42.4	42.0	40.6	24.2**
15 to 19	46.2	42.7	45.7	29.9**
19 to 24	41.3	39.4	44.0	32.4*
24 to 29	23.8	32.9*	36.3**	30.8
6 to 29	39.4	40.6	42.1	34.7

\* Statistically significant ( $p < 0.05$ )

\*\* Statistically significant ( $p < 0.01$ )

Caesarean-sectioning and litter observations: Pregnancy occurred in 24 animals in each group. Caesarean-sectioning observations were based on 23, 23, 15, and 8 pregnant animals with one or more live foetuses in the 0, 250, 500 and 1000 mg/kg/day groups, respectively, which survived to DG 29.

Foetal weights were reduced by 10% in the 1000 mg/kg/day group male and female foetuses and significantly reduced for male foetuses only when compared to controls. No other Caesarean-sectioning or litter parameters were affected by doses as high as 1000 mg/kg/day. The litter averages for corpora lutea, implantations, litter sizes, live foetuses, early and late absorptions, percent resorbed conceptuses and percent live male foetuses were comparable among the groups and did not significantly differ. No animals had a litter of only resorbed conceptuses and there were no dead foetuses. All placentae appeared normal.

**Table 6.6.2/04-4 Caesarean-sectioning and litter observations**

<b>Dose</b> <b>Findings</b>	<b>0</b> <b>(mg/kg/day)</b>	<b>250</b> <b>(mg/kg/day)</b>	<b>500</b> <b>(mg/kg/day)</b>	<b>1000</b> <b>(mg/kg/day)</b>
Pregnant	24 (96 %)	24 (96%)	24 (96%)	24 (96%)
Found dead	0	0	<b>7 (29%)**</b>	<b>8 (33%)**</b>
Moribund sacrificed	1 (4%)	1 (4%)	2 (8%)	6 (25%)
Abortet and sacrificed	0	0	0	2 (8%)
No. of caesarean-sectioned animal	23	23	15	8
Corpora lutea	9.0 ± 1.7	9.0 ± 2.1	8.1 ± 1.6	9.6 ± 2.7
Live foetuses	195	194	119	72
Implantation	8.8 ± 1.5	8.7 ± 2.4	8.1 ± 1.6	9.2 ± 3.0
Litter sizes	8.5 ± 1.6	8.4 ± 2.2	7.9 ± 1.8	9.0 ± 3.1
Resorption	0.3 ± 0.6	0.3 ± 0.7	0.2 ± 0.4	0.2 ± 0.7
Does with any resorptions	7 (30.4%)	5 (21.7%)	3 (20%)	1 (12.5%)
Live male foetuses	104	84	59	30
Live foetal bodyweight (g)	42.31 ± 5.33	42.19 ± 5.80	43.90 ± 5.50	38.15 ± 7.22
Bodyweight –live male foetuses/litter	43.20 ± 5.56	43.21 ± 6.07	44.98 ± 5.74	<b>36.60 ± 6.80*</b>
Bodyweight –live female foetuses/litter	41.16	41.60	43.05	38.25

\* Statistically significant (p < 0.05)

\*\* Statistically significant (p < 0.01)

The number of fetuses with alterations was significantly increased in the 1000 mg/kg/day group. This increase included a significant increase in the number of fetuses with thickened ribs. The average number of ossified forelimb phalanges was significantly reduced in this group. No other gross external, soft tissue or skeletal foetal alterations (malformations or variations) or differences in ossification sites per litter were caused by the test material. The percentages of total fetuses with alternations observed were 6.7%, 8.8%, 10.1% and 19.4% in groups I to IV, respectively.

**Table 6.6.2/04-5 Foetal soft tissue and skeletal alterations**

<b>Dose</b> <b>Findings</b>	<b>0</b> <b>(mg/kg/day)</b>	<b>250</b> <b>(mg/kg/day)</b>	<b>500</b> <b>(mg/kg/day)</b>	<b>1000</b> <b>(mg/kg/day)</b>
Litter evaluated	23	23	15	8
Foetuses evaluated	195	194	119	72
Foetuses with any alteration observed	13 (6.7 %)	17 (8.8 %)	12 (10.1%)	<b>14 (19.4%)**</b>
Foetuses with any alteration/litter (%)	7.3 ± 9.7	8.7 ± 11.4	10.8 ± 12.1	20.0 ± 17.0
<b>Soft tissue-litter incidence</b>				
Heart: Interventricular septal defect	0 (0%)	1 (4.3%)	0 (0%)	0 (0%)
Vessels: Aorta distended	0 (0%)	1 (4.3%)	0 (0%)	0 (0%)
Vessels: Pulmonary artery constricted	0 (0%)	1 (4.3%)	0 (0%)	0 (0%)
<b>Skeletal alterations-litter incidence</b>				
Skull: Irregular ossification	5 (21.7%)	6 (26.1%)	6 (40.0%)	5 (62.5%)
Cervical vertebrae: Cervical rib present at 7th cervical vertebra	0 (0%)	1 (4.3%)	0 (0%)	0 (0%)
Thoracic vertebrae: Hemivertebra	0 (0%)	1 (4.3%)	1 (6.7%)	0 (0%)
Thoracic vertebrae: Centrum, bifid	0 (0%)	1 (4.3%)	0 (0%)	0 (0%)
Sacral vertebrae: Fused	0 (0%)	0 (0%)	0 (0%)	1 (12.5%)

Caudal vertebrae: Fused	0 (0%)	1 (4.3%)	0 (0%)	1 (12.5%)
Caudal vertebrae: 13 present	0 (0%)	0 (0%)	0 (0%)	1 (12.5%)
Caudal vertebrae: Misaligned	2 (8.7%)	1 (4.3%)	1 (6.7%)	2 (25.0%)
Ribs: Thickened	2 (8.7%)	1 (4.3%)	0 (0%)	1 (12.5%)
Ribs: Thickened- Foetal incidence	2 (1.0%)	1 (0.5%)	0 (0%)	<b>5 (6.9%)**</b>
Manubrium: irregularly shaped	0 (0%)	0 (0%)	1 (6.7%)	0 (0%)
Sternal centra: Fused	1 (4.3%)	0 (0%)	2 (13.3%)	0 (0%)
Sternal centra: Incompletely ossified	0 (0%)	1 (4.3%)	1 (6.7%)	0 (0%)
Sternal centra: Asymmetric	0 (0%)	0 (0%)	1 (6.7%)	0 (0%)
Xipnoid: Irregularly shaped	0 (0%)	0 (0%)	1 (6.7%)	0 (0%)
Scapulae: Ala, angulated	0 (0%)	0 (0%)	0 (0%)	1 (12.5%)

\* Statistically significant ( $p < 0.05$ )

\*\* Statistically significant ( $p < 0.01$ )

**Table 6.6.2/04-6 Foetal ossification sites**

<b>Dose</b>	<b>0</b>	<b>250</b>	<b>500</b>	<b>1000</b>
<b>Findings</b>	<b>(mg/kg/day)</b>	<b>(mg/kg/day)</b>	<b>(mg/kg/day)</b>	<b>(mg/kg/day)</b>
Forelimb-phalanges	13.99 ± 0.04	13.82 ± 0.27	13.85 ± 0.37	13.65 ± 0.27**

\*\* Statistically significant ( $p < 0.01$ )

#### **Conclusion:**

The maternal no-observable-effect-level (NOEL) is 250 mg/kg/day (the 500 and 1000 mg/kg/day doses caused adverse clinical observations and mortality). The developmental NOAEL is 500 mg/kg/day based on only a slight reduction in ossification and foetal weight on a litter basis occurring at 1000 mg/kg/day.

**RMS 2018:** The NOAEL for maternal toxicity is supposed to be set at 250 mg/kg bw/day based on the increased mortality and clinical findings including soft and/or liquid faeces, hyperpnoea, convulsions, and hyperactivity observed in females of higher dose groups (500 and 1000 mg/kg bw day). In the top dose group, abortions

appeared in two animals, foetal weight was reduced by 10 %, and the overall incidence of foetal alterations (manifesting mainly as irregular or reduced ossification) was significantly increased. Therefore, the NOAEL for developmental toxicity is supported to be 500 mg/kg bw/day. No treatment-related teratogenic effects were observed.

#### Alar-Teratology study in rabbits

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Reference	Alar-Teratology study in rabbits, [REDACTED] 1985; Report No. A.7.6.9
Guideline	The study was conducted according to OECD TG 414 and US EPA Guideline 83-3
Deviations	Yes
GLP	Yes
Acceptability	Yes
Previous evaluation	Yes, study already peer-reviewed in original DAR

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**RMS comment 2018:** The study has been checked for compliance. The following deviations from OECD guideline 414 (adopted on 22nd January 2001) were found:

- 1) More animals should have been enrolled in the study. At the end of the study only 12, 14, 15, and 8 pregnant females were alive in the 0, 50, 150, and 300 mg/kg group, respectively. At necropsy, the groups consisting of approximately 20 females with implantation sites are recommended.
- 2) Animals were not weighed and food consumption was not recorded every 3 days during the dosing period
- 3) Gravid uteri including the cervix were not weighed

#### **Material and method:**

Inseminated Dutch Belted rabbits (source: [REDACTED]) assigned to one control and three treatment groups of 16 animals each were used to determine the teratogenic potential of Alar® (purity: 99%, stability: not reported). Dosage levels of 50, 150, and 300 mg/kg/day were administered orally by gavage as a single daily dose on days 7 through 19 of gestation at a volume of 6 mL/kg. The control group received the vehicle only, 0.5% carboxymethylcellulose, on a comparable regimen.

Throughout the study the females were observed twice daily for mortality and overt changes in appearance and behaviour. They were observed once daily for clinical signs of toxicity on days 7 through 28 of gestation. Individual maternal bodyweights were recorded on gestation days 0, 7, 13, 20, 24 and 28. Individual food consumption was recorded on gestation days 0, 7, 13, 20, 24 and 28 and calculated as g/animal/day and g/kg of bodyweight/day for the following intervals: days 0-7, 7-13, 13-20, 20-24, 24-28, 7-19 and 0-28.



On gestation day 28, all surviving females were sacrificed, the uteri and ovaries were exposed by an abdominal incision. The number and location of viable and nonviable foetuses, early and late resorptions and the number of total implantations and corpora lutea were recorded. The uterus was then excised and the foetuses removed. The abdominal and thoracic cavities and organs were examined for grossly evident morphological changes.

All foetuses were individually weighed, tagged and examined for external malformations and variations including the palate and eyes. Each foetus was dissected, internally sexed and examined for visceral malformations and variations, including the brain by a mid-coronal slice. The heart was dissected by a modification of the method described by Staples. The eviscerated skinned foetuses were fixed in alcohol, macerated in potassium hydroxide, stained with Alizarin Red S and cleared with glycerine by a method similar to that described by Dawson for subsequent skeletal examination. Foetal findings were classified as malformations or developmental variations.

### Results:

Observations: One 300 mg/kg/day doe died early in the treatment regimen (gestation day 12) and the cause of death was undetermined. Three females aborted and were subsequently sacrificed: one each in the control, 150 and 300 mg/kg/day groups on gestation days 20, 22 and 23, respectively. Survival to scheduled sacrifice was 100% in the 50 mg/kg/day group.

Ante-mortem and necropsy observations of the doe that died on study included ocular and nasal discharge, wet/matted hair coat (eyes, nose, mouth, ventral neck, forelimbs), small amount of stool, a thick white substance in the pan beneath the cage, and congested lungs.

The occurrence of diarrhoea and absent, soft and/or small amount of stool was observed more frequently in the treated animals than the control animals.

The remainder of the ante-mortem and necropsy observations were within the normal profile of observations for this species, or were observed at random incidence and thus, not representative of a treatment-related effect.

**Table 6.6.2/05-1 Clinical observation (No. of animal with observation)**

<b>Dose</b>	<b>0 (mg/kg/day)</b>	<b>50(mg/kg/day)</b>	<b>150 (mg/kg/day)</b>	<b>300 (mg/kg/day)</b>
<b>Findings</b>				
No visible abnormalities	3 (18.8%)	3 (18.8%)	1 (6.3%)	4 (25.0%)
Found dead	0	0	0	1 (6.3%)
Aborted and sacrificed	1 (6.3%)	0	1 (6.3%)	1 (6.3%)
Soft feces	3 (18.8%)	7 (43.8%)	5 (31.3%)	6 (37.5%)
Hair loss	3 (18.8%)	7 (43.8%)	7 (43.8%)	5 (31.3%)
Appears thin	0	0	1 (6.3%)	0

Small amount of stool	3 (18.8%)	7 (43.8%)	10 (62.5%)	10 (62.5%)
Diarrhea	0	0	1 (6.3%)	1 (6.3%)
Stool absent	0	0	2 (12.5%)	3 (18.8%)
Thick white substance in pan	0	0	0	1 (6.3%)
Red fluid in pan	1 (6.3%)	0	0	1 (6.3%)

**Bodyweight and food consumption:** Bodyweight gain of the 300 mg/kg/day animals was slightly inhibited during the overall treatment interval (gestation days 7-19) relative to the control; however, gains during the overall gestation interval (gestation days 0-28) were not similarly disparate. The 50 and 150 mg/kg/day females gained weight at a rate comparable to that of the control animals throughout both the treatment and overall gestation intervals.

**Table 6.6.2/05-2 Maternal bodyweight changes (kg)**

<b>Dose</b> <b>Days</b>	<b>0</b> <b>(mg/kg/day)</b>	<b>50</b> <b>(mg/kg/day)</b>	<b>150</b> <b>(mg/kg/day)</b>	<b>300</b> <b>(mg/kg/day)</b>
0 to 7	98 ± 89.2	132 ± 67.0	63 ± 73.9	141 ± 106.1
7 to 13	27 ± 46.9	10 ± 59.8	14 ± 98.8	17 ± 72.9
13 to 20	-9 ± 110.7	45 ± 115.2	49 ± 98.2	-16 ± 160.0
20 to 24	32 ± 57.2	-4 ± 101.3	16 ± 95.3	-23 ± 123.0
24 to 28	43 ± 74.0	-23 ± 92.3	8 ± 83.0	36 ± 162.6
7 to 19	88 ± 125.5	55 ± 118.5	63 ± 96.0	1 ± 120.2
0 to 28	186 ± 137.7	159 ± 184.0	149 ± 140.2	158 ± 260.4

There were no meaningful differences between the food intake of the treated and control animals during either the overall gestation or treatment intervals.

**Pregnancy data:** Treatment-induced effects on maternal and foetal observations at Caesarean section were not evident with respect to the numbers of corpora lutea, total implantations, preimplantation and post-implantation losses, and viable foetuses per dam, and foetal bodyweight and sex distribution values.

Table 6.6.2/05-3 Caesarean-sectioning and litter observations

<b>Dose</b> <b>Findings</b>	<b>0</b> <b>(mg/kg/day)</b>	<b>50</b> <b>(mg/kg/day)</b>	<b>150</b> <b>(mg/kg/day)</b>	<b>300</b> <b>(mg/kg/day)</b>
Pregnant	13 (81%)	14 (88%)	16 (100%)	10 (63%)
Found dead	0	0	0	1
Moribund sacrificed	1 (4%)	1 (4%)	2 (8%)	6 (25%)
Aborted and sacrificed	1	0	1	1
No. of caesarean-sectioned animal	15	16	15	14
Corpora lutea	12.6 ± 3.15	10.6 ± 3.37	11.1 ± 2.30	10.6 ± 3.25
Does with viable foetuses	12	13	14	8
Viable foetuses/doe	7.1 ± 3.37	6.9 ± 3.02	6.8 ± 3.03	6.9 ± 3.40
Implantation/doe	8.1 ± 3.26	8.3 ± 2.67	8.3 ± 2.55	8.5 ± 4.11
Preimplantation loss(%)	35.8	21.6	20.6	20.0
Postimplantation loss (%)	12.4	16.4	18.4	19.1
Does with resorptions only	0	1	1	0
Live foetal bodyweight (g)	32.3 ± 5.27	32.9 ± 4.27	32.3 ± 4.86	31.5 ± 10.68
Sex ratio (males/females, %)	54.1/45.9	54.6/45.4	53.9/46.1	42.9/57.1

Foetal data and abnormalities: The incidence of foetal malformations and developmental variations in the treated groups was not meaningfully different from the control. Fused skull bones and forked scapula were the most frequently observed malformations and occurred at respective frequencies of zero and one times in the 300 mg/kg/day group. The remaining malformations - ethmocephaly, exencephaly, ablepharia, cleft palate, hydrocephaly, omphalocele, absent spleen, and vertebral anomaly occurred in no more than one foetus per litter per group. With respect to developmental variations, 13th rudimentary ribs and unossified sternebra 5 or 6 occurred most often and at comparable frequencies in the control and treated groups.

Table 6.6.2/05-4: Foetal alterations

<b>Dose</b> <b>Findings</b>	<b>0</b> <b>(mg/kg/day)</b>	<b>50</b> <b>(mg/kg/day)</b>	<b>150</b> <b>(mg/kg/day)</b>	<b>300</b> <b>(mg/kg/day)</b>
Litter evaluated	12	13	14	8
Foetuses evaluated	85	97	102	56
Foetuses with any alteration observed	5 (5.9%)	5 (5.2 %)	3 (2.9%)	3 (5.4%)
Ethmocephaly	1 (1.2%)	0 (0%)	0 (0%)	0 (0%)
Exencephaly	0 (0%)	1 (1.0%)	0 (0%)	0 (0%)
Ablepharia	0 (0%)	1 (1.0%)	0 (0%)	0 (0%)
Cleft palate	0 (0%)	1 (1.0%)	0 (0%)	0 (0%)
Hydrocephaly	1 (1.2%)	0 (0%)	0 (0%)	1 (1.8%)
Omphalocele	0 (0%)	1 (1.0%)	0 (0%)	1 (1.8%)
Spleen absent	0 (0%)	0 (0%)	0 (0%)	1 (1.8%)
Fused skull bones	1 (1.2%)	2 (2.1%)	2 (2.0%)	0 (0%)
Forked scapula	2 (2.4%)	2 (2.1%)	0 (0%)	1 (1.8%)
Vertebral anomaly	0 (0%)	0 (0%)	1 (1.0%)	0 (0%)

**Original DAR conclusions**

- Since the observed stool changes were not found to be of toxicological relevance the NOAEL for maternal toxicity was established at  $\geq 300$  mg/kg bw/day.
- The NOAEL for developmental toxicity was established at  $\geq 300$  mg/kg bw/day.
- No teratogenic effects were observed.

**RMS 2018:** The original overall conclusion of this study is supported by the RMS. No teratogenic effects which could be related to the treatment were observed.

**B 6.6.3 Summary of reproductive toxicity**

In a dietary two-generation reproduction study in rats, the NOAEL for parental toxicity was established at 50 mg/kg bw/day, based on effects on food consumption and bodyweight at 500 mg/kg bw/day. No developmental effects or effects on fertility were observed (NOAEL 500 mg/kg bw/day, the highest dose tested). In a second gavage two-generation reproduction study, the NOAEL for parental effects was set at 360 mg/kg bw/day, based on

clinical signs (loose faeces, perianal fur staining, excessive post-dose salivation) and increased water consumption at the highest dose level (1200 mg/kg bw/day). The NOAEL for developmental effects and effect on fertility was established at 1200 mg/kg bw/day.

Teratogenicity studies with daminozide were conducted in rabbits (two studies) and rats (two studies of which one was not suitable for evaluation). In the rabbit, taking both studies into account the overall NOAEL for maternal toxicity can be established at 300 mg/kg bw/day (the highest dose tested in ██████████ 1985 and within the maternal LOAEL in ██████████ 2006b). Based on the most recent rabbit study (██████████ 2006b) the developmental NOAEL was 500 mg/kg based on occurrence of abortions, slight reduction in ossification and foetal weight. In a teratogenicity study with rats a maternal NOAEL of 150 mg/kg bw/day was established, based on decreased bodyweight gain. No developmental effects were observed. In an additional supplementary teratogenicity study with rats, no maternal and developmental effects were observed at dose levels up to 1000 mg/kg bw/day. No indications of any teratogenic potential were observed in these studies.

In Table 6.6-1 the results of the reproduction and teratogenicity studies with daminozide are summarized.

**Table 6.6-1: Reproduction toxicity and teratogenicity studies**

Species	Route	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Critical effects	Notifier, Reference
<b>Reproduction studies</b>					
rats	oral	Parental: 50 (1000 ppm)  Developmental: 500 (10000 ppm) Fertility: 500	Parental: 500  Developmental: >500  Fertility: > 500	Parental: changes in bodyweight  Developmental: No adverse effect  Fertility: No adverse effect	Uniroyal, ██████████ 1987
rats	oral	Parental: 360  Developmental: 1200 Fertility: 1200	Parental: 1200  Developmental: >1200 Fertility: > 1200	Parental: clinical signs (loose faeces, excessive post-dose salivation, perianal fur staining) and increased water consumption  Developmental: No adverse effect  Fertility: No adverse effect	██████████, ██████████ 1994

Species	Route	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Critical effects	Notifier, Reference
<b>Teratogenicity studies</b>					
rats	oral	maternal: 1000  developmental: 1000 teratogenicity: 1000	maternal: $\geq 1000$  developmental: $\geq 1000$ teratogenicity: $\geq 1000$	maternal: No adverse effect  developmental: No adverse effect teratogenicity: No adverse effect	██████████ ██████████ 1979
rats	oral	maternal: 150  developmental: 1500 teratogenicity: 1500	maternal: 750  developmental: $\geq 1500$ teratogenicity: $\geq 1500$	maternal: reduced bodyweight gain, developmental: No adverse effect teratogenicity: No adverse effect	██████████ ██████████ 1993
rabbits	oral	maternal: 300  developmental: 300  teratogenicity: 300	maternal: $\geq 300$ developmental: $\geq 300$ teratogenicity: $\geq 300$	-  -  -	Uniroyal, ████████████████████ ██████████
rabbits	oral	maternal: 250  developmental: 500  teratogenicity: 1000	maternal: 500  developmental: 1000  teratogenicity: $\geq 1000$	maternal: mortality, clinical signs (soft/liquid faeces, hyperpnoea, hyperactivity, convulsions) developmental: slight reduction in ossification and litter weight	████████████████████ 2006b

**B 6.7 Neurotoxicity studies****B 6.7.1 Neurotoxicity studies in rodents****An acute neurotoxicity study in rats**

Reference	An acute neurotoxicity study in rats, [REDACTED] Report No. 399-232
Guideline	The study was conducted according to OECD 424 and OPPTS 870.6200 guidelines
Deviations	No
GLP	Yes
Acceptability	Yes
Previous evaluation	No

**RMS 2018:** The study has been checked for compliance. No deviations from OECD guideline 424 (adopted on 21st July 1997) were found.

#### Material and method:

The study was conducted to evaluate the acute neurotoxicity of the test article, daminozide technical (100%), when administered to rats as a single dose via oral gavage. Three treatment groups of 10 male and 10 female CD [REDACTED] CD(SD)] rats (source: [REDACTED]) received the test article once by gavage at dose levels of 500, 1000 and 2000 mg/kg. One additional group of 10 animals/sex served as the control and received the vehicle, 0.5% carboxymethylcellulose (CMC), in deionised water. The vehicle or test article was administered to all groups via oral gavage once on day 1 at a dose volume of 10 mL/kg.

Observations for morbidity, mortality, injury and the availability of food and water were conducted twice daily for all animals. Clinical observations were conducted on days 1 (pre-dose and 2 to 4 hours post dose), 7 and 14. Functional observational battery (FOB) and motor activity evaluations were conducted pre-test, 2 to 4 hours post dose on day 1 and on days 7 and 14. FOB included but was not limited to, evaluation of activity and arousal, posture, rearing, bizarre behaviour, clonic and tonic movements, gait, mobility, stereotypy, righting reflex, response to stimulus (approach, click, tail pinch, and touch), palpebral closure, pupil response, piloerection, exophthalmus, lacrimation, salivation, and respiration. The amount, qualitative and/or quantitative measures, of defecation and urination were also recorded. Forelimb and hindlimb grip strength was measured and hindlimb splay was quantitatively measured. Pain perception was assessed by measuring the latency of response to a nociceptive (thermal) stimulus when each animal was placed on a hot plate apparatus set to 52°C. Bodyweights were measured and recorded on days -1, 1, 7 and 14. Food consumption was measured and recorded daily. At study termination, the first five animals/sex/group were euthanized, a necropsy examination was conducted and tissues and organs were collected for possible future processing and microscopic evaluation. The last five animal/sex/group were designated for neuropathology evaluations. The following tissues were collected from each animal: brain, proximal sciatic nerve, sural nerve, tibial nerve and tibial nerve calf branches, fibular nerve (peroneal nerve), the spinal cord at cervical swelling (C3 to C6) and lumbar swelling (L1 to L4), trigeminal ganglia, dorsal root ganglia (C3 to C6 and L1 to L4), dorsal and ventral root fibers (C3 to C6 and L1 to L4), and skeletal muscle (gastrocnemius).

#### Results:

Clinical and FOB observations: There were no clinical findings of systemic toxicity at any dose level. Basic movement, fine movement, and total distance were statistically significantly lower among males at 2000 mg/kg in the 10-20 min bin only on Day 1 (no change was noted over the entire 0 to 60 minute collection period). Rears were also statistically significantly lower on Day 1 in the motor activity portion but a similar change was not evident in the FOB portion. In females, the statistically significant decrease in rearing was observed over the entire 0-60 minute collection period of the locomotor activity assessment on Day 14 as well as on Day 2 of FOB examination. The total distance was also significantly lowered on Day 14.

**Table 6.7.1/01-1: An acute neurotoxicity study in rats; Summary of locomotor activity (0-60 minute study interval)**

Parameter	Dose [mg/kg/day]							
	0		500		1000		2000	
	Male	Female	Male	Female	Male	Female	Male	Female
Basic movement (count)								
Pre-test	4207.9	3629.0	3941.0	3648.6	4181.5	3918.0	3864.8	3646.9
Day1	2316.2	2984.8	2269.7	2817.9	2486.2	2645.4	1683.2	2790.2
Day7	3373.6	4065.4	3447.3	3902.7	3635.3	4034.2	3168.6	4136.9
Day14	4757.4	5286.0	4730.8	4358.6	4151.3	4474.2	3691.2	3250.9
Fine movement (count)								
Pre-test	3282.4	2698.7	3094.5	2651.7	3257.2	2878.0	2975.1	2697.7
Day1	1873.0	2135.3	1872.8	2067.8	1987.2	1997.9	1322.3	2099.2
Day7	2758.9	2992.1	2832.1	2999.5	2942.4	3014.1	2608.8	3115.2
Day14	3798.3	3864.4	3753.8	3321.4	3302.4	3275.1	2923.2	2476.8
Rearing (count)								
Pre-test	184.8	115.3	175.1	115.4	188.0	129.8	148.4	117.1
Day1	98.6	93.9	112.6	96.9	107.0	90.9	63.5	84.0
Day7	194.8	138.6	218.9	145.7	208.3	135.2	175.2	139.6
Day14	286.9	217.6	273.7	179.2	228.3	176.4	217.7	126.2*
Total distance (cm)								
Pre-test	7488.8	6362.5	6901.0	6522.6	7391.8	6862.9	6893.7	6359.4
Day1	4024.4	5339.1	3920.1	4919.9	4369.1	4635.9	3029.0	4892.6



Day7	5898.3	7152.1	6058.2	6827.7	6359.3	7014.0	5575.5	7297.7
Day14	8304.9	9319.8	8195.2	7623.5	7240.3	7785.4	6533.5	5685.7*

\*p<0.05

#### Necropsy:

##### *Macroscopic observations*

No test article-related macroscopic observations were made in male or female rats at necropsy. All tissues of all females were within normal limits. An enlarged testis was observed in one male at 1000 mg/kg; microscopic examination would be required to determine the cause. A diverticulum of the jejunum of a male at 2000 mg/kg was a developmental anomaly.

##### *Neuropathology evaluations*

There were no lesions in any of the examined neural tissues that indicated a test article-related effect. The only lesions in the central nervous system of any of the animals in this study was minimal mononuclear cell infiltrate in the meninges of the midbrain in one control Group 1 female and minimal axonopathy of a lumbar spinal cord cauda equina nerve in one control Group 1 female. The cause of the mononuclear cell infiltrate in the meninges was not apparent and is considered an incidental finding. The minimal axonopathy consisted of single fiber degenerative changes of a cauda equina nerve. There was fragmentation of the axon and formation of digestion chambers in the affected region. Minimal axonopathy is found occasionally in control rats, although more commonly in animals older than those in this study. No lesions were present, in any animal of any group, in the cortex, hippocampus, globus pallidus, caudate/putamen, thalamus, hypothalamus, pons, medulla oblongata, cerebellum, cervical spinal cord, gastrocnemius nor in any of the methacrylate sections of proximal sciatic nerve, tibial nerve, sural nerve, dorsal & ventral root fibers with dorsal root ganglia (C3-C6), or trigeminal ganglia.

#### **Conclusion:**

Under the conditions of this study, where male and female rats received a single oral (gavage) dose of daminozide technical at 0, 500, 1000 and 2000 mg/kg, the No Observed Effect Level (NOEL) was 2000 mg/kg, the highest dose tested. No signs of systemic or neurotoxicity were evident.

**RMS 2018:** Overall conclusion is not supported. The RMS suggests the setting of the NOAEL for neurotoxicity at 1000 mg/kg bw/day based on the decreased locomotor activity (total distance, basic and fine movement) over the entire 0 to 60 minute collection period in rats of the top dose group (on Day 1 and 14 in males; on Day 14 in females) when compared to the control.

**Daminozide: A 90-day oral (gavage) neurotoxicity study in rats**


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Reference	<b>Daminozide: A 90-day oral (gavage) neurotoxicity study in rats, [REDACTED]</b> [REDACTED] Report No. 399-232
Guideline	The study was conducted according to OECD 424 and OPPTS 870.6200 guidelines
Deviations	No
GLP	Yes
Acceptability	Yes
Previous evaluation	No

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**RMS 2018:** The study has been checked for compliance. No deviations from OECD guideline 424 (adopted on 21st July 1997) were found.

**Material and method:**

Ten female and male rats CD [REDACTED]:CD(SD)] were allocated to each of 3 groups, and gavaged with daminozide technical (purity: 100%) at doses of 100, 300 and 1000 mg/kw bw/day for 90 days. One additional group of 10 animals/sex served as the control and received the vehicle, 0.5% carboxymethyl cellulose (CMC).

All animals were observed for morbidity, mortality, injury and the availability of food and water twice daily.

Clinical examination of each animal was performed daily. Observations included, but were not limited to, evaluation of the skin, fur, eyes, ears, nose, oral cavity, thorax, abdomen, external genitalia, limbs and feet, respiratory and circulatory effects, autonomic effects such as salivation and nervous system effects including tremors, convulsions, reactivity to handling and unusual behaviour.

Detailed clinical examinations of each animal were performed weekly. On occasion, detailed clinical observations were recorded at unscheduled intervals for the first 4 weeks. Observations were made outside the animal's home cage beginning in Week 5 in a standard arena using an applicable scoring system at approximately the same time and day each week. The observations included, but were not limited to changes in the skin, fur, eyes, mucous membranes, occurrence of secretions and excretions, and autonomic activity (e.g., lacrimation, piloerection, pupil size, and unusual respiratory pattern). Changes in gait, posture, and reactivity to handling, as well as the presence of clonic or tonic movements, stereotypy (e.g., excessive grooming, repetitive circling), difficult or prolonged parturition or bizarre behaviour (e.g., self-mutilation, walking backwards) were also recorded. Examinations were not performed during the week of FOB evaluations.

FOB evaluations, including those conducted in the home-cage, during handling, in the open field and others, were conducted on all designated animals pre-test and at 2 to 4 hours post dose on days 1 and 28 and during weeks 8 and 13. The observations included, but were not limited to, evaluation of activity and arousal, posture, rearing,

bizarre behaviour, clonic and tonic movements, gait, mobility, stereotypy, righting reflex, response to stimulus (approach, click, tail pinch, and touch), palpebral closure, pupil response, piloerection, exophthalmus, lacrimation, salivation, and respiration. The amount, qualitative and/or quantitative measures, of defecation and urination were also recorded. Forelimb and hindlimb grip strength was measured using the procedure described by Meyer *et al*, and hindlimb splay was quantitatively measured as described by Edwards and Parker. Pain perception was assessed by measuring the latency of response to a nociceptive (thermal) stimulus when each animal was placed on a hot plate apparatus set to 52°C ( $\pm 1^\circ\text{C}$ ).

Motor activity evaluations were conducted on all animals pre-test and at approximately 2 to 4 hours post dose on days 1 and 28 and during weeks 8 and 13. The duration of the monitoring was 60 minutes with the data summarised into 10 minute segments. A range of different activities were assessed in a three dimensional array and were recorded. Only basic movement, fine movement, distance and rearing were used in comparisons between treated and control animals as the most representative activity parameters.

Bodyweights for all animals were measured and recorded 3 days after receipt, prior to randomisation and weekly during the study. Food consumption was measured and recorded weekly during the study. Ophthalmoscopic evaluations were conducted on all animals pre-test and during week 13.

At necropsy, the animals were examined carefully for external abnormalities including palpable masses. The skin was reflected from a ventral midline incision and any subcutaneous masses were identified and correlated with ante-mortem findings. The abdominal, thoracic, and cranial cavities were examined for abnormalities. The organs were removed, examined, and, where required, placed in fixative. Bodyweights and organ weights were recorded for all animals.

At termination, the last five animals/sex/group were designated for neuropathology evaluations. The following tissues were harvested and fixed: brain, sciatic nerve, sural nerve, proximal tibial nerve and tibial nerve calf branches, eye with optic nerve, fibular nerve, the spinal cord at cervical swelling (C3 to C6) and lumbar swelling (L1 to L4), trigeminal ganglia, dorsal root ganglia (C3 to C6 and L1 to L4), dorsal and ventral root fibers (C3 to C6 and L1 to L4), and skeletal muscle (gastrocnemius).

## Results:

**Table 6.7.1/02-1: Clinical observation**

<b>Dose</b> <b>Parameter</b>	<b>0</b> <b>(mg/kg/day)</b>	<b>100</b> <b>(mg/kg/day)</b>	<b>300</b> <b>(mg/kg/day)</b>	<b>1000</b> <b>(mg/kg/day)</b>
No. with no abnormalities-males	6	8	7	3
No. with no abnormalities-females	10	7	8	10

Salivation (♂/♀)	0/0	0/0	0/0	1/0
Soft feces (♂/♀)	0/0	0/0	0/0	4/0
Red material in pan (♂/♀)	0/0	0/0	0/0	1/0
Red material around eyes (♂/♀)	0/0	0/0	0/0	1/0
Hair sparse (♂/♀)	4/0	2/3	3/2	3/0

Bodyweight and food consumption: There were no test material-related effects on bodyweight. Bodyweight among females at 1000 mg/kg/day was slightly lower during week 8 (8%) compared to control values. This value was considered a spurious transient event and not test material-related as no pattern was evident over the 13-week dosing period. No test material-related effects on food consumption were noted. Female food consumption at 1000 mg/kg/day was transiently lower (9%) at week 6. This value was considered a spurious event and not test material-related as no pattern was evident over the 13-week dosing period.

**Table 6.7.1/02-2: Daminozide: A 90-day oral (gavage) neurotoxicity study in rats; Group mean bodyweight and food consumption in females**

Dose [mg/kg/day] Parameter	0	100	300	1000
<b>Bodyweight [g]</b>				
<b>Week -1</b>	179.0	181.1	178.9	178.9
<b>Week 1</b>	202.4	205.7	200.3	197.6
<b>Week 2</b>	232.7	230.3.	227.1	221.8
<b>Week 4</b>	250.2	250.5	252.4	237.1
<b>Week 6</b>	268.3	266.6	262.6	250.8
<b>Week 8</b>	275.9	269.9	269.6	253.3*
<b>Week 10</b>	287.7	284.6	282.6	265.6
<b>Week 12</b>	295.9	291.6	292.2	273.5
<b>Week 13</b>	293.7	287.4	290.2	273.5
<b>Bodyweight gain [g] (Week -1 – Week13)</b>	114.7	106.3	113.3	94.6

Food consumption [g/animal/day]				
<b>Week 1</b>	20.08	20.60	20.08	19.02
<b>Week 2</b>	21.46	21.09	21.29	19.53
<b>Week 3</b>	21.47	23.53	21.47	19.94
<b>Week 4</b>	20.66	20.27	20.39	19.19
<b>Week 5</b>	20.60	20.40	19.87	20.27
<b>Week 6</b>	21.97	21.70	21.19	19.97*
<b>Week 7</b>	21.71	21.66	21.61	20.23
<b>Week 8</b>	21.49	21.01	21.04	20.43
<b>Week 9</b>	21.16	21.83	21.93	20.66
<b>Week 10</b>	21.59	22.54	21.67	20.53
<b>Week 11</b>	20.64	21.03	20.93	20.44
<b>Week 12</b>	20.46	21.00	20.56	20.00
<b>Week 13</b>	18.38	18.47	18.90	17.60

\*p<0.05

**Haematology and biochemistry:** There were no test material-related effects on haematology parameters in either sex at any dose level. There were procedural related alterations among individual haematology (neutrophils, lymphocytes and total leukocytes) that were considered secondary to intraperitoneal perfusion and cardiac puncture at termination and had no relation to the test material. There were sporadic moderate to marked reductions in platelets in two males (one receiving vehicle control and one receiving 300 mg/kg/day) and one female (receiving 100 mg/kg/day). These changes were potentially also procedural related. There were no test material-related effects on coagulation times in either sex at any dose level. All mean and individual values were considered within an acceptable range for biologic and/or assay-related variation. There were no test material-related effects among clinical chemistry analytes in either sex at any dose level. All mean values were considered within an acceptable range for biologic variation. There were procedural related alterations among individual clinical chemistry analytes (aspartate aminotransferase (AST) and alanine aminotransferase (ALT) that were considered secondary to intraperitoneal perfusion and cardiac puncture at termination and had no relation to the test material.

**Urinalysis:** In both sexes and at all dose levels, there were mild reductions in urine pH relative to controls. These changes were generally dose-dependent and considered test material-related but of minor biologic relevance. There were occasionally other mild fluctuations in urine volume and specific gravity that were not considered toxicologically meaningful due to their sporadic nature and the inherent variability of these endpoints. There were some variations between treatment groups among biochemical (protein, ketones, glucose etc.) urinary components;

however, all findings were considered within an acceptable range for biological and/or procedure-related variability.

**Table 6.7.1/02-3: Urinalysis values (males/females)**

<b>Dose</b> <b>Parameter</b>	<b>0</b> <b>(mg/kg/day)</b>	<b>100 (mg/kg/day)</b>	<b>300 (mg/kg/day)</b>	<b>1000 (mg/kg/day)</b>
pH	7.85 / 7.10	7.55 / 7.00	7.45 / 7.05	6.40* / 6.61
Specific gravity	1.074 / 1.061	1.058 / 1.058	1.066 / 1.057	1.074 / 1.051
Volume, mL	4.90 / 3.35	5.65 / 3.50	5.20 / 4.50	6.05 / 5.12

\* Statistically significant ( $p < 0.05$ )

FOB and locomotor activity: There were no test material-related changes noted in any FOB parameter or in the motor activity assessment. Basic movement, fine movement, rearing and total distance were statistically significantly lower among males at 300 mg/kg/day in the 0-10 min bin resulting in a lower value over entire 0-60 min interval on day 1. These changes were not considered test material-related as there was no dose-response. On day 28, basic movement, fine movement, rearing and total distance were higher among males at 1000 mg/kg/day in the 4-50 min bin (no changes was evident over the entire 0-60 min collection period). A similar effect was not present among females. Therefore, these findings were considered spurious as no other parameters appeared affected in either the FOB or motor activity assessments at any other interval.

**Table 6.7.1/02-4: Locomotor activity (Day1 / Day28)**

<b>Dose</b> <b>Findings (Score)</b>	<b>0</b> <b>(mg/kg/day)</b>	<b>100</b> <b>(mg/kg/day)</b>	<b>300</b> <b>(mg/kg/day)</b>	<b>1000</b> <b>(mg/kg/day)</b>
<b>Locomotor activity-Basic movement (count, males)</b>				
0-10 min	1804.2 / 1934.6	1537.9 / 1967.4	1387.6* / 1862.2	1617.9 / 1861.5
10-20 min	742.4 / 1233.3	618.4 / 920.7	483.1 / 1080.7	399.7 / 1024.6
20-30 min	165.1 / 514.3	103.8 / 384.7	100.9 / 507.1	185.9 / 492.8
30-40 min	146.7 / 223.5	61.9 / 259.5	58.4 / 353.4	170.9 / 505.1
40-50 min	62.9 / 154.4	64.8 / 306.4	113.5 / 147.6	161.5 / 568.2*
50-60 min	146.5 / 177.4	160.6 / 135.9	89.8 / 167.4	179.9 / 342.5

0-60 min	3067.8 / 4237.5	2547.4 / 3974.6	2233.3* / 4118.4	2715.8 / 4794.7
<b>Locomotor activity-Basic movement (count, females)</b>				
0-10 min	1638.5 / 1907.7	1797.4 / 1948.8	1808.3 / 2124.3	1916.9 / 2059.5
10-20 min	577.3 / 863.9	712.5 / 850.1	624.6 / 861.1	712.8 / 1013.9
20-30 min	270.2 / 624.7	153.5 / 438.2	247.3 / 553.0	214.7 / 366.4
30-40 min	104.9 / 175.2	122.7 / 285.4	282.0 / 288.7	102.9 / 316.2
40-50 min	89.0 / 412.3	273.9 / 241.3	134.3 / 255.8	80.6 / 204.6
50-60 min	131.0 / 116.8	280.1 / 316.1	68.3 / 262.9	199.5 / 204.4
0-60 min	2810.9 / 4100.6	3304.1 / 4079.9	3164.8 / 4345.8	3227.4 / 4165.0
<b>Locomotor activity-Fine movement (count, males)</b>				
0-10 min	1230.3 / 1416.2	1061.1 / 1367.5	944.5* / 1304.4	1109.2 / 1349.9
10-20 min	623.1 / 975.0	537.4 / 750.2	412.4 / 889.0	373.0 / 838.3
20-30 min	145.6 / 450.0	103.2 / 349.0	100.3 / 438.3	182.8 / 415.9
30-40 min	140.8 / 214.5	61.9 / 215.4	58.3 / 307.3	156.9 / 418.1
40-50 min	56.6 / 141.8	64.8 / 253.0	113.4 / 132.3	136.6 / 482.0*
50-60 min	133.4 / 152.0	155.9 / 118.1	89.8 / 148.0	162.4 / 297.0
0-60 min	2329.8 / 3349.5	1984.3 / 3053.2	1718.7* / 3219.3	2120.9 / 3801.2
<b>Locomotor activity-Fine movement (count, females)</b>				
0-10 min	1116.7 / 1392.9	1185.1 / 1346.1	12.01.4 / 1459.2	1280.5 / 1379.1
10-20 min	473.5 / 710.5	585.1 / 672.3	482.3 / 681.1	590.8 / 791.9
20-30 min	230.0 / 524.1	143.2 / 354.8	199.2 / 448.6	205.5 / 295.6
30-40 min	93.7 / 163.4	111.6 / 233.9	224.5 / 236.3	102.4 / 260.9
40-50 min	77.2 / 334.7	194.3 / 210.0	116.5 / 205.1	80.3 / 168.0

50-60 min	115.5 / 109.1	236.7 / 261.0	68.3 / 225.5	166.3 / 160.6
0-60 min	2106.6 / 3234.7	2456.0 / 3078.1	2292.2 / 3255.8	2425.8 / 3056.1
<b>Locomotor activity-Rearing (count, males)</b>				
0-10 min	78.7 / 78.3	70.2 / 95.0	56.3* / 88.3	72.3 / 82.5
10-20 min	23.4 / 54.4	19.5 / 56.9	19.2 / 70.4	14.1 / 55.1
20-30 min	4.8 / 29.7	2.1 / 25.4	1.7 / 44.8	3.6 / 35.3
30-40 min	1.4 / 12.4	0.0 / 13.9	0.0 / 24.9	3.9 / 30.3
40-50 min	0.4 / 10.7	0.1 / 14.9	0.3 / 9.6	4.5 / 31.9*
50-60 min	2.7 / 15.9	1.3 / 8.1	1.3 / 11.6	4.3 / 19.9
0-60 min	111.4 / 201.4	93.2 / 214.2	78.8 / 249.6	102.7 / 255.0
<b>Locomotor activity-Rearing (count, females)</b>				
0-10 min	64.6 / 96.8	70.2 / 95.1	66.8 / 94.1	72.8 / 88.3
10-20 min	18.2 / 47.7	26.6 / 46.3	17.8 / 44.7	21.2 / 46.9
20-30 min	5.9 / 31.9	3.0 / 21.2	7.0 / 28.2	4.3 / 14.2
30-40 min	1.4 / 6.6	1.6 / 14.9	5.8 / 10.4	0.4 / 10.3
40-50 min	1.7 / 19.8	6.3 / 10.5	2.4 / 13.2	0.9 / 6.2
50-60 min	1.4 / 7.7	5.2 / 15.0	0.1 / 10.2	4.2 / 8.9
0-60 min	93.2 / 210.5	112.9 / 203.0	99.9 / 200.8	103.8 / 174.8
<b>Locomotor activity-Total distance (cm, males)</b>				
0-10 min	3215.0 / 3353.0	2772.7 / 3466.1	2527.0* / 3286.6	2905.3 / 3284.7
10-20 min	1279.1 / 2158.5	1039.8 / 1605.2	844.8 / 1894.3	667.6 / 1754.2
20-30 min	294.1 / 871.4	168.5 / 658.0	180.1 / 899.7	320.3 / 870.5
30-40 min	282.1 / 380.2	119.1 / 455.4	112.9 / 634.1	303.6 / 898.9



40-50 min	110.1 / 277.4	111.3 / 545.6	220.1 / 256.2	290.8 / 1000.1*
50-60 min	274.3 / 340.2	292.7 / 247.9	163.0 / 315.6	346.0 / 607.6
0-60 min	5454.7 / 7380.7	4504.1 / 6978.2	4047.9* / 7286.5	4833.6 / 8416.0
<b>Locomotor activity-Total distance (cm, females)</b>				
0-10 min	2900.4 / 3283.2	3203.4 / 3428.3	3138.3 / 3718.8	3396.7 / 3649.1
10-20 min	964.3 / 1491.7	1202.5 / 1479.1	1065.2 / 1499.4	1219.8 / 1786.9
20-30 min	472.3 / 1088.8	256.6 / 777.1	427.9 / 956.4	346.7 / 663.9
30-40 min	192.6 / 313.1	211.7 / 517.3	470.6 / 502.2	177.1 / 581.4
40-50 min	164.3 / 735.0	415.9 / 397.4	229.4 / 459.5	147.5 / 382.9
50-60 min	241.0 / 211.6	498.3 / 553.2	115.7 / 457.1	368.4 / 361.1
0-60 min	4934.9 / 7123.4	5788.4 / 7152.4	5447.1 / 7593.4	5656.2 / 7425.3

\* Statistically significant ( $p < 0.05$ ) in compare with control group

Organ weights: No test material-related target organ weight effects were identified in male or female rats. The only statistically significant mean weight value was for a slightly greater kidney to bodyweight ratio in females at 1000 mg/kg/day. This finding was attributable to lower mean final bodyweights in this group.

**Table 6.7.1/02-5: Organ weight in females - kidney**

<b>Dose</b>	<b>0</b>	<b>100 (mg/kg/day)</b>	<b>300 (mg/kg/day)</b>	<b>1000 (mg/kg/day)</b>
<b>Parameter</b>	<b>(mg/kg/day)</b>			
Kidney/bodyweight, %	0.7180	0.7809	0.7856	0.8227*
Final bodyweight, g	289	265	276	249*
Kidney weight, g	2.072	2.066	2.161	2.049

\* Statistically significant ( $p < 0.05$ )

Macroscopic observations: No test material-related macroscopic observations were made in male or female rats. All tissues of all male rats examined at necropsy were within normal limits. All tissues of all female rats examined at necropsy were within normal limits except for one 300 mg/kg/day female which had a mild enlargement of a uterine horn.

Neuropathological lesions: There were no treatment-related neuropathological lesions observed in this study. Minimal axonopathy observed in spinal cord and peripheral nerve of occasional Control and Group 4 animals as well as neuronal ectopia of the sural nerve and one example of an epidermoid cyst of the cortical meninges were all judged to be incidental findings consistent with occasional spontaneous lesions of rats.

### Conclusion:

Under the conditions of this study, where male and female rats received 13 weeks of oral (gavage) doses of daminozide technical at 0, 100, 300 and 1000 mg/kg/day, the highest No Observed Adverse Effect Level (NOAEL) was 1000 mg/kg/day, the highest dose level tested. No adverse signs of systemic toxicity or any evidence of neurotoxicity were evident.

**RMS 2018:** The RMS supports the NOAEL for neurotoxicity set at 1000 mg/kg bw/day (top dose).

### B 6.7.2 Delayed polyneuropathy studies

No data available.

### B 6.7.3 Summary of neurotoxicity studies

In the acute neurotoxicity study (■■■■■, 2012a), the neurotoxic potential of daminozide was evaluated after a single administration of 500, 1000, and 2000 mg/kg bw to rats via gavage followed by 14-day observation period. The NOAEL for neurotoxicity derived from this study was set at 1000 mg/kg bw/day based on the decreased locomotor activity (total distance, basic and fine movement).

In the subchronic neurotoxic study (■■■■■, 2012b), the rats were exposed to daminozide by gavage at dose of 100, 300, and 1000 mg/kg bw/day for 90 days. The NOAEL for neurotoxicity was established at 1000 mg/kg bw/day (top dose). No treatment-related neuropathological lesions were observed. The NOAEL for the systemic toxicity was set at 1000 mg/kg bw/day.

The results of neurotoxicity studies are summarized in Table 6.7.3-1.

**Table 6.7.3-1: The results of neurotoxicity studies**

Study	Species	Route	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Critical effects	Reference Notifier
Acute neurotoxicity	rat	oral	1000	2000	decreased locomotor activity (basic and fine	■■■■■ 2012a

					movement, total distance)	
Subchronic neurotoxicity (90 days)	rat	oral	neurotoxicity: 1000 systemic toxicity: 1000	neurotoxicity: >1000 systemic toxicity: >1000	neurotoxicity: no adverse effects systemic toxicity: no adverse effects	2012b

## B 6.8 Other Toxicological Studies

### B 6.8.1 Toxicity studies on metabolites

#### Unsymmetrical Dimethyl Hydrazine (UDMH, CAS 57-14-7)

##### 13 week toxicity study in rats

Reference	13 week toxicity study in rats, 1987a; Report No. A.7.2.4
Guideline	The study was conducted according to OECD 408
Deviations	Yes
GLP	Yes
Acceptability	Supplementary
Previous evaluation	Yes, study already peer-reviewed in original DAR

**RMS comment 2018:** The study has been checked for compliance. The following deviations from OECD guideline 408 (adopted on 21st September 1998) were found:

- 1) The purity of the test substance was not stated
- 2) Blood clotting time/potential was not evaluated
- 3) Thymus, uterus, and epididymides were not weighed at necropsy

#### **Material and method:**

The test article, UDMH, was administered in the drinking water ad libitum to 10 rats at dosage level of 10, 25, 75 and 125 ppm for thirteen weeks. The control rats received the vehicle (deionized water with citrate buffer) on the same regimen. The rats were observed for moribundity and mortality, signs of overt toxicity twice daily throughout the study. Detailed observations of appearance and condition,

behaviour and activity, excretory functions, respiration, orifices, eyes and palpable masses were conducted at least once each week. Individual bodyweights were recorded weekly beginning with the pre-test period. Individual food and water consumption values were recorded weekly. All rats were subjected to an ophthalmoscopic examination prior to study initiation and again at study day 86, prior to the termination of dosing. Haematological and biochemical (leukocyte, erythrocyte count, haematocrit, haemoglobin, MCV, MCH, MCHC, platelets, reticulocytes, ALT, ALP, AST, bilirubin, blood urea nitrogen, Na, K, Ca, P, Cl, cholesterol, creatinine, creatine phosphokinase, glucose, cholesterol, protein), and urologic parameters were determined on samples obtained from all surviving animals during the last week of the study (week 14).

At necropsy, adrenals, brain, heart, kidneys, liver, ovaries, testes were weighed. Histopathological examination was performed on the following organs/tissues: adrenals, aorta, brain, caecum, colon, duodenum, epididymides, eyes, heart, ileum, jejunum, kidneys, liver, lungs, mammary gland, mediastinal and mesenteric lymph node, oesophagus, optic nerve, ovaries, pancreas, pituitary, prostate, rectum, salivary gland, sciatic nerve, seminal vesicles, skeletal muscle, skin, spinal cord [3 levels], spleen, sternum, stomach, testes, thymus, thyroid with parathyroid, trachea, urinary bladder, uterus.

### Results:

The mean weight gain of females in the high dose group was slightly reduced but the difference with the controls was not statistically significant. Water consumption was statistically reduced for the males in the high dose group and for females in the 75 and 125 mg/l groups. This effect is most probably due to the poor palatability of the test substance and is considered of no toxicological relevance since no renal effects (pathological changes) were observed even after prolonged exposure to the test substance. Increases in specific gravity of urine and decreases in urine volume were considered the results of the decreased water consumption. Statistical significance was achieved in the 125 mg/l males and the 25 and 125 mg/l females.

**Table 6.8.1/01-1: 13 week toxicity study in rats; Bodyweight and bodyweight gain [g];**

Dose[ppm] Body weight [g]	Males					Females				
	0	10	25	75	125	0	10	25	75	125
<b>Allocation</b>	124	126	122	125	125	86	86	86	87	88
<b>Week 3</b>	212	210	204	211	206	133	130	130	133	132
<b>Week 6</b>	258	260	248	258	255	157	152	153	152	154
<b>Week 9</b>	289	289	274	286	284	175	169	166*	168	170
<b>Week 13</b>	316	319	305	318	313	188	182	180	185	180
<b>Bodyweight gain[g]</b>	192	193	183	193	188	102	96	94	98	92

\*p<0.05

Table 6.8.1/01-2: 13 week toxicity study in rats; Food consumption [g/animal/day]; \*p&lt;0.05, \*\*p&lt;0.01

Dose[ppm] Food consumption [g/animal/day]	Males					Females				
	0	10	25	75	125	0	10	25	75	125
Week 1	16.4	16.5	15.8	15.7	15.5	13.6	13.1	13.1	13.0*	12.7**
Week 3	18.4	17.8	17.6	18.2	17.9	14.3	13.7	13.9	14.2	14.5
Week 6	19.2	18.4	17.3**	18.6	18.1	15.2	13.8**	14.3	14.2	13.9**
Week 9	17.8	17.4	16.8	17.1	17.2	13.6	12.8*	12.6**	12.7*	12.8
Week 13	17.3	17.3	16.8	17.4	17.4	12.7	12.5	12.1	12.8	12.2

Table 6.8.1/01-3: 13 week toxicity study in rats; Water consumption [g/animal/day]; \*p&lt;0.05, \*\*p&lt;0.01

Dose[ppm] Water consumption [g/animal/day]	Males					Females				
	0	10	25	75	125	0	10	25	75	125
Week 1	17.9	17.6	17.5	17.3	15.5*	17.4	16.0	15.5	14.0**	13.1**
Week 3	20.1	19.6	19.0	19.7	16.9**	20.0	17.4*	16.6**	15.1**	13.8**
Week 6	20.5	20.9	19.5	19.2	17.5*	21.8	19.1	19.0	16.0**	13.7**
Week 9	21.2	21.3	20.7	20.2	18.9*	20.6	18.6	18.0	16.2**	14.1**
Week 13	19.8	20.4	19.5	19.5	19.4	18.6	17.4	17.4	16.1	14.6**

**Conclusion:** All effects observed were considered of no toxicological relevance therefore a provisional NOAEL of 125 mg/l (8.98 mg/kg bw/day) is established by the reviewer for UDMH with unknown purity.

**Note:** The purity of the test substance was not reported, therefore this study is not considered suitable for the overall toxicological evaluation, but will be used as supplementary information.

**RMS 2018:** RMS accepts the overall conclusion of this study, which is considered to be supplementary due to the unknown purity of the test substance.

#### 13 week toxicity study in mice

Reference

13 week toxicity study in mice [REDACTED] 1987b; Report No. A.7.2.4

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Guideline	The study was conducted according to OECD 408 guideline
Deviations	Yes
GLP	Yes
Acceptability	Supplementary
Previous evaluation	Yes, study already peer-reviewed in original DAR

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**RMS comment 2018:** The study has been checked for compliance. The following deviations from OECD guideline 408 (adopted on 21st September 1998) were found:

- 1) The purity of the test substance was not stated
- 2) Blood clotting time/potential was not evaluated
- 3) Plasma/serum levels of sodium and potassium were not measured
- 4) Thymus, uterus, and epididymides were not weighed at necropsy

#### **Material and method:**

The test article, UDMH, was administered in the drinking water ad libitum to 10 CD-1 mice sex at dosage level of 10, 25, 100 and 250 ppm for thirteen weeks. The control rats received the vehicle (deionized water with citrate buffer) on the same regimen. The mice were observed for moribundity and mortality, signs of overt toxicity twice daily throughout the study. Detailed observations of appearance and condition, behaviour and activity, excretory functions, respiration, orifices, eyes and palpable masses were conducted at least once each week. Individual bodyweights were recorded pre-test and weekly during the study. Individual food and water consumption values were recorded weekly.

All mice were subjected to an ophthalmoscopic examination prior to study initiation and again at study day 88, prior to the termination of dosing. Haematological and biochemical (leukocyte, erythrocyte count, haematocrit, haemoglobin, MCV, MCH, MCHC, platelets, reticulocytes, ALT, ALP, AST, total bilirubin, blood urea nitrogen, cholesterol, creatinine, creatine phosphokinase, glucose, cholesterol, protein, Ca, P), and urologic parameters were determined on samples obtained from all surviving animals during the last week of the study (week 13).

At necropsy, adrenals, brain, heart, kidneys, liver, ovaries, testes were weighed. Histopathological examination was performed on the following organs/tissues: adrenals, aorta, brain, caecum, colon, duodenum, epididymides, eyes, heart, ileum, jejunum, kidneys, liver, lungs, mammary gland, mediastinal and mesenteric lymph node, oesophagus, optic nerve, ovaries, pancreas, pituitary, prostate, rectum, salivary gland, sciatic nerve, seminal vesicles, skeletal muscle, skin, spinal cord [3 levels], spleen, sternum, stomach, testes, thymus, thyroid with parathyroid, trachea, urinary bladder, gallbladder, uterus.

#### **Results:**

Mortality: Survival was 100% in the control and treated groups.

There were no treatment-related differences between the appearance and behaviour of the mice in the control and treated groups.

The mean weekly bodyweights and mean weight gains of the treated mice were comparable to those of the control mice throughout the study.

**Table 6.8.1/02-1: 13 week toxicity study in mice; Group mean bodyweight [g];**

Dose[ppm] Week of study	Males					Females				
	0	10	25	100	250	0	10	25	100	250
-1	27	27	26	26	26	21	21	21	21	20
1	28	29	28	29	27	23	22	23	23	22
3	30	32	30	31	29	25	24	25	25	24
6	32	34	32	33	31	28	27	27	27	26
9	34	36	34	35	33	27	27	28	27	27
12	36	37	34	35	33	28	29	29	28	28
13	34	36	34	35	33	30	29	30	29	28
Bodyweight gain[g] (week 13 – week -1)	7	9	8	9	7	9	8	9	8	8

Reduced food consumption (g/animal/day and g/kg/day) relative to the control values, statistically significant in some cases, occurred during several weeks for the 250 ppm treated males and during a few weeks for the 100 ppm males. There was also a decrease in g/kg/day food consumption noted for the males in the 10 ppm dose group during weeks 10 and 11 in comparison to the control values. The food consumption of the males in the 25 ppm dose group was comparable to that of the control males during the study. Reductions in food consumption were present during a few weeks for the females in the 25, 100 and 250 ppm dose groups relative to the control values. However, none of the differences were statistically significant.

**Table 6.8.1/02-2: 13 week toxicity study in mice; Group mean food consumption [g/kg/day]; \*p<0.05**

Dose[ppm] Week of study	Males					Females				
	0	10	25	100	250	0	10	25	100	250

<b>1</b>	173.5	175.8	186.1	175.7	179.2	209.9	211.8	205.0	208.4	198.7
<b>3</b>	177.7	173.2	177.5	160.4*	165.8	223.8	227.9	219.5	213.3	217.6
<b>6</b>	168.5	154.6	164.9	155.9	157.4	210.3	220.8	192.0	201.9	206.7
<b>9</b>	150.3	147.9	156.5	141.6	145.2	200.6	207.5	189.5	189.3	192.9
<b>12</b>	142.8	133.6	143.9	139.9	145.6	196.5	202.8	179.5	182.5	188.4
<b>13</b>	146.5	135.5	149.1	138.0	141.6	185.7	175.6	183.0	168.5	180.4

**Water consumption:** There were decreases in g/animal/day and g/kg/day water consumption during weeks 1-5 for the 100 ppm treated males and during weeks 1-13 for the 250 ppm treated males in comparison to the control values. These differences were statistically significant during the majority of weeks for the 250 ppm treated males. Reductions in water consumption, statistically significant in some instances, occurred during the majority of study weeks for the females in the 100 and 250 ppm dose groups in comparison to that of the control group. Similarly, there were decreases in water consumption relative to the control values during several weeks for the 25 ppm treated females. The water consumption of the males and females in the 10 ppm dose group and of the males in the 25 ppm dose group was comparable to that of the control group.

**Table 6.8.1/02-3: 13 week toxicity study in mice; Group mean water consumption [g/kg/day]; \*p<0.05, \*\*p<0.01**

Dose[ppm] Week of study	Males					Females				
	0	10	25	100	250	0	10	25	100	250
<b>1</b>	232.3	222.4	236.1	204.0	163.9**	302.4	297.8	254.2	262.8	251.4
<b>3</b>	226.5	206.7	221.7	193.3	145.2**	325.1	317.2	283.2	248.3*	259.8
<b>6</b>	233.0	219.6	227.6	227.2	156.3**	324.1	326.1	289.7	273.2	261.1
<b>9</b>	195.9	195.6	211.8	197.1	172.8	336.6	317.5	281.2	273.1	262.8
<b>12</b>	196.3	182.0	200.2	184.0	163.8	321.7	303.7	270.5	250.9	238.2*
<b>13</b>	179.9	170.4	183.0	168.7	149.2	266.5	290.9	262.0	234.8	238.8

**Haematology, biochemistry, and urinalysis:** Animals in the 250 ppm group exhibited signs of anaemia. Females had significantly decreased erythrocytes, haemoglobin and haematocrit values and elevated reticulocytes. Males had decreased erythrocytes and an elevated MCV. There was a statistically significant reduction in urinary volume with a slight elevation in specific gravity (not statistically significant) in the 250 ppm males. This was probably due to the decreased water consumption, although a similar pattern was not noted in females.



**Table 6.8.1/02-4: 13 week toxicity study in mice; Group mean haematological values at the end of the study;**  
 \*p<0.05, \*\*p<0.01

Dose[ppm] Parameter	Males					Females				
	0	10	25	100	250	0	10	25	100	250
Leucocytes x 10 <sup>3</sup> /cmm	5.4	5.1	5.4	4.5	6.7	4.3	2.6	4.5	4.5	4.9
Erythrocytes x 10 <sup>6</sup> /cmm	8.47	8.34	8.41	8.27	7.73*	8.54	8.26	8.10	7.97	7.53**
Haemoglobin [g/dl]	15.8	16.1	16.2	15.5	15.0	16.8	16.5	16.2	16.2	15.2**
Haematocrit [%]	43.7	45.0	44.9	43.4	42.2	46.3	45.1	44.3	44.3	41.6**
MCV [microns <sup>3</sup> ]	52	54	54	52	55*	54	55	55	56	56
MCH [pg]	18.7	19.4	19.3	18.8	19.5	19.7	20.0	20.0	20.3	20.3
Platelets x 10 <sup>3</sup> /cmm	956	1083	1130	1164	1168	891	826	952	982	1037
Reticulocytes/100 RBC	2.3	2.6	3.3	2.8	2.7	2.1	2.1	2.7	2.3	4.0**

**Table 6.8.1/02-5: 13 week toxicity study in mice; Group mean urinalysis values at the end of the study;**  
 \*p<0.05

Dose[ppm] Parameter	Males					Females				
	0	10	25	100	250	0	10	25	100	250
Specific gravity	1.029	1.033	1.029	1.028	1.039	1.023	1.019	1.027	1.024	1.024
pH	6	7	6	6	6	7	6	7	7	6
Volume [mL]	4.1	2.1	0.7	7.8	0.4*	4.9	4.7	7.2	8.7	3.2

**Pathology:** A test article related macroscopic finding was observed in both male female mice consisting of an accentuation of the lobulation of the liver. Accentuation of the lobulation of the liver was more pronounced in the male than in the female mice. It was not seen in the controls of either sex and was present in 10 of 10 male mice in the groups receiving 100 ppm and 250 ppm. All other macroscopic findings in the mice were considered to be incidental or spontaneous in nature and unrelated to administration of the test article. Statistically significant test article related increases occurred only in the liver/gallbladder/bodyweight ratios of males in both the 100 ppm and the 250 ppm dose groups compared to the controls. This was consistent with the microscopic findings of karyomegaly and hypertrophy seen in the liver. Test article related lesions were only seen in the liver and were more pronounced in the male than in the female mice. These included brown pigment in the liver and an increase in both hepatic cell size (hypertrophy) and nuclear size (karyomegaly) in the hepatocytes around the central veins (centrilobular). The single macroscopic nodule seen in the lung of a high dose (250 ppm) male mouse was an

alveolar/bronchiolar adenoma. Two additional microscopic alveolar/bronchiolar adenomas were also seen (one in another high dose male and one in a 100 ppm dose female). Although three alveolar/bronchiolar adenomas are considered unusual in a subchronic study, these neoplasms are sometimes seen as spontaneous lesions in young mice. The small number does not permit conclusions being drawn as to their being treatment related, even if all three did occur in treated animals.

**Table 6.8.1/02-6: 13 week toxicity study in mice; Macroscopic and microscopic findings in the liver**

Dose[ppm]	Males (10 animals)					Females (10 animals)				
	0	10	25	100	250	0	10	25	100	250
<b>Accentuation of liver lobulation</b>										
<b>Mild</b>	0	4	4	2	2	0	0	1	5	2
<b>Moderate</b>	0	0	5	8	8	0	0	0	0	1
<b>Brown pigment</b>										
<b>Trace</b>	0	0	4	3	1	0	0	0	6	8
<b>Mild</b>	0	0	2	6	5	0	0	0	1	1
<b>Moderate</b>	0	0	0	1	4	0	0	0	0	0
<b>Karyomegaly/Hyperthrophy</b>										
<b>Trace</b>	0	0	0	0	0	0	0	0	0	0
<b>Mild</b>	0	2	1	4	7	0	1	0	0	1
<b>Moderate</b>	0	0	0	1	2	0	0	0	0	0

**Table 6.8.1/02-7: 13 week toxicity study in mice; Incidence of alveolar/bronchiolar adenomas**

Dose[ppm]	Males (10 animals)					Females (10 animals)				
	0	10	25	100	250	0	10	25	100	250
<b>Alveolar/bronchiolar adenomas</b>	0	0	0	0	2	0	0	0	1	0

**Original DAR conclusion:**

A NOAEL could not be established based on the bodyweight gain reduction in females and accentuation of liver lobulation in males in all dose levels. The maximum tolerated level for the subsequent chronic study is recommended by the author as approximately 10 mg/l. The NOAEL in this study is smaller than 10 mg/l (< 2 mg/kg bw/day). It should be noticed that alveolar/bronchiolar adenomas were observed at 100 mg/l in females and at 250 mg/l in males.

**Note:** The purity of the test substance was not reported, therefore this study is not considered suitable for the overall toxicological evaluation, but will be used as supplementary information.

**RMS 2018:** As the test substance related liver hypertrophy, karyomegaly, and accentuation of lobulation occurred in all treated male groups, RMS agrees that NOAEL could not be derived from this study. In addition, this study represents only supplementary material because the purity of the test substance was not provided. Based on the results of two year oncogenicity studies with UDMH in mice (██████████, 1989b; ██████████ 1990) strongly indicating the carcinogenic potential of UDMH, alveolar/bronchiolar adenomas observed in 100 ppm and 250 ppm groups during this subchronic study could be related to the treatment.

**Two year oncogenicity study in rats**

Reference	Two year oncogenicity study in rats, ██████████ 1989a; Report No. A.7.3.18
Guideline	The study was conducted according to Guideline 83-2 and OECD 451
Deviations	Yes
GLP	Yes
Acceptability	Yes
Previous evaluation	Yes, study already peer-reviewed in original DAR

**RMS comment 2018:** The study has been checked for compliance. The following deviations from OECD guideline 451 (adopted on 7th September 2009) were found:

- 1) The addition of the fourth test group is recommended if large intervals (6-10fold) between dosages are used
- 2) Historical control data are not involved in the report

**Material and method:**

Groups of 70 male and 70 female ██████████ Fischer 344 rats were offered UDMH (purity: 100 mg UDMH/mL 1N HCl) in the drinking water at concentrations of 0 (control), 1, 50 or 100 ppm. After 12 months, satellite groups

of 20 males and 20 females were used for interim sacrifice and the remaining survivors sacrificed after 24 months of treatment.

The rats were observed for moribundity, mortality, and signs of overt toxicity twice daily. Detailed observations of appearance and condition, behaviour and activity, excretory functions, respiration, orifices, eyes and palpable masses were conducted at least once a week. Individual bodyweights were recorded pre-initiation, once weekly for weeks 1-16 and every 4 weeks thereafter. Individual food consumption measurements were recorded pre-initiation, once weekly for weeks 1-16 and every 4 weeks thereafter. Individual water consumption values were determined weekly (based on the three times a week offering of fresh solution) for the first 16 weeks and once every four weeks thereafter.

Haematological evaluations were conducted on 10 randomly selected animals/sex/group at 6, 12, 18 and 24 months of study. The same animals were used for all time intervals. The following parameters were determined: leukocyte, erythrocyte count, haemoglobin, haematocrit, MCV, MCH, MCHC, platelets, differential leucocyte count.

External abnormalities and palpable masses were examined. Histopathological examination was performed on the following organs/tissues: adrenals, aorta, brain, caecum, colon, duodenum, epididymides, eyes, femur, heart, ileum, jejunum, kidneys, liver, lungs, mediastinal lymph node, mammary gland, lymph nodes, oesophagus, ovaries, pancreas, pituitary, prostate, rectum, salivary gland, sciatic nerve, seminal vesicles, skeletal muscle, skin, spinal cord, spleen, sternum, stomach, testes, thymus, thyroid with parathyroid, trachea, urinary bladder, uterus.

## Results:

Observations: There were no signs of test article-related toxicity noted in animals in any treated group during the study. There were a number of clinical observations seen in all groups, including controls, but all were judged to be of the type that occurs spontaneously in rats. Fewer mortalities were seen at the 100 ppm dosage level, but mortality was similar between the control group and the 1 and 50 ppm groups.

Bodyweight and food consumption: During the two years of study, statistically significant reductions in mean bodyweights, relative to the control values, were noted for the 50 ppm females and the 100 ppm males and females, at several intervals. In the males, the effects were seen primarily after the first 10 weeks, whereas in females, the differences were first noted during study week 20. These effects were seen throughout the remainder of the study. The differences from control ranged from approximately 2 to 5%.

Mean food consumption values on a g/animal/day basis showed statistically significant differences from the control group for all treatment groups occasionally during the study. During the first 52 weeks of study the differences were observed as decreased food consumption values (g/animal/day) for the 50 and 100 ppm dosage groups. After this time period all of the treatment groups showed overall comparable food consumption values to those of the control groups, even though occasional statistically significant differences from the control group were noted. Food consumption values, on a g/kg/day basis, were variable between the control and treated groups, with no discernible treatment-related effects.

Since the food consumption decreases were slight and variable by week, the slight decreases in bodyweight were probably related to the decrease in water consumption.

**Table 6.8.1/03-1: Two year oncogenicity study in rats; Group mean bodyweight [g]; \*p<0.05, \*\*p<0.01**

Dose [ppm] Week of study	Males				Females			
	0	1	50	100	0	1	50	100
-1	113	114	112	114	93	93	92	91
1	146	148	145	144	112	112	112	110
3	212	214	210	207	141	142	140	138**
6	264	267	263	259	164	166	164	163
9	302	305	300	296	179	179	180	178
12	328	330	328	320**	190	191	190	190
15	349	351	348	339**	198	199	198	196
20	370	372	366	358**	207	208	205	202**
24	386	388	385	376**	213	213	212	209*
28	392	393	390	382**	216	215	215	211**
32	401	402	400	392*	219	218	217	214**
36	406	409	404	395**	225	224	220*	217**
40	416	417	413	404**	230	229	224**	222**
44	421	421	420	412*	236	234	230**	227**
48	429	429	427	420*	242	240	233**	231**
52	429	430	429	422	243	243	237*	234**
56	438	436	433	425**	247	248	241	238**
60	444	445	439	429**	255	257	249	245**
64	446	446	441	433*	261	263	255	248**
68	449	448	444	438	269	268	262	256**
72	443	446	441	432	273	273	267	258**
76	441	443	437	430	275	281	272	265*
80	436	442	438	431	278	284	274	269*
84	435	441	434	428	283	288	282	273**
88	431	427	418*	412**	281	282	274	269**
92	427	421	416	410**	283	283	276	271**
96	408	402	397*	396*	280	276	267*	261**
100	393	399	392	393	280	281	272	266*

<b>104</b>	382	383	388	381	279	276	263	267
<b>Bodyweight gain [g] (week 104 - week -1)</b>	269	269	276	267	186	183	171	176

**Table 6.8.1/03-2: Two year oncogenicity study in rats; Group mean food consumption [g/kg/day]; \*p<0.05, \*\*p<0.01**

<b>Dose [ppm] Week of study</b>	<b>Males</b>				<b>Females</b>			
	<b>0</b>	<b>1</b>	<b>50</b>	<b>100</b>	<b>0</b>	<b>1</b>	<b>50</b>	<b>100</b>
<b>1</b>	95.0	95.9	94.8	93.2*	104.1	104.0	103.0	102.2*
<b>3</b>	78.6	79.8	79.2	79.2	87.0	87.2	88.7	89.4**
<b>6</b>	61.5	61.2	60.9	61.4	71.1	71.3	72.2	72.0
<b>9</b>	53.8	53.1	53.6	53.8	61.7	61.4	65.0**	62.9
<b>12</b>	49.4	48.6	49.3	49.8	58.0	57.7	61.4**	58.9
<b>15</b>	47.1	47.0	47.4	47.0	57.1	57.4	57.6	57.0
<b>20</b>	41.7	41.7	42.2	43.2**	52.4	52.6	53.8**	53.9**
<b>24</b>	41.2	40.9	41.0	42.0*	51.7	51.4	52.4	52.5
<b>28</b>	42.1	42.7	41.8	42.9**	53.2	53.7	53.7	53.7
<b>32</b>	43.8	42.7**	42.3	43.1	54.4	54.1	54.1	56.1**
<b>36</b>	41.7	41.1	41.0*	41.8	54.3	54.9	56.3**	55.7**
<b>40</b>	40.9	40.2	39.1**	39.7**	53.0	52.1	52.6	51.8
<b>44</b>	40.7	41.0	40.3	40.9	53.1	53.9	54.4	53.8
<b>48</b>	39.5	39.7	39.9	39.9	49.6	49.5	50.3	50.9*
<b>52</b>	40.9	41.2	40.7	40.7	51.4	50.1	52.2	52.4
<b>56</b>	42.2	41.4*	41.6*	41.7	53.8	52.6	55.4	55.4*
<b>60</b>	40.4	41.0	40.8	41.2	52.6	52.7	54.0	54.0
<b>64</b>	40.7	38.8**	39.7*	39.9	51.5	52.0	52.0	52.8
<b>68</b>	39.5	37.8**	39.5	39.2	49.6	49.2	51.2*	51.9**
<b>72</b>	38.8	37.9	38.7	39.0	48.3	48.4	50.7**	51.2**
<b>76</b>	38.4	38.2	39.1	40.2**	47.8	48.5	51.5**	55.4**
<b>80</b>	39.2	39.0	38.9	40.1	47.6	47.4	48.2	49.8*
<b>84</b>	37.6	38.2	38.1	40.3*	47.2	47.2	47.4	48.8
<b>88</b>	40.4	39.0	37.6*	39.9	46.9	46.2	47.0	49.2**

<b>92</b>	42.2	41.3	40.1*	41.8	49.2	48.0	50.1	51.2
<b>96</b>	45.3	43.0	44.9	44.1	50.1	50.1	50.8	54.8**
<b>100</b>	43.5	44.0	45.1	43.6	50.1	51.9	52.3	53.7**
<b>104</b>	47.7	48.0	46.6	45.7	52.7	49.6*	51.9	52.0

Water and test article consumption: Variations in mean water consumption, statistically significant in some instances, were present for all of the treated groups relative to the control group values occasionally during the two year study. Mean water consumption values (g/animal/day) were reduced for the 50 ppm females and 100 ppm males and females consistently throughout the study, when compared to the control group. Reduced water consumption values, relative to the control group, were also noted for the 50 ppm males in the first 12 weeks of study and during the last year of study. On a g/kg/day basis, males in the 50 ppm group had reduced values primarily during the first 11 weeks, while males and females at the 100 ppm level had reduced values throughout the first year of study. The 50 ppm females had reduced values primarily during the first 16 weeks of study. After the first year of study, mean water consumption values on a g/kg/day basis were variable between the treated groups and the control group. However, during the last 3 to 6 months differences between the high dose and control values were notable.

Average mean compound consumption values (mg/kg/day) for the two year study is as follows.

**Table 6.8.1/03-3: Two year oncogenicity study in rats; Average mean compound consumption values [mg/kg/day]**

Dosage level (ppm)	Average mean compound consumption for two years (mg/kg/day)	
	Male	Female
0 (control)	0.000	0.000
1	0.065	0.093
50	3.168	4.533
100	6.200	7.895

**Table 6.8.1/03-4: Two year oncogenicity study in rats; Group mean water consumption [g/kg/day]; \*p<0.05, \*\*p<0.01**

Dose [ppm] Week of study	Males				Females			
	0	1	50	100	0	1	50	100
<b>1</b>	125.9	128.6	123.0	117.6**	149.4	148.1	141.8**	132.5**
<b>3</b>	108.3	109.6	104.6*	97.6**	141.2	136.1	127.4**	114.1**
<b>6</b>	84.0	83.4	80.2**	76.4**	116.4	113.6	108.8*	89.8**

9	67.6	67.0	66.2	61.6**	102.4	99.1	94.8*	76.5**
12	60.2	60.3	61.5	59.5	104.9	96.6*	90.0**	69.8**
15	58.1	58.9	59.8**	58.3	92.8	89.1	87.7	77.0**
20	49.3	47.8	50.3	46.9	81.0	75.5	80.4	65.1**
24	49.4	50.1	49.9	49.1	80.2	80.8	80.3	63.9**
28	49.2	50.5*	48.3	49.4	84.7	80.9	80.8	64.8**
32	49.9	50.2	48.5	48.2*	86.7	83.3	80.8	68.7**
36	48.0	48.1	46.6	46.9	79.7	77.8	78.6	64.7**
40	49.3	49.4	47.9**	48.9	82.3	78.1	79.5	67.5**
44	48.1	48.9	47.3	46.8*	82.8	78.7	77.8	69.6**
48	44.9	47.3**	46.6*	45.4	75.8	74.7	69.4*	63.3**
52	47.1	48.6	49.6	46.2	78.6	73.6	73.8	64.8**
56	46.6	46.5	45.9	43.6*	74.4	72.0	73.6	66.7*
60	45.9	46.3	46.5	46.5	78.3	73.5	73.1*	68.4**
64	48.6	49.1	47.7	48.8	78.4	78.2	77.2	71.7*
68	51.4	48.5**	48.3**	49.7	79.0	76.8	78.3	73.4
72	50.0	49.8	47.2	48.6	73.8	72.3	73.8	70.3
76	51.4	52.3	49.0	50.0	79.9	78.3	80.3	72.2**
80	52.7	54.2	52.1	52.9	77.7	77.6	75.9	68.1**
84	53.1	54.8	53.3	53.5	77.8	78.3	76.2	70.0**
88	63.9	62.6	57.1*	56.8*	81.5	78.7	80.1	74.1*
92	68.8	65.1	60.0**	62.8**	87.4	81.4	83.0	76.5**
96	84.3	67.8**	67.9**	64.6**	85.9	90.0	81.3	87.3
100	86.9	77.9	75.2*	73.2**	90.2	92.8	89.5	85.9
104	91.7	77.2*	78.2*	71.4**	101.1	91.8	94.5	87.1**

Haematology: Statistical significance was noted for a few parameters when comparing the treated and control groups. However, the differences were considered to be due to normal animal variation. In all cases, the affected values were within the normal range of historical control values for this species in this laboratory.

Pathology:

*Macroscopic findings*

A variety of macroscopic lesions were seen in all dose groups. The most frequently seen macroscopic lesions in both the interim and terminal sacrifice groups included: cloudy corneas in both males and females; cysts in the



ovaries of the females; distended uteri in the females; and small testes in the males. In the animals that died after 12 months, or the animals that were sacrificed at 24 months, additional common macroscopic findings included: granular kidneys in the males, accentuated lobulation/mottling of the liver in both males and females; nodules/masses in the liver of both males and females; enlarged pituitary in both males and females; small seminal vesicles in the males; tan/yellow/white foci in the tests of the males; masses in the skin/subcutis in both the males and females; and enlarged spleen in both males and the females. Most of these findings were considered to be spontaneous and not related to the administration of the test article. The incidence of cloudy corneas in females in the 50 and 100 ppm dosage groups appeared to be slightly increased compared to the controls.

**Table 6.8.1/03-5: Two year oncogenicity study in rats; Group incidence of cloudy corneas and corneal mineralization; number of affected animals/number of examined animals**

Dose[ppm]	Males				Females			
	0	1	50	100	0	1	50	100
<b>Cloudy cornea</b>								
<b>0-12 months</b>	1/20	2/20	1/20	0/20	0/20	3/20	2/20	1/20
<b>12-24 months</b>	14/50	12/49	13/50	18/50	19/48	20/50	25/50	29/50
<b>Mineralization</b>								
<b>12-24 months</b>	24/50	28/49	29/50	24/50	24/48	23/50	31/50	29/50

#### *Microscopic findings*

A variety of neoplastic and non-neoplastic lesions were evident in both sexes of all dose groups. Chronic progressive nephropathy was common in the male and female rats that died or were sacrificed after 12 months and was much more severe in the males than in the females. The macroscopic granular kidneys in the males often correlated microscopically with chronic progressive nephropathy. Chronic progressive nephropathy was characterised by the presence of fibrosis, proteinaceous casts, cysts and lymphoid accumulations. Chronic progressive nephropathy is a common spontaneous lesion in old rats and is more prominent in the males than the females.

Multifocal mineralisation of the renal cortex was a common finding in both male and female rats. Multifocal mineralisation of the vascular tissue of the lungs and of the aorta was also a common finding in both male and female rats.

Chronic myocarditis was a common microscopic finding in the mandibular lymph node of both male and female rats, but was much less common in other lymph nodes.

Macroscopic corneal opacity occurred frequently and often correlated microscopically to corneal mineralisation. Macroscopic corneal opacity appeared to be slightly more common in females in the 50 and 100 ppm dosage groups, and microscopic corneal mineralisation also appeared to be increased in the same groups.

Vacuolar change of the adrenal cortex was a common microscopic finding in both male and female rats.

Mononuclear cell leukaemia was a common microscopic finding in both male and female rats and often correlated macroscopically with the enlarged spleens and the granular/mottled livers. The lymphoma actually occurred at a higher incidence in the controls than in the treated animals. This disease is one of the more common haematopoietic neoplasms in rats, especially the Fischer rat.

Ovarian cysts and hydrometra of the uterus were common findings in the female rats and often correlated to macroscopic ovarian cysts and macroscopically enlarged/distended uteri, respectively. Polyps of the uterus were also a common finding in the uterus of the female rats. Interstitial cell tumours of the testes were a common finding in the male rats and often correlated macroscopically with coloured foci in the testes. Macroscopic small/soft testes in the male rats often correlated microscopically with testicular atrophy.

Pituitary adenomas were a common finding in both male and female rats, but were more common in female rats. They often correlated macroscopically with pituitary enlargement. Pituitary adenomas are a common spontaneous lesion in female Fischer rats.

Bile duct hyperplasia in the liver was a common finding in the male and female rats, but was equally prominent in all dose groups. Inflammation of the liver was also a common finding in the male and female rats and was characterised by small multifocal areas of chronic inflammation. The chronic inflammation of the liver appeared to be slightly more prominent in the treated rats compared to the controls. A variety of cytologic alterations occurred in the livers of both male and female rats and included clear, eosinophilic and basophilic and vacuolated cell foci. The basophilic cell foci were the most common, but the incidence of these foci did not appear to be influenced by the administration of the test article.

Most of the above lesions occurred across dose levels and appeared to be spontaneous in origin and consistent with what would normally be expected in the 24 month chronic study in Fischer rats. None of the neoplastic lesions described above appeared to be related to administration of the test article.

The incidence of hepatocellular neoplasms appeared to be increased in the treated females, but not in the treated males. This is shown in Table 6.8.1/03-6. The historical incidence of hepatocellular neoplasms in female Fischer rats at International Research and Development Corporation (IRDC) in two year chronic studies is extremely low. In six chronic two-year studies, only two hepatocellular adenomas and no hepatocellular carcinomas occurred out of a total of 370 control female Fischer rats (0.5%). However, the incidence of hepatocellular neoplasms reported for the Fischer rat used in chronic studies conducted by the National Toxicology Program (NTP) is much higher.

Haseman *et al.*<sup>1</sup> reported that the incidence of hepatocellular neoplasms averaged 2.7% in female Fischer rats (range 0-10%).

**Table 6.8.1/03-6: Two year oncogenicity study in rats; Tumour incidence (overall rate); \*p<0.05, \*\*p<0.01**

Dose[ppm]	Males				Females			
	0	1	50	100	0	1	50	100
<b>LIVER</b>								
<b>Hepatocellular adenoma</b>	2/70 (2.9%)	0/70 (0%)	1/70 (1.4%)	2/70 (2.9%)	0/70 (0%)	1/70 (1.4%)	2/70 (2.9%)	1/70 (1.4%)
<b>Hepatocellular carcinoma</b>	1/70 (1.4%)	0/70 (0%)	0/70 (0%)	1/70 (1.4%)	0/70 (0%)	0/70 (0%)	3/70 (4.3%)	4/70 (5.7%)
<b>Hepatocellular adenoma/carcinoma</b>	3/70 (4.3%)	0/70 (0%)	1/70 (1.4%)	3/70 (4.3%)	0/70 (0%)	1/70 (1.4%)	5/70* (7.1%)	5/70* (7.1%)
<b>ADRENAL</b>								
<b>Pheochromocytoma (benign)</b>	3/70 (4.3%)	5/70 (7.1%)	1/70 (1.4%)	6/70 (8.6%)	-	-	-	-
<b>HAEMOLYMPHORETICULAR SYSTEM</b>								
<b>Mononuclear cell leukemia</b>	33/70 (47.1%)	21/70* (30.0%)	21/70* (30.0%)	16/70** (22.9%)	21/70 (30.0%)	18/70 (25.7%)	8/70** (11.4%)	10/70* (14.3%)
<b>MAMMARY REGION</b>								
<b>Fibroadenoma</b>	-	-	-	-	6/70 (8.6%)	5/70 (7.1%)	5/70 (7.1%)	3/70 (4.3%)
<b>PITUITARY</b>								
<b>Pituitary adenoma</b>	16/70 (22.9%)	15/70 (21.4%)	18/70 (25.7%)	13/70 (18.6%)	17/69 (24.6%)	23/69 (33.3%)	19/70 (27.1%)	32/70** (45.7%)
<b>SKIN</b>								
<b>Fibroma</b>	3/70 (4.3%)	2/70 (2.9%)	3/70 (4.3%)	2/70 (2.9%)	-	-	-	-
<b>TESTIS</b>								
<b>Interstitial cell tumour (benign)</b>	47/70 (67.1%)	42/70 (60.0%)	50/70 (71.4%)	45/70 (64.3%)	-	-	-	-
<b>THYROID</b>								

<b>Parafollicular cell adenoma</b>	6/70 (8.6%)	6/70 (8.6%)	3/70 (4.3%)	6/70 (8.6%)	2/70 (2.9%)	4/70 (5.7%)	3/70 (4.3%)	4/70 (5.7%)
<b>UTERUS</b>								
<b>Polyp</b>	-	-	-	-	9/70 (12.9%)	9/70 (12.9%)	4/70 (5.7%)	10/70 (14.3%)

#### Original DAR conclusions

- 2 years of oral exposure of rats to UDMH resulted in intermittent changes in bodyweights and water and food consumption between the two highest dose groups and control groups.
- An increased incidence of cloudy corneas was observed in the 50 mg/l and 100 mg/l females. This frequently correlated microscopically to corneal mineralization.
- Microscopically, chronic inflammation of the liver occurred in all groups. Statistically significant differences were found in the 50 mg/l and 100 mg/l females and the 100 mg/l males. There were no other non-neoplastic lesions considered to be treatment related.
- The incidence of hepatocellular neoplasms (adenomas and carcinomas) appeared to be increased in treated females, but not in treated males. An analysis of each tumour type individually did not provide statistically evidence of an oncogenic effect. When hepatocellular adenomas and carcinomas were combined, statistical significance was not achieved by the Fischer's exact test (two-sided) but significant dose related trends were determined by the Cochran Armitage trend analysis. The incidence of these tumours combined (>1%) is higher than in the historical controls. The increase in hepatocellular carcinomas, a rare neoplasm in female F344 rats at doses almost devoid of other toxic effects strongly suggests an oncogenic effect of UDMH in female rats.
- The present study is a carcinogenicity study and as such no NOAEL for chronic toxicity should be established. However, since the dose without effects was the lowest NOAEL in repeated dose studies with UDMH in the present dossier, a provisional NOAEL of 1 mg/l (0.1 mg/kg bw/day) is established.

**RMS 2018:** The RMS agrees that the occurrence of hepatocellular carcinomas in females at the dose of 50 and 100 ppm strongly indicates the oncogenic effect of UDMH. The RMS suggests that NOAEL for carcinogenicity cannot be established due to the occurrence of hepatocellular adenoma in all treated female groups. However, setting the provisional NOAEL at 0.1mg/kg bw/day is supported.

**Two year oncogenicity study in mice (UDMH) – low dose**

Reference	<b>Two year oncogenicity study in mice (UDMH) – low dose,</b> [REDACTED] <i>1989b</i> ; Report No. A.7.3.19
Guideline	The study was conducted according to OECD guideline 451
Deviations	Yes
GLP	Yes
Acceptability	Supplementary
Previous evaluation	Yes, study already peer-reviewed in original DAR

**RMS comment 2018:** The study has been checked for compliance. The following deviations from OECD guideline 451 (adopted on 7th September 2009) were found:

- 1) The purity of the test substance was not stated

**Material and method:**

Groups of 90 male and 90 female CD-1 mice were offered UDMH in the drinking water ad libitum at concentrations of 0 (control), 1, 5, 10 (only males), and 20 (only females) ppm. The control mice received the vehicle, 0.2M Na<sub>2</sub>HPO<sub>4</sub>/0.1M citric acid in deionized water, on the same regimen.

The mice were observed for moribundity, mortality, and overt toxicity three times daily on Monday through Friday, and twice daily on weekends and holidays. Detailed observations of appearance and condition, behaviour and activity, excretory functions, respiration, orifices, eyes and palpable masses were conducted at least once a week.

Individual bodyweights were obtained pre-initiation, weekly for the first 16 weeks and once every four weeks thereafter. Individual food and water consumption measurements were determined weekly for the first 16 weeks and once every four weeks thereafter.

Haematological evaluations (leukocyte count, differential leukocyte count, erythrocyte count, haemoglobin, haematocrit, MCV, MCH, MCHC, platelets) were conducted at 6, 12, 18 and 24 months of study on 10 randomly selected animals/sex/group. At 24 months biochemical analysis (alkaline phosphatase, total bilirubin, AST, ALT, SDH) were performed on these animals.

All animals in the 8 month and 12 month interim (20 mice/sex/group) and terminal sacrifice, and all animals not surviving to termination received a complete post-mortem examination.

Histopathological examination was performed on the following organs/tissues: adrenals, aorta, brain, caecum, colon, duodenum, stomach, epididymides, eyes, femur, heart, ileum, jejunum, kidneys, liver, lungs, mediastinal lymph node, mammary gland, lymph nodes, oesophagus, ovaries, pancreas, pituitary, prostate, rectum, salivary gland, sciatic nerve, seminal vesicles, skeletal muscle, skin, spinal cord, spleen, sternum, testes, thymus, thyroid with parathyroid, trachea, urinary bladder, gallbladder, uterus.

**Results:**

**Mortality:** During the study 25, 27, 26, and 34 males died/were sacrificed *in extremis* in the control, low- (1 ppm), mid- (5 ppm) and high- (10 ppm) dose groups, respectively. Thirty, 25, 32 38 females died or were sacrificed *in extremis* during the study in the control, low-, mid- and high- (20 ppm) dose groups, respectively. The majority of this mortality occurred during the second year of the study, when the animals were nearing the end of their normal lifespan.

**Clinical findings:** An increased incidence of moribundity, decreased activity, tremors, hypothermia and labored breathing were noted between weeks 79 and 91 for the high-dose females in comparison to the control group. With the exception of these agonal findings, there were no treatment-related differences in the appearance or behaviour of the mice in the treated groups relative to those of the control mice.

**Bodyweight, food and water consumption:** During the first 20 weeks of study, statistically significant reductions in weekly bodyweights relative to the control values were noted for the treated males for several weeks. However, these differences were comparatively slight and did not follow a dose-related trend. Sporadic statistically significant differences from the mean control bodyweight values were noted for the high-dose females. However, with the exception of an 11% reduction at study week 100, these differences were too slight to be meaningful. Variations in mean water consumption, statistically significant in some instances, were present for the treated animals relative to the control values. However, no consistent dose-related trends were apparent.

**Table 6.8.1/04-1: Two year oncogenicity study in mice (UDMH) – low dose; Group mean bodyweight [g];**

\*p<0.05, \*\*p<0.01

Dose [ppm] Week of study	Males				Females			
	0	1	5	10	0	1	5	20
-1	29	28**	29	28**	22	22	22	22
1	30	30*	30	29**	24	23	23	23**
3	33	31**	32*	31**	25	24	25	25
5	34	33*	33	33	27	26	26	26
9	36	34**	35	34**	28	28	29	28
12	37	36**	36**	36**	29	28**	29	29*
15	38	36**	37**	36**	30	29	30	29
20	39	37**	38*	38**	31	31	31	30*
24	39	38	38	38	31	31	32	31
28	39	38**	38*	38	31	31	32	31*
32	39	38	39	38	32	32	32	31
36	40	39	39	39	32	32	32	32

40	39	38	39	38	32	32	32	32
44	40	39	40	39	33	32	32	32
48	40	40	40	40	33	33	32	33
52	40	39	40	40	33	33	32	33
56	40	38	40	39	34	34	33	32*
60	41	39	40	39	34	34	34	32*
64	40	39	40	39	34	33	33	32**
68	40	38	40	39	34	34	33	32
72	39	38	39	38	33	33	33	32
76	40	38	40	38	34	34	33	33
80	39	38	39	37	33	34	33	33
84	39	37	39	38	33	33	34	33
88	38	38	39	38	34	34	34	33
92	38	37	39	38	34	34	33	33
96	39	39	40	38	36	35	35	34
100	38	38	39	37	35	34	33	31**
104	38	38	38	36	34	35	35	32
Bodyweight gain [g] (week 104 - week -1)	9	10	9	8	12	13	13	10

**RMS comment 2018:** The results of statistics in the table documenting the group mean bodyweights do not seem to be plausible. Although the mean value of bodyweight in the control and one of the treated groups is during several time periods identical, statistically significant difference is marked by asterisk.

**Table 6.8.1/04-2: Two year oncogenicity study in mice (UDMH) – low dose; Group mean food consumption [g/kg/day]; \*p<0.05, \*\*p<0.01**

Dose [ppm] Week of study	Males				Females			
	0	1	5	10	0	1	5	20
1	165.7	163.4	165.1	171.5**	185.6	195.8**	197.1**	193.7**
3	148.1	146.8	146.6	150.0	182.5	187.5	181.3	188.4
6	140.0	136.0	142.5	144.1*	171.1	171.3	173.6	171.5
9	138.4	137.2	136.8	142.0*	172.6	175.0	174.2	176.5
12	128.1	128.5	128.9	133.8**	154.9	168.1**	165.2**	163.3*
15	131.3	130.4	135.3*	137.7**	164.1	165.7	172.0	170.0

20	133.8	133.7	135.2	143.0**	162.7	168.9	171.6**	179.4**
24	137.4	132.5**	131.7**	135.7	168.8	172.4	168.2	170.3
28	129.2	126.9	127.8	131.0	158.0	160.0	161.8	160.6
32	127.0	125.8	121.8**	125.0	154.5	150.9	156.5	165.3**
36	126.0	120.5**	119.4**	121.7*	151.8	155.9	149.7	152.4
40	124.1	120.4*	120.7*	123.8	152.0	154.0	153.3	156.4
44	116.8	113.3*	113.7	120.2*	141.6	143.6	146.0	148.5
48	111.4	106.8**	110.0	112.0	137.5	135.5	138.9	138.3
52	108.7	108.0	112.5*	112.9*	136.5	134.9	141.7	144.2*
56	117.1	119.3	117.3	119.9	139.6	141.3	151.9**	142.5
60	111.0	114.2	111.9	114.1	134.7	138.5	140.7	136.3
64	113.5	112.7	110.8	116.1	133.1	138.6	140.0*	138.1
68	115.8	117.3	113.3	121.5**	134.9	136.5	143.4*	141.7
72	134.0	137.5	135.1	136.8	161.3	171.5	168.7	164.1
76	133.9	135.0	136.8	137.9	156.5	163.6	163.7	162.5
80	136.3	134.5	138.8	137.9	157.7	164.9	163.4	157.8
84	129.5	134.0	129.2	126.2	153.0	157.4	155.2	146.8
88	138.8	133.4	138.4	139.0	161.3	165.4	159.1	158.0
92	129.3	129.8	127.8	128.4	143.9	149.0	143.4	145.1
96	124.2	125.4	123.9	121.9	140.9	142.8	140.4	142.1
100	124.9	128.9	124.2	125.7	144.2	146.0	142.0	149.7
104	130.9	131.5	130.3	135.6	149.7	148.9	157.5	154.9

**Table 6.8.1/04-3: Two year oncogenicity study in mice (UDMH) – low dose; Group mean water consumption [g/kg/day]; \*p<0.05, \*\*p<0.01**

Dose [ppm] Week of study	Males				Females			
	0	1	5	10	0	1	5	20
1	244.9	257.9*	269.2**	247.0	279.8	310.0**	287.7	326.8**
3	237.7	239.6	245.2	248.3	297.1	294.1	295.1	300.9
6	200.9	189.9*	213.7*	216.5*	255.2	252.3	248.1	260.7
9	201.0	190.4*	199.9	200.2	275.1	265.4	270.2	265.6
12	173.5	175.0	178.8	189.6*	230.3	269.7**	245.3	249.1*



15	178.9	164.9*	178.3	170.3	230.3	231.9	224.3	240.3
20	168.0	165.4	174.7	179.5	245.1	242.3	234.0	250.4
24	156.7	160.4	187.5**	177.7**	241.7	230.3	240.5	267.8*
28	174.9	159.8**	172.1	144.0**	249.8	244.9	252.2	249.8
32	167.2	163.7	171.0	153.2*	250.4	243.3	258.7	260.8
36	162.6	156.1	164.7	153.5	240.2	249.0	259.3	254.5
40	174.0	164.2	175.4	167.2	265.1	269.3	287.3	268.2
44	150.3	138.4*	152.9	153.5	213.4	233.5	267.9**	242.0
48	150.9	135.7**	149.8	145.0	229.8	224.4	258.0	246.9
52	158.1	145.2*	166.2	151.3	223.6	244.5	267.2*	258.3
56	156.2	155.3	161.2	156.1	232.0	262.6	293.1*	249.4
60	161.0	155.9	164.3	171.3	251.6	263.5	300.3	293.7
64	168.7	165.4	165.8	165.2	248.8	282.5	306.2	263.0
68	168.2	172.5	169.6	170.9	239.2	282.2	310.8**	301.0*
72	173.9	193.3	193.7	192.7	270.2	296.6	332.6	315.4
76	183.1	207.7	198.7	187.9	295.6	295.2	342.4	278.3
80	186.6	207.9	201.8	195.7	273.0	304.2	342.1	293.3
84	200.0	201.9	203.5	192.6	267.3	298.7	343.1	222.1
88	192.4	214.2	199.2	224.0	280.7	290.3	342.0	273.0
92	158.6	192.7	179.3	169.8	208.9	216.0	286.2*	205.2
96	161.7	187.0*	187.2	207.9	228.8	267.2	286.5*	269.1
100	176.4	198.2	182.3	196.0	247.1	245.7	266.7	261.4
104	202.3	211.6	226.9	206.4	267.7	260.8	337.3	255.3

Haematology and biochemistry: Possible test article related haematological findings occurred at the 18 month interval where females in the 5 and 20 ppm groups had decreased erythrocyte counts (statistically significant). The 5 ppm females also had statistically significant decreased haemoglobin and haematocrit at this interval. There were no test article related effects on biochemistry noted at the 24 month interval.

**Table 6.8.1/04-4: Two year oncogenicity study in mice (UDMH) – low dose; Group mean haematological values [g/kg/day]; \*p<0.05; M/F = males/females**

Dose [ppm]	0				1				5				10/20 (M/F)			
Mont	6	12	18	24	6	12	18	24	6	12	18	24	6	12	18	24

Dose [ppm]	0				1				5				10/20 (M/F)			
h																
<b>Erythrocytes x 10<sup>6</sup>/cmm</b>																
♂	7.84	7.71	7.67	8.63	7.63	7.50	7.47	7.98	7.78	7.56	7.54	7.91	7.57	7.40	7.16	8.21
♀	7.90	7.72	7.66	7.36	7.63	7.08	7.07	7.64	7.82	7.48	6.87*	7.37	7.89	7.50	6.80*	7.51
<b>Haemoglobin [g/dl]</b>																
♂	15.2	14.5	15.3	16.5	15.1	14.5	15.3	15.6	15.0	14.3	14.9	15.6	14.9	14.3	14.9	15.7
♀	15.5	14.9	15.8	15.1	15.2	14.0	15.0	15.5	15.6	14.8	14.4*	14.8	15.8	14.7	14.6	15.6
<b>Haematocrit [%]</b>																
♂	42.2	43.1	43.8	46.2	42.6	42.6	43.8	44.1	41.5	37.9	42.8	43.5	41.5	42.3	42.6	43.9
♀	43.6	43.9	44.9	42.7	42.7	40.6	42.3	43.7	43.7	43.2	40.7*	41.5	44.5	43.4	41.1	44.7
<b>MCV [microns<sup>3</sup>]</b>																
♂	54	56	57	54	56	57	59	55	54	55	57	55	55	57	59	54
♀	55	57	59	59	56	57	60	57	56	58	59	57	56	58	61	59
<b>MCH [pg]</b>																
♂	19.3	18.8	20.0	19.1	19.8	19.3	20.4	19.6	19.3	18.9	19.7	19.7	19.7	19.4	20.8	19.3
♀	19.6	19.3	20.7	21.1	20.0	19.8	21.1	20.3	20.0	19.8	21.0	20.1	20.1	19.6	21.6	20.7

**Pathology:***Macroscopic findings*

The only common macroscopic lesions seen in the 0 to 8 month animals included cysts in the ovaries and enlarged/cyst/cystic uteri in the females. In the 8 to 12 month animals, common macroscopic lesions included cyst/cystic uteri in the females, nodules/masses in the livers of the males and nodules in the lungs of both males and females. The cystic uteri, liver nodules/masses and the lung nodules occurred randomly across dose levels and did not appear to be related to the administration of the test article. A variety of macroscopic findings were evident in the 12 to 24 month old animals. Some of the more frequently seen macroscopic lesions included cloudy corneas of the eyes in the males and females, cysts in the kidneys of the males, granular surface of the kidneys of both the males and females, masses/nodules in livers and the lungs of both males and females, enlarged seminal vesicles in the males, ovarian cysts in the females, cyst/cystic uteri in the females, enlarged spleens in the females

and masses in the uteri of the females. The only macroscopic finding possibly related to the administration of the test article included masses/nodules in the lungs.

#### *Microscopic findings*

In the 0 to 8 month animals, brown pigment occurred in livers of three high dose (10 ppm) males and may have been related to the administration of the test article. A small number of hepatocellular adenomas and a small number of alveolar/bronchiolar adenomas were also seen, but a dose response was not evident. In the 8 to 12 month animals, some of the common microscopic findings included cystic endometrial hyperplasia in the female mice, amyloidosis of the ileum in female mice, A-cell hyperplasia in the adrenal cortex in both males and females, brown pigment in the cortex of the adrenal glands of both male and female mice, lymphocytic infiltrations in the kidneys of both male and female mice, focal calcification of the cerebrum in both males and females, inflammation of the liver in both males and females, and hyperplasia of the mucosa of the glandular stomach in both males and females. Brown pigment and cellular hypertrophy (enlargement) occurred in the livers of 1 died on study and 3 sacrificed high dose males and may have been related to the administration of the test article. None of the neoplastic findings at the 8 to 12 month interval appeared to be related to the administration of the test article.

A variety of neoplastic and non-neoplastic microscopic lesions were evident in both sexes across dose levels in the 12 to 24 month animals. Some of the more common non-neoplastic lesions that occurred in both males and females included A-cell hyperplasia of the adrenal cortex, amyloidosis in a variety of organs, brown pigment in the adrenal cortex, mineralization of the cerebrum, mineralization of the cornea of the eyes, chronic nephritis in the kidneys, focal mineralization of the kidneys, alveolar macrophages in the lungs and chronic inflammation and hyperplasia of the glandular stomach. Common non-neoplastic lesions restricted to the males included mineralization and atrophy of the testes, and common non-neoplastic lesions restricted to the females included cystic endometrial hyperplasia of the uterus and ovarian cysts. Macroscopic granular kidneys often correlated microscopically to either chronic nephritis or renal amyloidosis. None of the above non-neoplastic lesions appeared to be increased by the administration of the test article. Chronic inflammation of the liver was a common microscopic finding in both male and female mice. It occurred across dose levels and did not appear to be affected by the administration of the test article. Brown pigment in the liver was also a common finding in the livers of both male and female mice and was sometimes associated with the focal areas of inflammation.

Some of the more common neoplastic lesions included haematopoietic neoplasms, hepatocellular adenomas and carcinomas of the liver, and haemangiomas and polyps of the uterus.

None of the above neoplasms appeared to be affected by the administration of the test article.

Pulmonary neoplasms were the most common neoplasm seen in the study. The incidence of the neoplasm, as well as the ratio of carcinomas to adenomas, appeared to be increased in the Group 3 (5ppm) males and in all groups of the treated female mice.

**Table 6.8.1/04-5: Two year oncogenicity study in mice (UDMH) – low dose; Tumour incidence (overall rate); \*p≤0.05, \*\* p≤0.01, \*\*\*p≤0.001**

Dose[ppm]	Males				Females			
	0	1	5	10	0	1	5	20
<b>LIVER</b>								
<b>Hepatocellular adenoma</b>	12/90 13.3%	5/90 5.6%	7/90 7.8%	13/90 14.4%	4/90 4.4%	1/90 1.1%	0/90 0%	4/90 4.4%
<b>Hepatocellular carcinoma</b>	5/90 5.6%	2/90 2.2%	5/90 5.6%	8/90 8.9%	1/90 1.1%	0/90 0%	0/90 0%	1/90 1.1%
<b>Hepatocellular adenoma/carcinoma</b>	17/90 18.9%	7/90 7.8%	12/90 13.3%	21/90 23.3%	5/90 5.6%	1/90 1.1%	0/90 0%	5/90 5.6%
<b>Haemangiosarcoma</b>	0/90 0%	1/90 1.1%	0/90 0%	2/90 2.2%	3/90 3.3%	2/90 2.2%	1/90 1.1%	5/90 5.6%
<b>LUNG</b>								
<b>Alveolar/bronchiolar adenoma</b>	16/90 17.8%	14/90 15.6%	19/90 21.1%	16/90 17.8%	9/90 10.0%	13/89 14.6%	16/90 17.8%	24/90** 26.7%
<b>Alveolar/bronchiolar carcinoma</b>	4/90 4.4%	4/90 4.4%	7/90 7.8%	4/90 4.4%	1/90 1.1%	1/89 1.1%	1/90 1.1%	7/90* 7.8%
<b>Alveolar/bronchiolar adenoma/carcinoma</b>	20/90 22.2%	18/90 20.0%	26/90 28.9%	20/90 22.2%	10/90 11.1%	14/89 15.7%	17/90 18.9%	31/90*** 34.4%
<b>HAEMOLYMPHORETICULAR SYSTEM</b>								
<b>Malignant lymphoma (lymphocytic)</b>	1/90 1.1%	0/90 0%	0/90 0%	2/90 2.2%	3/90 3.3%	1/90 1.1%	5/90 5.6%	2/90 2.2%
<b>Histiocytic sarcoma</b>	0/90 0%	0/90 0%	1/90 1.1%	0/90 0%	2/90 2.2%	3/90 3.3%	1/90 1.1%	2/90 2.2%
<b>UTERUS</b>								
<b>Polyp</b>	-	-	-	-	3/90 3.3%	2/71 2.8%	0/76 0%	5/90 5.6%
<b>Haemangioma</b>	-	-	-	-	1/90 1.1%	4/71 5.6%	0/76 0%	2/90 2.2%

**Table 6.8.1/04-6: Two year oncogenicity study in mice (UDMH) – low dose; Macroscopic masses/nodules in the lungs (Incidence/Number of animals; 12 to 24 months; doses in males and females: 1, 5, 10 ppm and 1, 5, 20 ppm, respectively)**

Dose[ppm]	0		1		5		10/20	
	DOS	SAC	DOS	SAC	DOS	SAC	DOS	SAC
<b>Males</b>	5/22 (22.7%)	5/25 (20.0%)	7/23 (30.4%)	8/23 (34.8%)	6/19 (31.6%)	14/24 (58.3%)	7/30 (23.3%)	5/16 (31.3%)
<b>Females</b>	5/29 (17.2%)	1/20 (5.0%)	1/23 (4.3%)	5/25 (20.0%)	7/30 (23.3%)	4/18 (22.2%)	17/37 (45.9%)	4/12 (33.3%)

DOS=died on study, SAC=scheduled sacrifice

#### Original DAR conclusions

- The increased mortality, the decreased bodyweight gain, and the changes in haematological findings observed at 18 months were indicative of treatment-related effects in the 5 and 20 mg/l females.
- There was a statistically significant reduction in survival in the 10 mg/l males.
- The incidence of pulmonary masses, alveolar/bronchiolar adenomas and/or carcinomas was increased in treated mice. These findings were evident at levels of 5 mg/l and above.
- Increased incidence of brown pigment in the livers was noted in males and females at 5 mg/l and above. This represents a toxic response.

**RMS 2018:** As the purity of the test substance was not stated, this study uses as a supplementary material. However, the increased incidence of alveolar/bronchiolar adenomas combined with carcinomas with dose-response relationship in treated female groups strongly supports the results of other carcinogenicity studies in mice with UDMH.

#### Two year oncogenicity study in mice (UDMH) – high dose

Reference	<b>Two year oncogenicity study in mice (UDMH) – high dose,</b> [REDACTED] 1990; Report No. A.7.3.21
Guideline	OECD 451
Deviations	Yes
GLP	Yes
Acceptability	Supplementary
Previous evaluation	Yes, study already peer-reviewed in original DAR

Reference: [REDACTED] 1990; Report No. A.7.3.21

The study was conducted according to OECD 451 guideline and was performed in compliance with GLP.

**RMS comment 2018:** The study has been checked for compliance. The following deviations from OECD guideline 451 (adopted on 7th September 2009) were found:

- 1) The purity of the test substance was not stated
- 2) At least three dose levels and a concurrent control should be used (two dose levels in the study)
- 3) The number of survivors in the lower dose or control group should not fall below 25%
- 4) Historical control data are not involved in the report

#### Material and method:

Groups of 90 male and 90 female CD-1 mice were offered UDMH in the drinking water ad libitum at concentrations of 0 (control), 40, 80 ppm. The control mice received the vehicle, 0.2M Na<sub>2</sub>HPO<sub>4</sub>/0.1M citric acid in deionized water, on the same regimen.

The mice were observed for moribundity, mortality, and overt toxicity three times daily on Monday through Friday, and twice daily on weekends and holidays. Detailed observations of appearance and condition, behaviour and activity, excretory functions, respiration, orifices, eyes and palpable masses were conducted at least once a week.

Individual bodyweights were obtained pre-initiation, weekly for the first 16 weeks and once every four weeks thereafter. Individual food and water consumption measurements were determined weekly for the first 16 weeks and once every four weeks thereafter.

Haematological evaluations (leukocyte count, differential leukocyte count, erythrocyte count, haemoglobin, haematocrit, MCV, MCH, MCHC, platelets) were conducted at 6, 12, 18 and 24 months of study on 10 randomLy selected animals/sex/group. At 12 and 24 months biochemical analysis (alkaline phosphatase, total bilirubin, AST, ALT, SDH, triglycerides, glucose, gamma glutamyltranspeptidase) were performed on these animals.

All animals in the 8 month and 12 month interim (20 mice/sex/group) and terminal sacrifice, and all animals not surviving to termination received a complete post-mortem examination.

Histopathological examination was performed on the following organs/tissues: adrenals, aorta, brain, caecum, colon, duodenum, stomach, epididymides, eyes, femur, heart, ileum, jejunum, kidneys, liver, lungs, mediastinal lymph node, mammary gland, lymph nodes, oesophagus, ovaries, pancreas, pituitary, prostate, rectum, salivary gland, sciatic nerve, seminal vesicles, skeletal muscle, skin, spinal cord, spleen, sternum, testes, thymus, thyroid with parathyroid, trachea, urinary bladder, gallbladder, uterus.

#### Results:

Mortality: During the study 63 control animals (35 males, 28 females), 84 animals at 40 ppm (38 male, 46 female), and 95 animals at 80 ppm (49 male, 46 female) died/were sacrificed *in extremis*. The majority of the mortality occurred during the second year of the study.

**Table 6.8.1/05-1: Two year oncogenicity study in mice (UDMH) – high dose; Survival at study termination**

Dose[ppm]	Males			Females		
	0	40	80	0	40	80
Survival	15 (17%)	12 (13%)	1 (1%)	21 (23%)	4 (4%)	4 (4%)

Clinical findings: There was an increased occurrence of laboured breathing relative to that in the control group for the 80 ppm males during weeks 53 through 65. During weeks 66 through 78, increased incidences of tremors and pale exposed skin areas were noted for these treated males in comparison to the control group.

Bodyweight, food and water consumption: During some weeks of the study, statistically significant reductions in mean weekly bodyweights relative to the control values were noted for the 40 ppm males and females and the 80 ppm females. These differences were slight and were not considered toxicologically significant. There were also decreases in mean weekly bodyweights for the 80 ppm females in relation to the control values during the last six months of the study. However, the sample size in this treated group was also reduced relative to that in the control group at this interval, and, in general, these decreases were not statistically significant. Although statistically significant reductions in mean food consumption occurred on some occasions, the differences from the control values were slight and not considered meaningful. Dose-related reductions in mean g/animal/day and g/kg/day water consumption, statistically significant in most instances, occurred for the males in the 40 and 80 ppm treated groups relative to the control values during the majority of study weeks.

**Table 6.8.1/05-2: Two year oncogenicity study in mice (UDMH) – high dose; Group mean bodyweight [g]**

Dose[ppm]	Males			Females		
	0	40	80	0	40	80
-1	27	26	26**	22	22	22*
1	28	27**	28	23	23	23
3	31	30	30	25	26	25*
6	33	32*	33	27	26	26
9	34	33	34	28	28	27*
12	34	34	35	28	29	28
15	34	34	35	29	28	28
20	37	36	36	30	30	29*
24	36	36	37	30	30	30
28	37	36*	37	31	31	30
32	38	37	37	32	31**	30**
36	38	37	37	32	32	30*
40	38	38	38	32	32	31*
44	38	38	38	33	33	32**

48	39	38	38	33	33	32**
52	39	37	38	33	32	31**
56	39	38	38	33	33	31*
60	39	38	38	34	34	32*
64	39	38	39	34	34	32*
68	39	37	38	34	33	32
72	39	38	38	34	34	32
76	38	37	37	34	33	32
80	38	37	37	34	34	31*
84	38	37	38	34	35	32
88	38	37	36	34	34	31
92	38	37	36	34	33	30
96	38	37	36	35	35	29*
100	38	37	36	35	34	30
104	38	36	31	34	35	30
<b>Bodyweight gain [g] (week 104 – week -1)</b>	11	10	5	12	13	8

\*p<0.05, \*\*p<0.01

**RMS comment 2018:** The results of statistics in the table documenting the group mean bodyweights do not seem to be plausible. Although the mean value of bodyweight in the control and one of the treated groups is in certain time periods identical, statistically significant difference is marked by asterisk.

**Table 6.8.1/05-3: Two year oncogenicity study in mice (UDMH) – high dose; Group mean food consumption [g/kg/day]; \*p<0.05, \*\*p<0.01**

Dose[ppm]	Males			Females		
	0	40	80	0	40	80
1	171.5	178.8**	165.5**	197.2	191.2	190.8*
3	162.4	158.8*	162.7	195.3	187.8*	198.4
6	150.2	160.1**	148.2	200.8	192.4	195.2
9	153.4	154.8	150.4	199.7	191.2	198.0



<b>12</b>	149.1	143.1**	144.4*	197.4	186.7*	204.6
<b>15</b>	150.3	146.4	146.8	193.1	193.1	199.8
<b>20</b>	138.6	139.6	141.4	181.1	182.2	190.0
<b>24</b>	141.1	140.9	136.6**	179.2	177.0	181.5
<b>28</b>	134.4	138.7**	134.6	178.0	175.5	181.0
<b>32</b>	132.5	132.9	133.2	169.9	178.2	178.2
<b>36</b>	137.5	133.9*	134.0*	169.5	169.1	169.1
<b>40</b>	130.6	129.7	126.7*	164.0	160.2	165.1
<b>44</b>	133.4	132.6	127.9**	166.6	155.3**	162.9
<b>48</b>	128.6	127.5	129.5	156.0	156.1	162.1
<b>52</b>	128.0	125.1	132.5*	150.1	133.4**	158.0
<b>56</b>	132.2	128.7	127.3	156.0	154.9	156.1
<b>60</b>	131.4	129.3	128.3	154.6	153.3	158.7
<b>64</b>	127.2	122.1*	119.5*	152.5	151.6	156.3
<b>68</b>	130.3	124.2*	128.6	150.2	146.1	155.8
<b>72</b>	133.2	129.3	127.3	155.2	157.9	154.9
<b>76</b>	132.2	129.9	124.7	155.1	161.6	164.4
<b>80</b>	134.1	130.6	133.6	157.0	150.1	166.2
<b>84</b>	140.7	135.0*	125.6**	160.9	156.4	166.3
<b>88</b>	136.0	136.0	132.6	160.7	163.3	167.5
<b>92</b>	142.6	140.7	127.2	169.7	167.7	167.6
<b>96</b>	129.5	124.0	121.1	147.7	158.8	159.0
<b>100</b>	125.2	120.5	119.0	140.2	131.6	140.9
<b>104</b>	129.7	123.3	96.8	153.0	136.6	135.5

**Table 6.8.1/05-4: Two year oncogenicity study in mice (UDMH) – high dose; Group mean water consumption [g/kg/day]; \*p<0.05, \*\*p<0.01**

\*p<0.05, \*\*p<0.01

Dose[ppm]	Males			Females		
	0	40	80	0	40	80
<b>1</b>	253.4	240.0	219.2**	295.2	275.1*	272.4*
<b>3</b>	230.4	213.4*	198.3**	285.3	245.3**	264.6**
<b>6</b>	206.2	188.9**	159.2**	284.4	250.8**	254.8**
<b>9</b>	209.1	200.7	180.6**	286.6	252.9**	270.4
<b>12</b>	175.0	165.1	163.7	253.0	235.1	246.5
<b>15</b>	200.2	167.3**	182.8**	283.7	268.2	257.9*
<b>20</b>	167.4	163.7	145.1**	255.8	230.1*	254.2
<b>24</b>	178.3	162.7**	138.3**	252.4	257.7	240.8
<b>28</b>	176.1	170.0	145.5**	259.9	265.9	275.4
<b>32</b>	168.3	153.3**	147.7**	262.6	295.7*	301.5*
<b>36</b>	172.6	167.0	160.0	273.0	302.7	297.2
<b>40</b>	169.7	151.1*	150.6*	276.3	291.9	282.8
<b>44</b>	183.9	164.8**	163.3*	289.9	306.5	291.3
<b>48</b>	167.7	161.3	165.8	273.2	296.5	303.6
<b>52</b>	176.8	164.7	177.6	292.1	316.9	315.3
<b>56</b>	172.5	167.0	188.6	315.9	358.6	340.4
<b>60</b>	184.5	171.7	180.5	307.7	363.6	322.7
<b>64</b>	196.8	170.4*	183.1	318.3	355.2	300.9
<b>68</b>	205.2	179.3	168.8	331.2	324.2	323.8
<b>72</b>	204.6	184.7	170.3	340.8	316.0	292.8
<b>76</b>	229.2	189.1*	148.6**	344.4	361.5	246.2*
<b>80</b>	246.8	185.6**	177.6*	311.2	340.0	311.7
<b>84</b>	244.0	179.7**	147.7**	313.4	362.6	327.7
<b>88</b>	238.6	184.8**	141.7**	357.1	369.9	354.9

<b>92</b>	233.8	194.4	154.7	301.5	360.9	271.3
<b>96</b>	191.7	175.4	139.3	301.8	308.3	231.6
<b>100</b>	172.7	173.0	11.9	308.5	225.7	226.2
<b>104</b>	224.7	203.1	92.2	316.3	250.1	231.1

Haematology and biochemistry: Males in both of the 40 and 80 ppm groups had statistically significant decreases in mean erythrocytes, haemoglobin and haematocrit at 12 and 18 months and at 24 months in the 40 ppm animals. There was only one male available at 24 months in the 80 ppm dosage level and therefore no relevant comparisons can be made with that group at that time interval.

At 12 months, results of the biochemical tests correlated with the microscopic evidence of liver toxicity. This was particularly true in values for alanine aminotransferase and sorbitol dehydrogenase where statistically significant increases were noted in both males and females from both treatment groups.

**Table 6.8.1/05-5: Two year oncogenicity study in mice (UDMH) – high dose; Biochemical values; \*p<0.05, \*\*p<0.01**

Dose[ppm]	0		40		80	
	12months	terminal	12months	terminal	12months	terminal
<b>Alanine aminotransferase [IU/l]</b>						
<b>Males</b>	35	142	127**	267	224	267**
<b>Females</b>	31	41	78**	105**	72*	244
<b>Sorbitol dehydrogenase [IU/l]</b>						
<b>Males</b>	79.6	23.9	148.8**	23.6	139.7**	23.77
<b>Females</b>	69.4	22.0	122.2**	29.7	116.5**	25.8

#### Pathology:

##### *Macroscopic findings*

The most frequently seen macroscopic lesions included cloudy corneas, cystic uteri, ovarian cysts, accentuated lobulation of the liver in the males, nodules or masses in the lungs, nodules and masses in the livers of the male mice, red fluid or blood in the abdominal cavity, enlarged spleen, and masses in the uterus. Many of the macroscopic findings occurred randomly across dose levels and did not exhibit a dose response. The accentuated lobulation of the liver in the male mice at 0 to 8 months and 8 to 12 months appeared to be related to treatment.

**Table 6.8.1/05-6: Two year oncogenicity study in mice (UDMH) – high dose; Macroscopic nodules (0-24months) and masses (12-24 months) in the lungs (Incidence/Number of animals examined; DOS=died on study, IS=interim sacrifice, TS=terminal sacrifice**

Dose[ppm]	0		40		80	
	DOS	IS/TS	DOS	IS/TS	DOS	IS/TS
<b>0-8 months</b>						
<b>Males</b>	0/3 (0%)	2/20 (10%)	0/3 (0%)	2/20 (10%)	0/2 (0%)	3/20 (15%)
<b>Females</b>	0/3 (0%)	0/20 (0%)	0/3 (0%)	0/20 (0%)	0/4 (0%)	0/20 (0%)
<b>8-12 months</b>						
<b>Males</b>	0/2 (0%)	3/20 (15%)	0/2 (0%)	6/20 (30%)	4/11 (36%)	7/20 (35%)
<b>Females</b>	0/1 (0%)	2/20 (10%)	1/4 (25%)	4/20 (20%)	1/7 (14%)	11/20 (55%)
<b>12-24 months</b>						
<b>Males: Nodules</b>	5/29 (17%)	5/16 (31%)	17/34 (50%)	10/12 (83%)	14/36 (39%)	1/1 (100%)
<b>Males: Masses</b>	2/29 (7%)	0/16 (0%)	4/34 (12%)	0/12 (0%)	1/36 (3%)	1/1 (100%)
<b>Females: Nodules</b>	5/24 (21%)	5/21 (24%)	14/39 (36%)	3/4 (75%)	16/35 (46%)	2/4 (50%)
<b>Females: Masses</b>	0/24 (0%)	0/21 (0%)	3/39 (8%)	0/4 (0%)	2/35 (6%)	0/4 (0%)
<b>Total</b>						
<b>Males: Nodules</b>	15/90 (17%)		35/91 (38%)		29/90 (32%)	
<b>Females: Nodules</b>	12/89 (13%)		22/90 (24%)		30/90 (33%)	

#### *Microscopic findings*

Macroscopic cyst/cystic uteri correlated with microscopic cystic endometrial hyperplasia and macroscopic ovarian cysts correlated with microscopic ovarian cysts. These are common lesions in many strains of female mice, and the incidence tends to increase with age. Other microscopic lesions that occurred with some frequency included amyloidosis of a variety of organs including adrenal cortex, glandular stomach, duodenum, jejunum, ileum, kidneys, spleen, liver, testes, ovaries, thyroid, mesenteric lymph node and heart; A-cell hyperplasia in the adrenal cortex of the males and females; brown pigment in the cortex of the adrenal glands of both male and female mice; chronic nephritis and mineralization of the kidneys of both male and female mice; mineralization of the cerebrum in both male and female mice; atrophy and mineralization of the testes of the male mice; myelofibrosis of the bone marrow of female mice; hyperplasia of the mucosa of the glandular stomach in both male and female mice; inflammation of the liver in both males and females; necrosis in the livers of the male mice; hypertrophy (enlargement) of hepatocytes in the livers of male mice and brown pigment in the livers of both male and female mice. Hypertrophy (enlargement) and necrosis of hepatocytes appeared to be related to treatment.

**Table 6.8.1/05-7: Two year oncogenicity study in mice (UDMH) – high dose; Tumour incidence (overall rate); \*p≤0.05, \*\* p≤0.01, \*\*\*p≤0.001 (Fisher exact test)**

Dose[ppm]	Males			Females		
	0	40	80	0	40	80
<b>KIDNEY</b>						
<b>Adenoma (cortical)</b>	0/90 (0%)	0/90 (0%)	3/90 (3.3%)	1/90 (1.1%)	0/90 (0%)	0/90 (0%)
<b>LIVER</b>						
<b>Hepatocellular adenoma</b>	7/90 (7.8%)	8/90 (8.9%)	10/90 (11.1%)	2/89 (2.2%)	6/90 (6.7%)	1/90 (1.1%)
<b>Hepatocellular carcinoma</b>	3/90 (3.3%)	* 11/90 (12.2%)	0/90 (0%)	0/90 (0%)	0/90 (0%)	0/90 (0%)
<b>Hepatocellular adenoma/carcinoma</b>	10/90 (11.1%)	19/90 (21.1%)	10/90 (11.1%)	2/89 (2.2%)	6/90 (6.7%)	1/90 (1.1%)
<b>Haemangioma</b>	0/90 (0%)	2/90 (2.2%)	2/90 (2.2%)	1/89 (1.1%)	2/90 (2.2%)	2/90 (2.2%)
<b>Haemangiosarcoma</b>	4/90 (4.4%)	*** 29/90 (32.2%)	*** 39/90 (43.3%)	1/89 (1.1%)	** 10/90 (11.1%)	*** 38/90 (42.2%)
<b>Liver haemangioma/ haemangiosarcoma</b>	4/90 (4.4%)	*** 31/90 (34.4%)	*** 41/90 (45.6%)	2/89 (2.2%)	** 12/90 (13.3%)	*** 40/90 (44.4%)
<b>LUNG</b>						
<b>Alveolar/bronchiolar adenoma</b>	22/90 (24.4%)	* 35/90 (38.9%)	** 38/90 (42.2%)	17/89 (19.1%)	* 31/90 (34.4%)	*** 38/90 (42.2%)
<b>Alveolar/bronchiolar carcinoma</b>	3/90 (3.3%)	9/90 (10.0%)	4/90 (4%)	1/89 (1.1%)	5/90 (5.6%)	3/90 (3.3%)
<b>Alveolar/bronchiolar adenoma/carcinoma</b>	25/90 (27.8%)	** 44/90 (48.9%)	** 42/90 (46.7%)	18/89 (20.2%)	** 36/90 (40.0%)	*** 41/90 (45.6%)
<b>HAEMOLYMPHORETICULAR SYSTEM</b>						
<b>Malignant lymphoma (lymphocytic)</b>	2/90 (2.2%)	0/90 (0%)	1/90 (1.1%)	4/90 (4.4%)	3/90 (3.3%)	2/90 (2.2%)
<b>Malignant lymphoma (mixed)</b>	-	-	-	5/90 (5.6%)	1/90 (1.1%)	* 0/90 (0%)
<b>Histiocytic sarcoma</b>	2/90 (2.2%)	2/90 (2.2%)	0/90 (0%)	5/90 (5.6%)	2/90 (2.2%)	4/90 (4.4%)
<b>UTERUS</b>						
<b>Polyp</b>	-	-	-	3/89 (3.4%)	0/71 (0%)	4/90 (4.4%)
<b>MAMMARY REGION</b>						
<b>Adenocarcinoma</b>	-	-	-	2/90 (2.2%)	5/90 (5.6%)	3/90 (3.3%)
<b>OVARY</b>						
<b>Cystadenoma</b>	-	-	-	3/89 (3.4%)	3/69 (4.3%)	2/90 (2.2%)

**Table 6.8.1/05-8: Two year oncogenicity study in mice (UDMH) – high dose; Macroscopic and microscopic findings in the liver (Incidence/Number of animals); DOS=died on study, SAC=scheduled sacrifice**

Dose[ppm]	0		40		80	
	DOS	SAC	DOS	SAC	DOS	SAC
<b>Chronic inflammation (12-24 months)</b>						
<b>Males</b>	7/29	10/16	26/33	12/12	29/36	1/1
<b>Females</b>	5/24	17/21	17/39	3/4	22/35	4/4
<b>Liver cell hyperthrophy (8-12 months)</b>						
<b>Males</b>	0/2	0/20	0/2	16/20	4/11	19/20
<b>Females</b>	0/1	0/20	0/4	3/20	1/7	4/20
<b>Hepatic necrosis (12-24 months)</b>						
<b>Males</b>	0/29	1/16	11/33	0/12	17/36	0/1
<b>Females</b>	2/24	0/21	5/39	0/4	4/35	1/4
<b>Accentuated liver lobulation (8-12 months)</b>						
<b>Males</b>	0/2	0/20	0/2	11/20	1/11	9/20
<b>Females</b>	0/1	0/20	0/4	1/20	0/7	2/20

#### Conclusion:

Evidence of toxicity was observed at 40 and 80 ppm (increased mortality, effects on water consumption, haematology, etc.). Neoplastic lesions in liver (adenomas, carcinomas and haemangiosarcomas) and lung (alveolar/bronchiolar adenomas and carcinomas) were observed at 40 and 80 ppm.

**RMS 2018:** This study serves as a supplementary material because the purity of the test

substance was not provided. Nevertheless, statistically significant dose-related trends and pair-wise comparisons were detected for both male and female mice at the 40ppm, ( $\approx 7$  g/kg bw per day) and 80 ppm dose levels for liver haemangiosarcoma and haemangioma combined and for liver haemangiosarcoma alone and for lung alveolar bronchiolar adenoma/carcinoma combined and alveolar bronchiolar adenoma alone. Lung tumours are relatively common in two year old CD-1 mice but the incidence rates in the test substance treated groups were increased. Blood vessel tumors are not uncommon in old mice, but the incidence in treated mice in this study were clearly increased over both control and historical levels.

Furthermore, other observed effects at doses of 40 and 80 ppm were: decreased animal survival, hepatotoxicity (accentuated liver lobulation, liver cell hyperthrophy and necrosis, presence of chronic inflammation and brown pigment, elevated levels of alanine aminotransferase and sorbitol dehydrogenase).

**Ames/Salmonella plate incorporation assay**

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Reference	Ames/Salmonella plate incorporation assay, <i>Stankowski L.F., 1986</i> ; Report No. 301-UN-005-86
Guideline	OECD 471
Deviations	Yes
GLP	No
Acceptability	Supplementary
Previous evaluation	Yes, study already peer-reviewed in original DAR

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**RMS comment 2018:** The study has been checked for compliance. The following deviations from OECD guideline 471 (adopted on 21st July 1997) were found:

- 3) The purity of the test substance was not stated
- 4) Recommended combination of *Salmonella typhimurium* strains is the following: 4 strains with GC and 1 strain with AT base pair at the primary revision site in order to detect mutagens including oxidizing and cross-linking agents. The strain with AT base pair such as TA 102 was not used in the study.
- 5) 2-anthramine is not present in the list of recommended positive controls. The results of this report showed that 2-anthramine did not work as a positive control in the mutation test with the strain TA98. In addition, only historical negative data (spontaneous revertants), but not positive control data were provided.
- 6) The choice of used solvent (HCl) should be justified
- 7) It should be justified, why the confirmatory mutation test was not performed

**Material and method:**

UDMH of unknown purity was tested in Ames test for its ability to induce back mutations at selected loci of several strains of *Salmonella typhimurium* in the presence and absence of microsomal enzymes derived from Aroclor 1254-induced rat liver. Toxicity of UDMH was first evaluated in a pre-screen test by treating duplicate cultures of strains TA1538 and TA100 with five doses of UDMH (25.0, 83.3, 250, 833 and 2500 µg/plate). Based upon findings of pre-screen test, mutagenic potential of UDMH was evaluated in triplicate cultures in strains TA1535, TA1537, TA1538, TA98 and TA100 in the presence and absence of S9 at doses of 25.0, 83.3, 250, 833, 2500 and 5000 µg/plate. The following positive controls were used: 2-nitrofluorene for strains TA98 and TA1538, sodium azide for strains TA1535 and TA100 and 9-aminoacridine for strain TA1537 in the absence of S-9; and 2-anthramine for all strains in the presence of S-9 mix. The solvent (HCl) was used as the negative control. Following the 48-hour incubation, the background lawn and spontaneous revertants were scored for normal, inhibited or no growth. Inhibited growth was characterized by the absence of a confluent bacterial lawn and/or the

presence of pindot colonies. Following incubation for 48-72 hours, revertant colonies are enumerated with an automated colony counter.

**Evaluation criteria:** A positive result is defined as a statistically significant and/or dose-related increase in the number of histidine-independent colonies with at least one dose point inducing an average revertant frequency that is two-fold that of the solvent control. Alternatively, if the solvent control is within the 95% confidence limits of the historical mean for control values and the test chemical produces an increase greater than or equal to three times the solvent control value, the test chemical is considered positive. A negative result is defined as the absence of a two-fold increase in the number of histidine-independent revertants.

#### Results:

Inhibited growth and/or complete toxicity was observed at doses of 5000 µg/plate without S9, and at doses of 2500 and 5000 µg/plate with S9. Revertant frequencies for all doses of UDMH in all strains approximated or were less than those observed in the concurrent negative control cultures. All positive and negative control values were within historical limits.

**Table 6.8.1/06-1: Ames/Salmonella plate incorporation assay; Mean revertant colony counts per plate**

<b>Strain Dose level [µg/plate]</b>	<b>TA1535 -S9/+S9</b>	<b>TA1537 -S9/+S9</b>	<b>TA1538 -S9/+S9</b>	<b>TA98 -S9/+S9</b>	<b>TA100 -S9/+S9</b>
<b>25</b>	17/16	8/8	11/27	29/39	284/261
<b>83.3</b>	16/13	10/14	11/18	30/41	262/252
<b>250</b>	13/11	6/7	7/20	10/24	265/265
<b>833</b>	10/12	4/8	7/15	17/8	214/239
<b>2500</b>	10/6	4/6	6/16	17/11	141/148
<b>5000</b>	9/-	4/4	2/4	9/7	59/78
<b>Solvent (0.25N HCl)</b>	16/11	8/9	11/21	26/41	229/234
<b>SA (10)</b>	1359*/-	-/-	-/-	-/-	1333*/-
<b>9AA (150)</b>	-/-	688*/-	-/-	-/-	-/-
<b>2NF (5)</b>	-/-	-/-	893*/-	524*/-	-/-
<b>2A (2.5)</b>	-/157*	-/742*	-/1728	-/33	-/2168*

+ = presence, - = absence, SA=sodium azide, 9AA=9-aminoacridine, 2NF=2-nitrofluorene, 2A=2-anthramine, \*=positive response



**Original DAR conclusion:**

The test substance was not found to be mutagenic when tested in the reverse mutation assay in the presence and the absence of metabolic activation.

**RMS 2018:** The RMS agrees that the result of Ames test with the strains of *Salmonella thyphimurium* is negative. However, this study is considered to be only supplementary because the purity of UDMH was not stated. In addition, the confirmatory mutation test was not performed and the strain for detection of oxidizing and cross-linking agents was not used (see deviations from OECD TG 471 above).

**CHO/HPRT mammalian cell forward gene mutation assay**

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Reference	<b>CHO/HPRT mammalian cell forward gene mutation assay</b> , <i>Stankowski L.F., Tunman W., 1987</i> ; Report No. A.7.6.8
Guideline	OECD 476
Deviations	Yes
GLP	Yes
Acceptability	Supplementary
Previous evaluation	Yes, study already peer-reviewed in original DAR

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**RMS comment 2018:** The study has been checked for compliance. The following deviations from OECD guideline 476 (adopted on 29th July 2016) were found:

- 1) The purity of the test substance was not stated
- 2) The choice of Dimethylnitrosamine as a positive control should be justified because it is not involved in the list of recommended substances
- 3) Sufficient number of cells (but never less than 2 million) should be cultured during the expression period and plated for mutant selection, which was not met in the study
- 4) Results should include historical negative as well as positive control data with ranges, means, standard deviations, and confidence interval

**Material and method:**

Cytotoxicity: Cytotoxicity of the test article was determined by a reduction in colony forming ability of the Chinese hamster ovary cells, clone K1, subclone BH4 (CHO-K1-BH4) cells or cell density following a 5-hour treatment with UDMH in the presence and absence of S9. The test article was evaluated for cytotoxicity at concentrations of 0.00833, 0.0250, 0.0833, 0.250, 0.833, 2.50, 8.33, 25.0, 83.3, and 250 µg/mL with and without S9 metabolic activation.

**Mutation assay:** The assay was performed using duplicate cultures for each test article concentration, as well as positive and negative (solvent) controls. The test article was evaluated at doses of 50.0, 100, 250, 500, 750 and 1000 µg/ml with and without S9. Positive controls used in the assay were ethyl methanesulfonate (EMS), a mutagen not requiring S9 activation, and dimethylnitrosamine (DMN), a mutagen which does require S9 activation. The mutant frequency was calculated by dividing the total number of mutant clones by the number of cells plated, corrected for the cloning efficiency (average of three plates) of the cells at the time of mutant selection.

**Data evaluation:** A test article is considered to be positive if it exhibits a dose-dependent increase in mutation induction with at least one concentration producing an average mutant frequency greater than or equal to three times the average mutant frequency, and greater than or equal to the 95% confidence interval, of the pooled concurrent negative controls. A test article exhibiting only one of these two responses [i.e., dose-dependent increase ( $p \leq 0.05$ ) or three-fold, statistically significant increase in comparison to the negative control cultures] is considered to be a suspect mutagen. A test article exhibiting neither dose-dependent nor 3-fold, statistically significant increases relative to the pooled concurrent negative control cultures, is considered to be negative.

## Results:

**Cytotoxicity:** UDMH was not cytotoxic with or without metabolic activation.

**Mutation assay:** statistically significant (t-test,  $p < 0.05$ ) increases of approximately two- to three-fold (over the pooled negative controls,  $\pm$  S9) at concentrations of 500, 750 and 1000 µg/ml without S9, and at concentrations of 100 and 750 µg/mL with S9. In addition, the increases observed in the absence of S9 were dose-dependent ( $p < 0.005$ ). However, only one pair of cultures (500µg/mL -S9) exhibited an average mutant frequency that was outside the 95% confidence interval of the pooled concurrent negative control cultures ( $\pm$  S9). Comparison of the treated cultures to their respective negative controls (+ or - S9) indicates that none of the cultures treated with UDMH in the absence of S9 exhibited average mutant frequencies greater than the 95% confidence interval of the concurrent negative controls without S9. In contrast, almost all of the cultures treated with UDMH in the presence of S9 had average mutant frequencies greater than the 95% confidence interval of their respective negative controls with S9. In addition, all of the mutant frequencies of the UDMH-treated cultures ( $\pm$  S9) were within historical limits ( $\bar{x} = 5.21 \pm 4.74$ ,  $n = 2217$ ). Therefore, the minor increases in mutant frequencies observed, albeit statistically significant, may represent random fluctuation sampling errors.

**Table 6.8.1/07-1: Unsymmetrical dimethylhydrazide (UDMH) CHO/HPRT mammalian cell forward gene mutation assay; Cloning efficiency and mutant frequency**

Dose level [µg/mL]	Cloning efficiency [%] -S9/+S9	Mutant frequency (Mutants/10 <sup>6</sup> clonable cells) -S9/+S9	Average mutant frequency -S9/+S9
50	78.0/79.4 84.2/89.9	2.6/3.8 1.2/10.0	1.9/6.9

100	84.0/92.0 87.9/105.0	3.6/6.5 <1.2/9.5	<2.4/8.0*
250	104.0/71.5 106.9/87.9	5.8/2.8 <1.0/2.3	<3.4/2.6
500	92.7/94.0 82.0/94.0	6.5/4.3 12.2/8.5	9.4*/6.4
750	93.2/82.5 96.5/79.5	8.6/8.5 6.2/6.3	7.4*/7.4*
1000	80.0/81.4 90.7/83.0	10.0/1.2 6.6/8.4	8.3*/4.8
Untreated	77.0/80.9 72.9/76.0	10.4/1.2 2.7/1.3	6.6/1.3
0.25 N HCl (10)	78.4/74.5 70.9/82.4	<1.3/<1.4 4.2/<1.3	<2.8/<1.4
0.25 N HCl (40)	75.0/91.0 73.7/88.9	<1.4/4.4 2.7/<1.2	<2.1/<2.8
EMS (200)	57.4/- 72.0/-	228.2/- 266.7/-	247.5/-
DMN (100)	-/52.4 -/52.9	-/290.1 -/187.1	-/238.6

EMS= ethylmethanesulfonate, DMN= dimethylnitrosamine, \* p<0.05 (significantly greater than pooled negative controls)

### Conclusion:

The results for UDMH are considered equivocal in the CHO/HPRT Mammalian Cell Forward Gene Mutation Assay under the conditions of the assay. Additional evaluation, preferably including higher concentrations demonstrating some cytotoxicity, is required before a definitive assessment can be made.

### Unsymmetrical dimethylhydrazide (UDMH) CHO/HPRT mammalian cell forward gene mutation assay

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Reference	Unsymmetrical dimethylhydrazide (UDMH) CHO/HPRT mammalian cell forward gene mutation assay, <i>Stankowski L.F., 1988</i> ; Report No. A.7.6.17
Guideline	Guideline 84-2; OECD 476

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Deviations	Yes
GLP	Yes
Acceptability	Supplementary
Previous evaluation	Yes, study already peer-reviewed in original DAR

**RMS comment 2018:** The study has been checked for compliance. The following deviations from OECD guideline 476 (adopted on 29th July 2016) were found:

- 1) The purity of the test substance was not stated
- 2) The choice of Dimethylnitrosamine as a positive control should be justified because it is not involved in the list of recommended substances
- 3) For a spontaneous mutant frequency of  $5/10^6$  cells, it is recommended to treat at least  $20 \times 10^6$  cells, which was not met in the study
- 4) Sufficient number of cells (but never less than 2 million) should be cultured during the expression period and plated for mutant selection, which was not met in the study

#### Material and method:

**Cytotoxicity:** Cytotoxicity of the test article was determined by a reduction in colony forming ability of the Chinese hamster ovary cells, clone KI, subclone BH4 (CHO-KI-BH4) cells or cell density following a 5-hour treatment with UDMH in the presence and absence of S9. The test article was evaluated for cytotoxicity at concentrations of 0.167, 0.500, 1.67, 5.00, 16.7, 50.0, 167, 500, 1670 and 5000 µg/mL with and without S9 activation.

**Mutation assay:** The mutation assay was performed using duplicate cultures for each test article concentration, as well as positive and negative (solvent) controls. Positive controls used in the assay were ethyl methanesulfonate (EMS), a mutagen not requiring S9 activation, and dimethylnitrosamine (DMN), a mutagen which does require S9 activation. UDMH was evaluated at doses of 50.0, 100, 250, 500, 1000, 2500, 3750 and 5000 µg/mL with and without S9. Following treatment, relative initial cell survival was determined for each culture. After growth for a period of 8 days to allow expression of the mutant phenotype,  $10^6$  cells from each culture were plated in medium containing TG to select for mutant cells. UDMH was re-evaluated in a confirmatory assay at concentrations of 50.0, 100, 250, 500, 1000, 1500, 2000, 2500, 3000, 3500, 3750, 4000, 4500 and 5000 µg/mL with and without S9. The mutant frequency was calculated by dividing the total number of mutant clones by the number of cells plated, corrected for the cloning efficiency (average of three plates) of the cells at the time of mutant selection.

**Data evaluation:** A test article is considered to be positive if it exhibits a dose-dependent increase in mutation induction with at least one concentration producing an average mutant frequency greater than or equal to three times the average mutant frequency, and greater than or equal to the 95% confidence interval, of the pooled concurrent negative controls. A test article exhibiting only one of these two responses [i.e., dose-dependent

increase ( $p \leq 0.05$ ) or three-fold, statistically significant increase in comparison to the negative control cultures] is considered to be a suspect mutagen. A test article exhibiting neither dose-dependent nor 3-fold, statistically significant increases relative to the pooled concurrent negative control cultures, is considered to be negative.

#### Results:

Cytotoxicity: UDMH was cytotoxic in the presence and absence of metabolic activation. Relative initial cell survivals at a concentration of 5000 µg/mL were 43.7 and 42.4% with and without S9, respectively. In addition, the cell densities of those cultures treated at concentrations of 1670-5000 µg/mL were reduced, indicating that UDMH produced an appreciable degree of immediate cytotoxicity/cytostasis.

Mutation assay: A statistically significant increase in average mutant frequency was observed at a concentration of 3500 µg/mL without S9 (30.65 TG mutants/10 clonable cells). However, this increase was not three-fold the pooled concurrent negative control cultures. All other average mutant frequencies approximated those of the pooled concurrent negative control cultures, and no dose-dependent increases in average mutant frequencies were observed in confirmatory assay.

**Table 6.8.1/08-1: Unsymmetrical dimethylhydrazide (UDMH) CHO/HPRT mammalian cell forward gene mutation assay; Cloning efficiency and mutant frequency**

Dose level [µg/mL]	Cloning efficiency [%]		Mutant frequency (Mutants/10 <sup>6</sup> clonable cells)		Average mutant frequency	
	Test1 -S9/+S9	Test2 -S9/+S9	Test1 -S9/+S9	Test2 -S9/+S9	Test1 -S9/+S9	Test2 -S9/+S9
50	75.33/81.33 72.00/85.17	90.83/81.83 70.50/92.00	17.26/11.07 6.94/7.04	6.61/2.44 11.35/6.52	12.10/9.06	8.98/4.48
100	69.33/84.33 76.00/75.17	76.83/69.50 82.67/93.83	<1.44/9.49 6.58/3.99	11.71/27.34 22.98/18.12	<4.01/6.74	17.35/22.73
250	85.50/82.00 78.33/77.84	111.67/103.33 82.00/80.33	8.19/9.76 8.94/6.42	13.43/6.77 8.54/7.47	8.57/8.09	10.98/7.12
500	95.67/67.50 74.84/76.00	68.83/59.50 92.83/91.17	31.36/14.81 5.34/2.63	30.51/53.78 10.77/10.97	18.35/8.72	20.64/32.38
1000	78.17/71.50 78.00/74.50	77.83/88.33 79.17/107.83	11.51/6.99 14.10/28.19	11.56/37.36 16.42/12.06	12.81/17.59	13.99/24.71
1500	-/-	89.67/104.50 113.00/90.00	-/-	3.35/3.83 10.62/21.11	-/-	6.98/12.74

2000	-/-	79.33/78.33 79.33/78.33	-/-	12.61/5.11 3.78/7.66	-/-	8.19/6.38
2500	79.50/79.33 82.50/75.00	72.83/79.33 54.00/58.50	22.64/MC MC/15.0	20.59/11.34 16.67/20.51	NA/NA	18.63/15.93
3000	-/-	62.83/88.50 62.83/86.33	-/-	15.92/16.95 17.51/19.69	-/-	16.71/18.32
3500	-/-	91.83/80.50 83.83/92.33	-/-	32.67/12.42 28.63/20.58	-/-	30.65*/16.50
3750	73.33/78.17 80.84/80.00	83.83/87.33 78.00/81.00	8.18/23.03 17.01/53.75	16.70/16.03 26.92/17.28	12.60/38.39**	21.81/16.66
4000	-/-	72.00/83.50 80.00/86.50	-/-	4.17/25.15 3.75/5.78	-/-	3.96/15.47
4500	-/-	77.17/86.17 81.00/94.67	-/-	12.96/13.93 9.88/6.34	-/-	11.42/10.13
5000	68.50/71.50 78.67/80.33	83.67/119.00 71.00/92.00	5.84/8.39 <1.27/6.22	17.93/5.88 23.94/11.96	EC/7.31	20.94/8.92
Untreated	74.33/84.67 67.47/72.50	69.00/87.50 84.83/102.17	13.45/7.09 5.91/13.79	21.74/14.86 25.93/8.81	9.68/10.44	23.84/11.83
2xF12/di H <sub>2</sub> O (50% v/v)	58.17/64.84 67.00/80.67	101.00/87.33 56.00/87.17	15.47/9.25 2.99/4.96	10.89/3.44 23.21/3.44	9.23/7.11	17.05/3.44
2xF12/Tris (50% v/v)	73.17/55.84 69.67/73.67	89.67/85.50 89.67/67.67	<1.39/8.95 5.74/5.43	1.12/5.85 16.73/22.17	<3.57/7.19	8.92/14.01
EMS (200)	81.84/- 77.17/-	90.83/- 85.50/-	234.60/- 204.74/-	148.62/- 154.39/-	219.67**/-	151.50**/-
DMN (100)	-59.67 -57.84	-51.83 -44.33	-276.52 -204.01	-171.70 -175.94	-240.17**	-173.82**

EC=excluded from evaluation due to extreme cytotoxicity, MC=microbial infection, NA=not applicable, 2x F12/di-H<sub>2</sub>O and 2x F12/Tris= 2x F12 media and 50% (v/v) de-ionized water or 250mM Tris/HCl (pH=8.44), EMS= ethylmethanesulfonate, DMN= dimethylnitrosamine, \*p<0.05, \*\*p<0.01 (significantly greater than pooled negative controls)

#### Original DAR conclusion:

In the first study, the test substance induced an increase in mutant frequencies at concentrations of 500, 750 and 1000 µg/mL, in the absence of metabolic activation. Similar increases were found in all dose groups of the UDMH treated cultures in the presence of metabolic activation. According to the authors the minor increases in mutant frequencies observed, albeit statistically significant, may represent random fluctuation sampling errors. The authors considered the results equivocal under the conditions of the test, and proposed an additional study with higher concentrations demonstrating some cytotoxicity.

In the second study, the test substance induced a statistically significant increase in average mutant frequency at a concentration of 3750 µg/mL in the presence of metabolic activation. However, the mutant frequencies of the cultures treated with 2500 µg/mL, could not be evaluated because of microbial contamination. At all other test concentrations the mutant frequencies were comparable to control values.

In the confirmatory assay a statistically significant increase in mutant frequencies was observed at a concentration of 3500 µg/mL, in the absence of metabolic activation. At all other test concentrations, the mutant frequencies were comparable to control values.

According to the author, the increase in mutant frequencies observed (in the main test of the second study) at a concentration of 3750 µg/mL in the presence of metabolic activation, and at a concentration of 3500 µg/mL, in the absence of metabolic activation (in the confirmatory study), should be considered an incidental finding due to random fluctuations of the spontaneous mutant frequency. The reviewer agrees with the author, that UDMH was negative in the HPRT test in Chinese Ovary Hamster cells.

**RMS 2018:** The result of the HPRT test of the first study (*Stankowski 1987*) is equivocal. The RMS agrees that the result of the HPRT test of the second study (*Stankowski 1988*) is negative. However, this study is considered to be only supplementary because the purity of UDMH was not provided.

#### UDMH - Rat hepatocyte primary culture/DNA repair test

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Reference	UDMH - Rat hepatocyte primary culture/DNA repair test, <i>Barfknecht T.R., 1986</i> ; Report No. A.7.6.12
Guideline	The study was not conducted according to any guideline
Deviations	-

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GLP	Yes
Acceptability	Yes
Previous evaluation	Yes, study already peer-reviewed in original DAR

**Material and method:**

Test article, UDMH (purity: 97%), was supplied as a clear liquid and was dissolved in 0.25N HCl at the suggestion of the sponsor. The liver of a male Fischer 344 rat was perfused, excised and combed yielding  $2.53 \times 10^6$  cells per mL of media with 86% viability.

Triplicate cultures were seeded with  $1 \times 10^5$  viable cells and treated with 20 $\mu$ L of UDMH at doses of 0.0083, 0.025, 0.083, 0.25, 0.83, 2.5, 8.3, 25, 83, and 250 $\mu$ g/mL. The test article was supplied at a concentration of 25 mg/mL and this solution was employed as the top dose level in the study. A 0.25N HCl group and 2-acetamidofluorene (2-AAF) at a final concentration of  $1 \times 10^{-5}$  M, serving as the positive control were evaluated concurrently. The test article was nontoxic at lower dose levels therefore the highest dose scored in the DNA Repair Test was 250  $\mu$ g/mL which did exhibit cytotoxic effects based on the observation of abnormal cell morphology. Unscheduled DNA Repair synthesis was quantified by a net nuclear increase of black silver grains for 50 cells/coverslip. This value was determined by subtracting the highest of three adjacent cytoplasmic counts from the nuclear counts. Three coverslips at each dose point were evaluated for a total of 150 cells/dose. The coverslips were evaluated at a magnification of approximately 1500 $\times$ .

**Results:**

Autoradiographic analyses of DNA repair in the Hepatocyte Primary Culture/DNA Repair Test are found in the following table. Net nuclear grain counts represent the difference between the nuclear and the highest of three cytoplasmic grain counts. Net negative grain count values are expressed as such. Each treatment is representative of triplicate cultures. Due to the nontoxicity of the test article at the lower dose levels, the highest dose level evaluated in the study was 250 $\mu$ g/mL. This top dose level did exhibit cytotoxic effects based on the observation of abnormal cell morphology. Additional levels evaluated were 8.3, 25 and 83  $\mu$ g/mL. None of the treated cultures produced mean net nuclear grain counts that were substantially greater than the solvent control. The positive control value was within the acceptable range of mean historical data.



**Table 6.8.1/09-1: Autoradiographic analysis of DNA repair**

Dose level (µg/mL)	Net nuclear grains triplicate cultures (mean ± SD)
0 (control)	- 8.7 ± 7.0
8.3	- 12.2 ± 6.8
25	- 12.1 ± 7.2
83	- 6.0 ± 6.0
250	- 6.4 ± 6.4
<b>Positive controls</b>	
2AAF	22.3 ± 9.8

2AAF: 2-acetamidofluorene

**Conclusion:**

The results for UDMH were negative in the Rat Hepatocyte Primary Culture/DNA Repair Test under conditions of the assay.

**In Vitro chromosome aberration analysis in Chinese Hamster Ovary (CHO) cells**

	<b>In Vitro chromosome aberration analysis in Chinese Hamster Ovary (CHO) cells, <i>San Sebastian J.R.</i>, 1986; Report No. A.7.6.13</b>
Reference	
Guideline	OECD TG 473
Deviations	Yes
GLP	Yes
Acceptability	Yes
Previous evaluation	Yes, study already peer-reviewed in original DAR

The study has been checked for compliance. The following deviations from OECD guideline 473 (adopted on 29th July 2016) were found:

- 1) The following experimental conditions should be conducted: (i) a short term treatment with test substance in the absence of S9 mix; (ii) a short term treatment with test substance in the presence of S9 mix; (iii) a long term treatment with test substance in the absence of S9 mix;  
The long term treatment was not performed (i.e. continuous exposure of cells to the test or control substance until sampling at time equivalent to about 1.5 normal cell cycle lengths).
- 2) At least 300 well-spread metaphases should be scored per each concentration of test substance and control, which was not met in the study (100 metaphases instead).

- 3) RICC (relative increase in cell count) or RPD (relative population doubling) is recommended for the assessment of cytotoxicity in cytogenetic tests using cell lines. In the study, % increase in the average proliferation time (APT) was determined.
- 4) Despite the fact that the proliferation (reflecting cytotoxicity) was evaluated during initial (dose-finding) experiment, it should be also measured in the main experiment.
- 5) If no precipitate or limiting cytotoxicity is observed, 2 mg/mL should represent the highest test concentration. In the study, the concentration of 5 mg/mL was used.
- 6) As the used positive controls are not included in the list of recommended reference substances, their choice should be justified.
- 7) Results should include historical negative as well as positive control data with ranges, means, standard deviations, and confidence interval.

**Material and method:**

Cultures of Chinese hamster ovary cells (CHO-K1-BH4) were exposed to UDMH (unknown purity) with and without metabolic activation (liver preparations from Aroclor 1254-induced male Sprague-Dawley rats) at concentrations of 0 (solvent control: 0.25N HCl), 500, 1500, and 5000 µg/mL for 5 hours. Doses of 5, 40, 75, 125, 400, 750, 1250, 2500, and 5000 µg/mL, with and without metabolic activation, were tested in a dose-range finding study. As positive controls, N-methyl-N-nitro-N-nitrosoguanidine and N-nitrosodimethylamine were used for non-activation and metabolic activation series, respectively. After the treatment, 50 µl of BrdUrd was added to the cultures. And they were incubated for additional 27 hours at 37°C in 5% CO<sub>2</sub>. For the last 2-3 hours of incubation, colcemid was added to each culture to arrest cells in metaphase. At the end of incubation, cell suspensions were collected by the mitotic shake-off method and slides were prepared and stained for sister chromatid differentiation. A total of 100 metaphases were scored for each data point. Fifty metaphases were obtained per culture and data pooled for analysis.

Evaluation criteria: Assessment of the test article as positive was based upon its ability to produce a statistically significant increase in chromosome aberrations and proportion of aberrant metaphases as compared to solvent control or its ability to produce a dose response.

**Results:**

The tested article exerted no cytotoxic effect or significant increase in average proliferation time at any doses evaluated. Treatment with UDMH did not result in statistical increase in aberrations or proportion of aberrant metaphases at any doses evaluated with or without metabolic activation.

**Table 6.8.1/10-1: Results of chromosome aberration test in CHO cells; MNNG = N-Methyl-N-nitro-N-nitrosoguanidine; DMN = N-nitrosodiethylamine; \* =  $p \leq 0.05$**

Dose ( $\mu\text{g/mL}$ )	Cells scored	Total aberrations per cell (mean $\pm$ S.D.)	Cells with aberrations	Total aberrations (gaps excluded)
<b>-S9 mix</b>				
Untreated	100	0.020 $\pm$ 0.140	2	2
0.25N HCl	100	0.030 $\pm$ 0.171	3	3
UDMH 500	100	0.060 $\pm$ 0.238	6	6
UDMH 1500	100	0.070 $\pm$ 0.256	7	7
UDMH 5000	100	0.070 $\pm$ 0.293	6	7
MNNG 2	100	1.210 $\pm$ 1.200*	66*	121*
<b>+S9 mix</b>				
Untreated	100	0.030 $\pm$ 0.171	3	3
0.25N HCl	100	0.040 $\pm$ 0.196	4	4
UDMH 500	100	0.070 $\pm$ 0.256	7	7
UDMH 1500	100	0.020 $\pm$ 0.140	2	2
UDMH 5000	100	0.090 $\pm$ 0.287	9	9
DMN 1000	100	0.340 $\pm$ 0.606*	28*	34*

**Original DAR conclusion:**

Slightly increased incidences in cells with aberrations were observed in UDMH treated cultures at all doses in the absence of S-9 and at 500 and 5000  $\mu\text{g/mL}$  in the presence of S-9. However, because of the absence of a repeat test and dose-relationship the present study is considered inconclusive.

**Note:** The study did not comply with OECD guideline 473, as no independent repeat was performed and no information was provided on the purity of the test substance.

**RMS conclusion 2018:** UDMH did not induce chromosomal aberrations in Chinese hamster cells under conditions of this study. However, several deviations from OECD TG 473 were revealed, e.g. the long term treatment was not performed (see the RMS comment above). In addition, the purity of the test substance was not stated. Therefore, this study is considered to represent supplementary material.

#### Literature data

**Sagelsdorff (1988): DNA methylation in rat liver by daminozide, 1,1-dimethylhydrazine, and dimethylnitrosamine; *Fundamental and applied toxicology* 11, 723-730;**

The aim of the study was to measure the potential of UDMH to methylate the liver DNA *in vivo*.

#### Material and method:

Radio-labelled ( $^{14}\text{C}$ ) Daminozide (37 mg/kg; radiochemical purity: 97.3%), UDMH (19 mg/kg; radiochemical purity: 99.8%), and NDMA (N-nitrosodimethylamine; 0.1 mg/kg; radiochemical purity: 95.9%) were administered by gavage to 2 male Sprague-Dawley rats. After 24 hours, the liver DNA was purified to the constant specific radioactivity and enzymatically degraded to 3'-deoxynucleotides. The level of DNA methylation was determined by HPLC analysis. Radio-labelled 7-methylguanine (7mG) was identified by co-chromatography with unlabelled 7mG added as standard. To exclude the non-covalent interaction of the test substance with DNA and external contamination of DNA samples with radiolabels, the control experiment with untreated animals were performed.

#### Results:

All three tested compounds were found to methylate DNA. The relative methylation potency was approximately 50-fold lower for daminozide than UDMH, whereas 100-fold lower for UDMH than NDMA (1:47:4900 for daminozide, UDMH, and NDMA, respectively). The administration of UDMH resulted in the formation of 7mG with dose-response relationship. Authors showed that the most of observed DNA radioactivity could be attributed to the biosynthetic incorporation of metabolic breakdown products of  $^{14}\text{C}$ -labelled UDMH into DNA (in general, e.g. C6 of purine bases could be taken from  $^{14}\text{CO}_2$  molecule). Taking into account this fact, the extent of DNA damage expressed in the units of 7mG index, values of 0.55, 26 and 2700 were counted for daminozide, UDMH and NDMA, respectively. This means that a theoretical single dose of 1 mmol/kg bw/day of the test compound would result in 0.55, 26 and 2700 7mG molecules per  $10^6$  nucleotides, under the assumption of a linear dose-binding relationship.

**RMS 2018:** Since the level of 7-methylguanine was identical to the level of modified nucleotides, 7mG index for daminozide and UDMH is the same as the covalent binding index (CBI;  $\mu\text{mol}$  chemical bound per mol nucleotide/mmol chemical applied per kg bodyweight).

Compounds with CBI: (i)  $> 1000$  are regarded as potent carcinogens; (ii) of the order of 100 as moderately strong carcinogens; (iii)  $< 10$  weakly genotoxic carcinogens; If the CBI  $< 1$ , it is unlikely that the substance will induce tumours via DNA binding (*Sagelsdorff, 1986*; Report No. A.7.6.14, Uniroyal Chemical). Therefore, based on the data of this study, the genotoxic potential of UDMH cannot be excluded without any doubt.

*Cllet, 1989: In vivo micronucleus test using mouse hepatocytes; Mutation research/environmental mutagenesis and related subjects, 216, 6, 321-326;*

#### **Aim of the study:**

The bone-marrow micronucleus (BMM) test is highly specific for clastogenic effects but its sensitivity is determined to a great extent by the substances tested, particularly by their metabolism. Some compounds, such as unstable mutagens or those which generate short-lived metabolites, are not detected in this test because the metabolites produced in the liver do not reach the bone marrow.

In an attempt to provide qualitative and quantitative assessments of chromosomal mutations produced *in vivo* by genotoxic agents not detected in the mouse BMM test, a mouse-liver micronucleus test, adapted from Tates model, was developed.

#### **Material and method:**

The CD1/CR mice (5 per group) were treated with two intraperitoneal injections (24 h between treatments) and then subjected to partial hepatectomy (24 h after the second treatment). The test was performed with 5 substances: dimethylnitrosamine (DMN), diethylnitrosamine (DEN), 1,1-dimethylhydrazine (UDMH; 14, 28, 56 mg/kg bw/day, purity: unknown), 4-aminophenol (4-APOL), 4-aminobiphenyl (4-ABPYL) and  $\beta$ -propiolactone (BPL). As a negative control water and methylcellulose was used. The liver was perfused 96 h after hepatectomy (i.e. 120 h after the second treatment). The hepatocytes were isolated, spread on slides, fixed, stained, and evaluated under microscope (oil immersion). For each dose, 10 000 hepatocytes were examined.

#### **Results:**

All the groups treated with UDMH differed significantly ( $p \leq 0.01$ ) in the frequency of micronucleated hepatocytes compared with controls. The observed frequencies were comparable with the rest of tested compounds, known to be mutagenic.

**Table:** Incidence of micronucleated hepatocytes; DMN= dimethylnitrosamine, DEN= diethylnitrosamine, 4-ABPYL=4-aminobiphenyl, 4-APOL=4-aminophenol, BPL=  $\beta$ -propiolactone, # = all animals died in this group, \* =  $p \leq 0.01$

Group	Dose (mg/kg)	Micronucleated hepatocytes per thousand cells
Control (water)	0	3.3 $\pm$ 1.3
Control (methylcellulose)	0	3.9 $\pm$ 1.5
UDMH	14	7.1 $\pm$ 0.9*
	28	10.5 $\pm$ 0.9*
	56	8.4 $\pm$ 2.0*
DMN	1.5	4.0 $\pm$ 0.6
	3	9.1 $\pm$ 0.7*

	6	$11.0 \pm 3.7^*$
DEN	28	$16.2 \pm 3.3^*$
	56	$36.9 \pm 9.2^*$
	112	#
4-ABPYL	14	$7.8 \pm 1.4^*$
	28	$11.2 \pm 0.8^*$
	56	$15.8 \pm 0.6^*$
4-APOL	53	$4.0 \pm 0.3$
	107	$13.3 \pm 0.7^*$
	214	$9.5 \pm 2.1^*$
BPL	27	$6.4 \pm 2.2^*$
	54	$11.3 \pm 3.7^*$
	108	$17.8 \pm 2.4^*$

**RMS 2018:** *In vivo* micronucleus test using mouse hepatocytes is positive under conditions of this study. We agree with the authors that in some cases, the bone marrow may not be affected, and the mutagenic potential can be revealed in micronucleus test with hepatocytes.

#### B 6.8.2 Summary of toxicity studies on metabolites

Genotoxicity and repeat dose toxicity studies were provided for UDMH.

The CLP Regulation (Regulation (EC) No 1272/2008 as amended) lists UDMH (N,N-dimethylhydrazine, CAS 57-14-7, EC 200-316-0, index 007-012-00-5) with the following classification for toxicology: Carc. 1B, H350; Acute Tox. 3\*, H331; Acute Tox. 3\*, H301; Skin Corr. 1B, H314.

#### Genotoxicity

UDMH did not induce mutations in *Salmonella typhimurium* strains either in the presence or absence of the metabolic activation. However, the strain for detection of oxidizing and cross-linking agents was not used (see deviations from OECD TG 471 above).

Ames test with *Escherichia coli* was not performed. The results of HPRT test with CHO cells were equivocal in the first study (Stankowski 1987), whereas negative in the second one (Stankowski 1988). However, each of these studies serves only as a supplementary material because the purity of UDMH was not stated. UDMH was found negative in an *in vitro* UDS assay in rat hepatocytes. *In vitro* chromosome aberration assay (San Sebastian, 1986) was performed with several deviations from OECD TG 473, e.g. the long-term treatment was not performed,

therefore is regarded as a supplementary material. Nevertheless, under conditions of the study showed a negative result. *In vivo* genotoxicity studies with UDMH were not provided by the notifiers.

As for public literature, UDMH was found positive in an *in vivo* mouse-liver micronucleus test (Cllet *et al.*, 1989). Moreover, in a covalent binding study, UDMH and NDMA were found to bind to DNA of the liver (Sagelsdorff *et al.*, 1988). Based on the available data, the genotoxic potential of UDMH cannot be unequivocally excluded.

**Table 6.8.2/01-1: In vitro genotoxicity data**

Type of study		Result		Reference Notifier
Indicator cells	Endpoint	Without activation	With activation	
S. Typhimurium	point mutation	-*	-*	Uniroyal, Stankowski, 1986
Chinese Hamster Ovary Cell	gene mutation (HPRT)	-*	-*	Uniroyal, Stankowski and Tunman, 1987; Uniroyal, Stankowski, 1988
Primary rat hepatocytes	DNA repair (UDS)	-	-	Uniroyal, Barfknecht, 1986
Chinese Hamster Ovary Cells	chromosomal aberration	-*	-*	Uniroyal, San Sebastian, 1986

\*Since no information is provided on the purity of the test substance, the conclusions regard only the tested substance and not UDMH in general

#### Short-term, subchronic, and chronic toxicity studies

Two oral subchronic toxicity studies with UDMH were conducted in rats and mice. However, the both studies represent only supplementary material because the purity of UDMH was not stated. In the 90-day study with rats no treatment-related toxic effects were observed. The highest dose tested (8.98 mg/kg bw/day) was considered the NOAEL. The 90-day toxicity study in mice revealed accentuation of liver lobulation already at the lowest dose of 2 mg/kg bw/day. Moreover, alveolar and bronchial adenomas were observed. No NOAEL could be established in this study. The results of subchronic toxicity studies with the metabolite UDMH are summarised in Table 6.8.2/01-2.

A chronic carcinogenicity study with UDMH (86.4% pure) was conducted in rats. An increased incidence of hepatocellular neoplasms was observed in females at all dose levels (0.1 - 8 mg/kg bw/day). Therefore, only provisional NOAEL has been established. Toxic effects in this study were mainly confined to dose levels of 3.2 mg/kg bw/day and above and consisted of decreased water consumption, increased incidences of cloudy corneas and corneal mineralization, and an increased incidence of hepatocellular neoplasms. In all dose groups chronic inflammation of the liver was observed. Two oral long-term studies with UDMH of unknown purity were conducted in mice. In one study, conducted with dose levels ranging from 7.3-21.8 mg/kg bw/day, neoplastic lesions in liver (adenomas, haemangiomas and haemangiosarcomas) and lung (alveolar/bronchiolar adenomas and carcinomas) were observed at all dose levels. In addition, evidence of toxicity was observed at these levels as summarised in Table 6.8.2/01-2. In the second study, conducted with lower levels (ranging from 0.2-2.7 mg/kg

bw/day), the incidence of pulmonary masses and alveolar/bronchiolar adenomas and/or carcinomas was increased at 1.0 mg/kg bw/day and above. In addition, evidence of toxicity was observed at these levels as summarised in Table 6.8.2/01-2.

Findings in a 90-day study suggest that UDMH decreases the latency period of lung tumours in mice. An increased incidence of haemangiosarcoma in mice after exposure for 12 months was considered to be a clear effect of UDMH. An increased incidence of lung tumours but not of haemangiosarcomas was seen in a lower dose chronic study, however it was not considered possible to define a threshold for this effect.

**Table 6.8.2/01-2: Repeat dose toxicity studies with their NOAELs and critical effects**

Duration	Species	Route	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Critical effects	Reference/ Notifier
90 days	rat	oral	$\geq$ 8.98 <sup>1</sup> (provisional)	-	No treatment related toxicological effects	██████ (1987a), Uniroyal
90 days	mice	oral	<2	2	accentuation of liver lobulation, liver karyomegaly/hypertrophy - occurrence of alveolar/bronchiolar adenomas	██████ (1987b), Uniroyal
2 years	rat	oral	0.1 <sup>1</sup>	3.2 <sup>1</sup>	- chronic inflammation of the liver - decreases in water consumption - increased incidences of cloudy corneas and corneal mineralization - increased incidence of hepatocellular neoplasms in females	██████ (1989a), Uniroyal
2 years	mouse	oral	0.2 <sup>2</sup>	1.0 <sup>2</sup>	- increased incidence of nodules and masses in the lungs - increased incidence of lung tumours	██████ (1989b), Uniroyal
2 years	mouse	oral	- <sup>2</sup>	7.3 <sup>2</sup>	- increases in mortality - inflammation and brown pigment in the liver, hypertrophy of hepatocytes, accentuation of liver lobulation - elevated levels of alanine aminotransferase and sorbitol dehydrogenase - nodules/masses in the lung - increased incidences of liver haemangioma/haemangiosarcoma, and alveolar/ bronchiolar adenoma/ carcinoma	██████ (1990), Uniroyal

<sup>1</sup> carcinogenicity study with UDMH, also considered suitable to establish a NOAEL for chronic toxicity



<sup>2</sup> carcinogenicity study with UDMH of unknown purity

#### Supplementary study

### UDMH carcinogenicity conclusion

Based on the available data it could not be excluded that UDMH has intrinsic mutagenic properties and is genotoxicant. Three long term studies were conducted with UDMH. In the 2 years carcinogenicity study in rats, an increased incidence of hepatocellular neoplasms was observed in females already from the lowest dose. This effect was considered as related to the treatment. Furthermore, the low dose carcinogenicity study in mice revealed an association between exposure to UDMH and the occurrence of alveolar/bronchiolar adenomas combined with carcinomas. In the high dose mice study a statistically significant effects were observed for both male and female mice at all dose levels for liver haemangiosarcoma/haemangioma and for lung alveolar bronchiolar adenoma/carcinoma. However, the excessive mortality in this study indicates that the dosing was probably set over the MTD. In addition, in the 13 week subchronic study in mice, alveolar/bronchiolar adenomas were observed in 100ppm and 250ppm treated groups. As these neoplasms are rather rare, in particular in the subchronic study and taking into account the results of long term studies, the oncogenic potential of UDMH has been clearly demonstrated.

Generally, the results of chronic and subchronic studies conducted with UDMH strongly indicating a carcinogenic potential of this substance and are in line with the current classification. The EU, IARC and the EPA categorise UDMH as a Group 1B carcinogen (H350, H331, H301, H314).

### B 6.9 Supplementary studies on the active substance

#### Immunotoxicity Evaluation of Daminozide Technical in CD-1 Female Mice: Anti-Sheep Red Blood Cell (SRBC) Response

Immunotoxicity Evaluation of Daminozide Technical in CD-1 Female Mice: Anti-Sheep Red Blood Cell (SRBC) Response, [REDACTED] 2011; Report No. BRT 20110408	
Reference	
Guideline	The study was not conducted according to any guideline, but was performed in compliance with GLP
Deviations	-
GLP	Yes
Acceptability	Yes
Previous evaluation	No

**Material and method:**

Dose concentrations of 0, 1000, 4000 and 16000 ppm daminozide (purity: 100%) were administered to 10 mice (CD-1, source: [REDACTED]) per dose group for 28 days. Intraperitoneal doses of 25 mg/kg cyclophosphamide, the immunomodulatory positive control, were administered to a group of 10 mice during days 24 to 28.

On a weekly basis, all mice were weighed, food and water consumption measured and detailed clinical observations recorded. On day 24, a single intravenous dose of  $1 \times 10^8$  sheep red blood cells (SRBC) per mL in phosphate buffered saline was administered to all animals. Five days after immunisation (day 29), each mouse was weighed, euthanized, a serum sample collected for anti-SRBC IgM titer and the spleen and thymus weighed.

**Results:**

There were no test material related clinical observations. All animals survived to the scheduled necropsy.

Bodyweights, food and water consumption: There were no test material related or cyclophosphamide effects on bodyweights. Mean water consumption for the 16000 ppm group was increased at weeks 3 and 4. Mean water consumption for the 1000 and 4000 ppm groups was comparable to the control. There were no test material-related effects on mean weekly food consumption.

Organ weights: There were no test material-related effects in mean absolute spleen and relative spleen weights for the 1,000 and 4,000 ppm dose groups. Absolute and relative spleen weights for the 16,000 ppm group were significantly greater than the control. Cyclophosphamide treated animals had absolute and relative spleen weights significantly lower than control. Mean absolute thymus weight for the 1000 ppm group was greater than the control but the relative thymus weight was comparable to the control. The 4000 and 16000 ppm groups absolute and relative thymus weights were comparable to control. In the absence of a dose-response relationship, the absolute thymus weight increase is not considered to be toxicologically relevant. Cyclophosphamide treated animals had absolute and relative thymus weight comparable to the control.

Anti-SRBC IgM: was measured on day 29, five days post-immunisation with SRBC. Slight reductions of comparable magnitude were observed for all three concentrations of daminozide. However, these decreased values were not considered to be toxicologically significant in the absence of a dose-relationship and the absence of statistical significance of any test material treated group. Compared to the controls, cyclophosphamide mean anti-SRBC IgM production was reduced by a large magnitude (0.05x vehicle control).

**Conclusion:**

Cyclophosphamide treatment caused a reduction in spleen weight and a large reduction in anti-SRBC IgM production which is consistent with the immunosuppressive effects of this immunomodulatory control article.

Calculated systemic doses achieved were 178, 708 and 2879 mg/kg/day for the 1000, 4000 and 16000 ppm daminozide dose concentrations, respectively. An increase in absolute and relative spleen weight was observed

with the 16000 ppm treatment group. However, in the absence of statistically significant changes in anti-SRBC IgM, the immunotoxicity NOAEL for daminozide is 16,000 ppm (2879 mg/kg/day).

**Determination of the concentration of unsymmetrical dimethyl hydrazine (UDMH) in a mixture of daminozide and water over a storage period of 24 hours**

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Reference	<b>Determination of the concentration of unsymmetrical dimethyl hydrazine (UDMH) in a mixture of daminozide and water over a storage period of 24 hours, B.Connor, J. Hart, 2012; Report No. BRT 20110408</b>
Guideline	The study was not conducted according to any guideline, but was performed in compliance with GLP
Deviations	-
GLP	Yes
Acceptability	Yes
Previous evaluation	No

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**Material and method:**

A sample solution of approximately 3750 ppm daminozide technical (purity: 99.5%) in tap water was prepared by dissolving 3.75364 g of the test item in 1 litre of tap water. The solution was stored in a 1L HDPE bottle at ambient temperature. At Time 0, immediately after preparation of this solution, two aliquots were taken and tested for UDMH content as per analytical method GRL-GM-1281. At additional time intervals of approximately 1 hour, 2 hours, 4 hours and 6 hours, two aliquots of this solution were sampled and tested for UDMH content according to the method. At approximately the 24 hour time period and after the calibration solutions were analysed, one aliquot of the daminozide solution was prepared and analysed as a single test injection for UDMH content. Based on this injection, no dilution was necessary other than called for in the method. Two additional aliquots of the solution were taken and tested for UDMH content at the 24 hour time interval as per analytical method GRL-GM-1281. A blank sample of the tap water was prepared and analysed as per GRL-GM-1281 to test for non-analyte interference.

**Results:**

Mathematical transformations used to arrive at the numerical values presented in this report were conducted with affectively unrounded numbers. However, for increased clarity, some of the values presented in the report may be rounded. Therefore calculations based on these values may give results that differ slightly from those presented.

UDMH content over 24 hours:

At each time interval, two aliquots of the daminozide solution were taken and tested for UDMH content as per analytical method GRL-GM-1281. Results of these analyses are as follows:

Time Interval	UDMH (mg/L)	Avg. UDMH (mg/L)	Std. Deviation	% RSD
Time 0	0.035	0.043	0.011	26.5
	0.051			
1 Hour	0.15	0.16	0.010	6.01
	0.17			
2 Hours	0.35	0.36	0.012	3.38
	0.37			
4 Hours	0.65	0.67	0.022	3.28
	0.68			
6 Hours	1.04	1.1	0.020	1.88
	1.07			
24 Hours	3.70	3.7	0.019	0.5
	3.72			

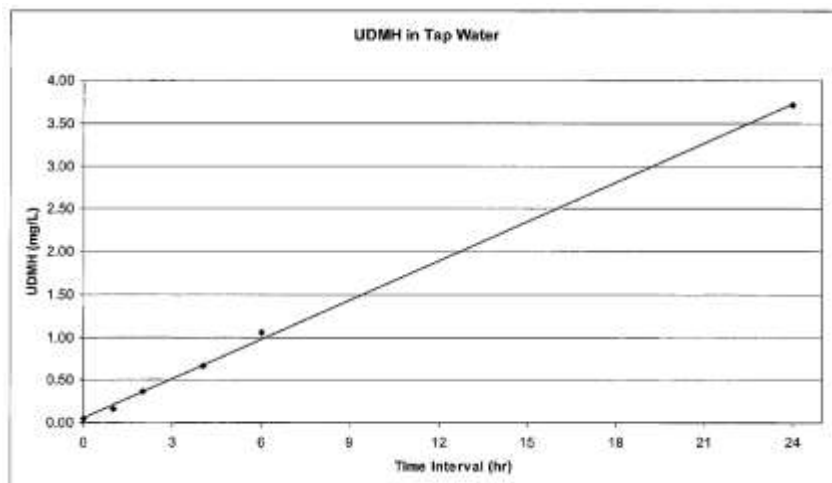
A t-test at the 95 % confidence interval was conducted comparing the results of each of the storage periods to time 0. The results are as follows:

Comparing Time 0 to	t <sub>absolute</sub>	t <sub>critical</sub>
1 Hour	10.9	4.30
2 Hours	27.0	4.30
4 Hours	35.8	4.30
6 Hours	62.5	4.30
24 Hours	237	4.30

The t-test indicate a significant difference in the UDMH concentration at each time interval form the Time 0 value.

Graphical representation of UDMH content over 24 hours:

A graphical representation of the amount of UDMH determined over the 24 hour storage period is displayed below:

**Conclusion:**

A sample solution of approximately 3750 ppm daminozide technical in 1L of tap water was prepared and stored in a 1L HDPE bottle for 24 hours at ambient temperature. The solution was tested for UDMH content in duplicate at Time 0 and after 1, 2, 4, 6 and 24 hours. The UDMH concentration at Time 0 was 0.043 mg/L and at 24 hours was 3.7 mg/L.

**B 6.10 Endocrine disrupting properties**

There were no effects in the repeat dose toxicity studies that could be interpreted as being mediated via the endocrine system.

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The potential of daminozide to induce adverse effects on components of the endocrine system has been assessed in short-term and long-term feeding studies, in reproduction studies, and in developmental toxicity studies. In these studies, there was no evidence to suggest that daminozide directly interferes with the function of the oestrogen, androgen, or thyroid pathways. No effects on fertility, reproduction, development, sexual maturation or reproductive organ toxicity were noted.

**B 6.11 Medical data****B 6.11.1 Data collected on humans**

No data available

**B 6.11.2 Direct observations**

No data available

**B 6.11.3 Epidemiological studies**

No data available

**B 6.11.4      Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical tests**

No data available

**B 6.11.5      Proposed treatment: first aid measures, antidotes, medical treatment**

Skin contact: remove contaminated clothing and wash with plenty of soap and water.

Eye contact: flush with flowing water or saline solution for at least 15 minutes.

Ingestion: Immediately drink plenty of warm water. If so to do so, induce vomiting. Seek medical attention immediately.

**B 6.11.6      Expected events of poisoning**

No data available

**B 6.12      Summary of mammalian toxicity and overall evaluation****B 6.12.1      Effects having relevance to human and animal health arising from exposure to the active substance or to impurities in the active substance or to their transformation products**

Daminozide is considered to be absorbed to at least 35% after oral administration, although it was considered that saturation of absorption could occur at a high dose level (45 mg/kg bw). Elimination is rapid (nearly complete within 48 hours) with the faeces being the main route of excretion, followed by the urine and expired air. Bile is not an important route of excretion. Daminozide is widely distributed within the body, with radioactivity found mainly in the liver in both rat and miniature swine.

Daminozide appears to be largely metabolised to unsymmetrical dimethylhydrazine (UDMH) in rats receiving an oral low dose (ca. 30% of administered dose). Approximately 40% of administered dose is found in urine and faeces of rats as the unchanged parent compound. In urine and faeces of miniature swine, daminozide is converted also to N-nitrosodimethylamine (NDMA) in addition to UDMH, and UDMH is found also in the liver.

Daminozide is of low acute toxicity, with oral and dermal LD<sub>50</sub> values greater than 5000 mg/kg and inhalation LC<sub>50</sub> higher than 2.1 mg/L (the highest attainable concentration). It is not a skin or eye irritant, and was negative for skin sensitisation using the Buehler method, with a more recent LLNA study (■■■■■ 2003) confirming daminozide not to be a skin sensitizer. A phototoxicity study is not triggered.

A subchronic 1 year oral toxicity study in dogs was available for Annex I inclusion, establishing the NOAEL at 80.5 mg/kg/day based on reduction of bodyweight. A more recent 90-d subchronic toxicity study performed in rats (■■■■■ 2005) established the NOAEL at the limit dose of 1000 mg/kg/day, even using oral gavage administration, as there was no evidence of any neurotoxicological effects, and no histopathological findings.

No adverse effects were encountered in a new 28-day dermal toxicity study (■■■■■ 2012) up to the top dose of 2000 mg/kg bw/day.

At the time of Annex I inclusion, it was considered that sufficient data were available to conclude “No genotoxic potential”. An additional Ames test (*Williams, 2006*) more recently performed using the highly hydrazine-sensitive tryptophan-requiring strain WP2uvrA of *Escherichia coli*, also confirmed no genotoxic potential. No further studies are considered necessary.

With respect to carcinogenicity, long term dietary studies with daminozide were conducted in rats and mice. In the two year rat study, increased incidence of pituitary adenomas was observed at females from the lowest dose. In the second study in mice, an increased incidence of pulmonary neoplasms (adenomas+carcinomas) was detected. The effects of both chronic studies were considered as treatment-related, indicating the oncogenic potential of Daminozide. In addition, Daminozide is metabolised to UDMH which has been shown to be carcinogenic in animal studies. The new *in vitro* comparative metabolism study was provided. However, due to the described deficiencies, no information on UDMH role in human metabolism can be extracted from this study.

Based on the results of the chronic toxicity/carcinogenicity studies and according to the CLP criteria, a classification as **Carc. 1B** should be considered warranted for Daminozide.

Previously available studies on reproductive and developmental toxicity offered no evidence of daminozide causing effects on fertility even at high dose levels. To rectify deficiencies in some of the previous studies, a prenatal developmental toxicity study is now available in New Zealand rabbits (██████, 2006) that confirms the lack of any teratogenic potential, even in the presence of severe maternal toxicity.

There is no obvious indication of neurotoxicity findings in toxicity studies of daminozide, including observations during a Functional Observational Battery (FOB) and motor activity assessment in a recent 13-week oral gavage study. In newly available acute neurotoxicity study in rats (██████, 2012a) decreased locomotor activity (total distance, basic and fine movement) was observed. The NOAEL for neurotoxicity derived from this study was set at 1000 mg/kg bw/day. In 90-day neurotoxicity study in rats (██████ 2012b) no adverse effects were encountered up to the dose of 1000 mg/kg bw/day.

In a newly conducted repeat dose immunotoxicity study in mice, daminozide showed no indications of immunotoxicity.

There were no indications in the studies conducted of any endocrine activity by daminozide. Therefore it can be concluded that Daminozide is not an endocrine disruptor.

A threshold approach may be adopted for UDMH with an extra factor 10 to cover the uncertainties.

**Table 6.12.1/01-1: Summary of toxicity studies critical in setting reference doses for daminozide**

Study	NOEL/NOAEL (mg/kg bw/day)	Adverse effects	Reference
90-day gavage study in the rat at 0, 40, 200, and 1000 mg/kg bw/day	1000	No adverse effects	██████ 2005

Study	NOEL/NOAEL (mg/kg bw/day)	Adverse effects	Reference
12-month dietary study in dogs at 0, 300, 3000 or 7500 ppm	80.5 (3000 ppm)	Renal cell adenoma, food-like emesis, soft stool	J■■■■■ 1988a
28-day dermal study in the rat at 0, 125, 500 or 1000 mg/kg bw/day	1000	No adverse effects	■■■■■ 2012
2-year rat study at 0, 100, 500, 5000, 10000 ppm	Provisional NOAEL of 5mg/kg/bw day	Pituitary adenomas, bile duct hyperplasia	■■■■■, 1988b
2-year study in mice at 0, 300, 3000, 6000, 10000 ppm	NOAEL cannot be established	Alveolar/bronchiolar adenomas, alveolar/bronchiolar adenomas+carcinomas	■■■■■ 1988c
Multigeneration (dietary) study in rats at 0, 100, 1000 and 10000 ppm	Parental: 50 (1000 ppm)  Developmental: 500 (10000 ppm) Fertility: 500	Parental: changes in bodyweight  Developmental: No adverse effect  Fertility: No adverse effect	■■■■■ 1987
Multigeneration (gavage) study in rats at 0, 60, 360 and 1200 mg/kg bw/day	Parental: 360  Developmental: 1200  Fertility: 1200	Parental: clinical signs (loose faeces, excessive post-dose salivation, perianal fur staining) and increased water consumption  Developmental: No adverse effect Fertility: No adverse effect	■■■■■ ■■■■ ■■■■, 1994
Developmental toxicity in rats at 0, 150, 750 and 1500 mg/kg bw/day	Maternal: 150  Developmental: 1500 Teratogenicity: 1500	Maternal: reduced bodyweight gain,  Developmental: No adverse effect Teratogenicity: No adverse effect	■■■■■ 1993
Developmental toxicity in rabbits at 0, 50 150 and 300 mg/kg bw/day	Maternal: 300 Developmental: 300 Teratogenicity: 300	Maternal: no adverse effect Developmental: no adverse effect Teratogenicity: no adverse effect	■■■■■■■■■ 1985



Study	NOEL/NOAEL (mg/kg bw/day)	Adverse effects	Reference
Developmental toxicity in rabbits at 0, 250, 500 and 1000 mg/kg bw/day	Maternal: 250  Developmental: 500  Teratogenicity: 1000	Maternal: mortality, clinical signs (soft/liquid faeces, hyperpnoea, hyperactivity, convulsions)  Developmental: slight reduction in ossification and litter weight	██████ 2006b
Acute neurotoxicity study in rats at 0, 500, 1000 and 2000 mg/kg	Neurotoxicity: 1000 Systemic: 2000	Decreased locomotor activity (basic and fine movement, total distance)	██████ 2012a
90-day oral (gavage) neurotoxicity study in rats at 0, 100, 300 and 1000 mg/kg bw/day	Neurotoxicity and systemic: 1000	Neurotoxicity and systemic: no adverse effect	██████ 2012b
28-day dietary immunotoxicity study in mice at 0, 178, 708 and 2,879 mg/kg bw/day	Immunotoxicity: 2879	No immunotoxicity was observed.	██████ 2011

#### B 6.12.2 Acceptable Daily Intake (ADI)

##### Daminozide

Based on the use pattern of formulations with daminozide, there is no concern for dietary exposure to daminozide (no uses on edible crops). However, since residues of daminozide in drinking water cannot be excluded the acceptable daily intake (ADI) is calculated.

The calculation of the ADI is based on the results of a carcinogenicity study in rat, which revealed a provisional NOAEL of 5 mg/kg bw/day. Application of an assessment factor of 100 and additional safety factor of 2 results in ADI of 0,025 mg/kg bw/day.

##### UDMH

ADI not established as none applicable because there are no uses on edible crops. In its 2012 MRL Reasoned Opinion, EFSA proposed a residue definition containing UDMH to allow monitoring and enforcement of potential illegal use. The following residue definition was adopted in Commission Regulation (EU) No 87/2014 of 31 January 2014: Daminozide (sum of daminozide and 1,1-dimethyl-hydrazine (UDMH), expressed as daminozide).

#### B 6.12.3 Acceptable Operator Exposure Limit (AOEL)

##### Daminozide

Usually, the AOEL for systemic exposure is set on basis of the lowest NOAEL from short term toxicity studies. However, due to the frequent use pattern of formulations based on daminozide, the provisional NOAEL from long-term studies being 5 mg/kg bw/day from the carcinogenicity study in rats is used for the derivation of the AOEL. By using a safety factor of 100, additional safety factor of 2 and adjustment for 35% oral absorption, this results in a long-term systemic AOEL of 0.009 mg/kg/day.

## UDMH

Using the lowest NOAEL for carcinogenicity of UDMH derived by the Scientific Panel on Plant Health, Plant protection products and their Residues (0.09 mg/kg bw/d, rats) an AOEL of 0.09 µg/kg bw/d can be derived.

### B 6.12.4 Acute Reference Dose (ARfD)

#### Daminozide and UDMH

Not applicable (not necessary).

### B 6.13 Proposal for a drinking-water limit

Exposure to daminozide through drinking water should account for not more than 10% of the ADI. If it is assumed that the average daily consumption of water amounts to 2 L per person of 60 kg, a drinking water limit of  $((60 \times 0.025)/10)/2$  mg/L, i.e. 0.075 mg/L, can be established.

According to Document 8064/VI/97 of the European Commission, the EU drinking water limit for pesticides of 0.1 µg/L drinking water is applicable for daminozide.

In the environmental studies only daminozide was found as a residue in soil and water. Therefore only a drinking water limit for daminozide was established.

### B 6.14 Literature search

The literature search was carried out by the notifier in accordance with Article 7, Paragraph 1(m) of Commission Implementing Regulation (EU) No. 844/2012.

The search was performed for the active substance daminozide, daminozide metabolites and relevant impurities:

- Daminozide (common name); 1596-84-5 (CAS Number)
- UDMH (unsymmetrical dimethyl hydrazine); 57-14-7 (CAS Number)
- NDMA (N,N-dimethylnitrosamine); 62-75-9 (CAS Number)
- Methanol; 67-56-1 (CAS number); 200-659-6 (EC number)

A series of searches were carried out using the STN and the Dialog platforms:

- an initial search covering studies published from 2004 to 2011;
- additional searches to cover the time periods 2012 to 2013, 2013 to 2014 and February to December 2014;

- a separate search for all *in vitro/in vivo* metabolism studies excluding hits preceding 2004 (to address the requirement under Regulation (EU) No. 283/2013 for comparative *in vitro* metabolism studies);
- a separate search covering the metabolite methanol from 2005 to 2015;

These literature searches were performed to cover 10 years prior to the expected submission of the AIR 3 dossier for daminozide which was submitted for review in April 2015.

**Table 6.14-1: Summary of the literature review**

Summary of the review	n
Total number of summary records retrieved after removing duplicates from all database searches	2134
Number of summary records excluded after rapid assessment for relevance (by title/abstract)	2074
Number of summary records of potential/unclear relevance assessed in further detail (by abstract/full-text)	60
Number of studies excluded from further consideration after detailed assessment for relevance (by abstract/full-text)	48
Number of studies not excluded for relevance after detailed assessment (i.e. relevant studies and studies of unclear relevance)	12
Number of relevant and reliable studies (Klimisch criteria 1-2) identified by the literature search and appraisal process	8
Number of studies considered relevant and reliable relating to the mammalian toxicology	0

The following criteria are considered to be fundamental when evaluating the relevance of an open-literature study:

- Studies should address the data requirements detailed in Commission Regulations (EU) No. 283/2013.
- Studies should be conducted with the well-identified test material (i.e. purity and impurity profile provided).
- Studies performed on vertebrate animals should use the relevant species (rodents are preferred, i.e. rats and mice); the number of animals per group must be sufficient to establish the statistical significance; several dose levels should be tested (at least 3) and negative control included to evaluate a dose-response relationship; the route and length of exposure should be appropriate in terms of risk assessment; a description of observations, examinations or necropsy as well as data analysis should be provided;

The criteria considered for the relevance of studies relating to the individual toxicology data requirements are detailed in the table below:

Table 6.14-2: Relevance criteria considered

Data requirement (data point)	Criteria for relevance
Studies on absorption, distribution, metabolism and excretion (KCA 5.1)	<ol style="list-style-type: none"> <li>1. Well-defined test material</li> <li>2. <i>In vivo</i> tests in relevant test species</li> <li>3. <i>In vitro</i> tests</li> <li>4. PBPK modelling</li> <li>5. Specific endpoint can be clearly related to this data requirement</li> </ol>
Acute toxicity (KCA 5.2)	<ol style="list-style-type: none"> <li>1. Well-defined test material</li> <li>2. Relevant test species</li> <li>3. Relevant route of exposure</li> <li>4. Specific endpoint can be clearly related to this data requirement</li> </ol>
Short-term toxicity (KCA 5.3)	<ol style="list-style-type: none"> <li>1. Well-defined test material</li> <li>2. Relevant test species</li> <li>3. Relevant route of exposure</li> <li>4. Specific endpoint can be clearly related to this data requirement</li> </ol>
Genotoxicity (KCA 5.4)	<ol style="list-style-type: none"> <li>1. Well-defined test material</li> <li>2. <i>In vitro</i> tests</li> <li>3. <i>In vivo</i> tests in relevant test species</li> <li>4. Specific endpoint can be clearly related to this data requirement</li> </ol>
Long-term toxicity and carcinogenicity (KCA 5.5)	<ol style="list-style-type: none"> <li>1. Well-defined test material</li> <li>2. Relevant test species</li> <li>3. Relevant route of exposure</li> <li>4. Specific endpoint can be clearly related to this data requirement</li> </ol>
Reproductive toxicity (KCA 5.6)	<ol style="list-style-type: none"> <li>1. Well-defined test material</li> <li>2. Relevant test species</li> <li>3. Relevant route of exposure</li> <li>4. Specific endpoint can be clearly related to this data requirement</li> </ol>

Neurotoxicity studies (KCA 5.7)	<ol style="list-style-type: none"><li>1. Well-defined test material</li><li>2. <i>In vivo</i> tests in relevant test species</li><li>3. Relevant route of exposure</li><li>4. Specific endpoint can be clearly related to this data requirement</li></ol>
Other toxicological studies (KCA 5.8)	<ol style="list-style-type: none"><li>1. Well-defined test material</li><li>2. <i>In vitro</i> tests</li><li>3. <i>In vivo</i> tests in relevant test species</li><li>4. Relevant route of exposure</li><li>5. Specific endpoint can be clearly related to this data requirement</li></ol>
Medical data (KCA 5.9)	<ol style="list-style-type: none"><li>1. Well-defined test material</li><li>2. Epidemiological studies</li><li>3. Poisonings, clinical cases</li><li>4. Relevant route of exposure</li></ol>

**RMS conclusion 2018:**

The RMS accepts the literature search performed by the notifier according to EFSA guidance on submission of scientific literature (EFSA Journal 2011; 9(2):2092). All the studies submitted in the document MCA 9, i.e. 4 articles relating to the mammalian toxicology deal with the toxicity of methanol. The RMS does not consider these studies to be relevant for daminozide assessment. In addition, their reliability is not unequivocal for the following reasons: e.g. not relevant route of exposure, no information on the test substance, insufficient number of tested animals, unclear description of the study design etc.). Thus, taking into account 10 year time period prior to the dossier submission (April 2015), no study related to the mammalian toxicology endpoints, being useful for regulatory use, was found.

**B 6.15 References relied on**

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
CA 5.1.1/01	████████	1966	Radiotracer metabolism study <sup>14</sup> C-ALAR in rats ████████. Report No. A.8.3.2 Non-GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.1.1/02	████████ ████████	1987	Metabolism of daminozide in miniature swine ████████. Report No. A.8.3.21 Non-GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.1.1/03	████████ ████████ ████████ ████	1987	Metabolism of daminozide in miniature swine. Analysis for UDMH in liver tissue Uniroyal Chemical Co., Inc. Report No. A.8.3.23 Non-GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.1.1/04	████████ ████████ ████████	1993	Metabolism and excretion of daminozide in male rats at a single oral dose level ████████ Report No. 64C-5315 GLP Unpublished	Y	N	Not applicable	Fine Agrochemicals Limited
CA 5.2.1/01	████████	1994a	Single dose oral toxicity in rats ████████, Inc. Report No. A.7.1.11 GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.2.2/01	████████	1994b	Acute dermal toxicity in rabbits ████████, Inc. Report No. A.7.1.12 GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.

CA 5.2.3/01	████████	1994a	An acute (4 hour) inhalation toxicity study of Alar Technical in the rat via nose only exposure ████████. Report No. A.7.1.15 GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.2.4/01	████████	1994	Primary dermal irritation in albino rabbits ████████, Inc. Report No. A.7.1.13 GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.2.5/01	████████	1994c	Primary eye irritation and/or corrosion in rabbits ████████, Inc. Report No. A.7.1.14 GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.2.6/01	████████	1994b	Closed patch repeated insult dermal sensitization study of Alar Technical in guinea pigs (Buehler method) ████████. Report No. A.7.1.16 GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.2.6/02	████████	2003	Daminozide Technical: Local Lymph Node Assay in the Mouse (Individual Method) ████████. Report No. 2242/012 GLP Unpublished	Y	Y	New data for AIR3 renewal	Fine Agrochemicals Limited
CA 5.3.2/02	████████	1988a	One Year Dietary Toxicity Study in Dogs ████████ ████████ Report No. A.7.3.12 GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.3.2/01	████████	2005	Daminozide: 13 week oral (gavage) administration toxicity study in the rat ████████. Report No. 2242/040 GLP Unpublished	Y	Y	New data for AIR3 renewal	Fine Agrochemicals Limited

CA 5.3.3/01	██████	2012	28 day dermal toxicity study in rats with daminozide technical ██████. Report No. 10519 GLP Unpublished	Y	Y	New data for AIR3 renewal	EU Daminozide Task Force
CA 5.4.1/01	Williams, L.	2006	Daminozide: reverse mutation in one Tryptophan-requiring strain of <i>Escherichia coli</i> Covance Laboratories Ltd. Report No. 2242/50-D6171 GLP Unpublished	N	Y	New data for AIR3 renewal	Fine Agrochemicals Limited
CA 5.4.1/02	Bootman, J., Lodge, D.C.	1983	P7642: Assessment of its ability to induce genetic damage in <i>Saccharomyces Cerevisiae</i> Life Science Research. Report No. A.7.6.7 Non-GLP Unpublished	N	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.4.1/03	Sans, R.H.C., Shelton, J.B.	1991	Salmonella/Mammalian-microsome plate incorporation mutagenicity assay (Ames test) with a confirmatory assay Microbiological Associates, Inc. Report No. A.7.6.18 GLP Unpublished	N	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.4.1/04	Bootman, J., Lodge, D.C., May, K.	1982a	P7642: Assessment of its ability to induce primary DNA damage in <i>Escherichia coli</i> Life Science Research. Report No. A.7.6.6 GLP Unpublished	N	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.4.1/05	Bootman, J., Rees, R., Anderson, C.	1982b	P7642: Investigation of mutagenic activity in the TK+/- mouse lymphoma cell mutation assay Life Science Research. Report No. A.7.6.5 GLP Unpublished	N	N	Not applicable	Arysta LifeScience Great Britain Ltd.



CA 5.4.1/06	Putman, D.L., Morris, M.J.	1991	Chromosome aberration in Chinese Hamster Ovary (CHO) cells Microbiological Associates, Inc. Report No. A.7.6.19 GLP Unpublished	N	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.4.1/07	Richold, M., Jones, E., Fenner, L.A.	1984	Ames metabolic activation test to assess the potential mutagenic effect of daminozide Huntingdon Research Centre. Report No. FNA 4/84222 GLP Unpublished	N	N	Not applicable	Fine Agrochemicals Limited
CA 5.4.2/01	██████ ██████████	2003	In vivo micronucleus and chromosome aberration assay in mouse bone marrow cells. Test article: B-Nine technical ██████ Report No. AA72HH.123108.BTL GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.4.2/02	██████████ ██████████ ██████████	1986	Investigation of the potential for covalent binding of daminozide (ALAR) to rat liver DNA ██████████ Report No. A.7.6.14 Non-GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.4.3/01	██████████ ██████████	1973	Dominant lethal assay of Alar in the male mouse ██████████ Report No. A.7.6.1 Non-GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.4.3/02	Epstein, S., Arnold, E., Andrea, J., Bass, W., Bishop, Y.	1972	Determination of chemical mutagens by the dominant lethal assay in the mouse Toxicology and Applied Pharmacology (1972) Vol. 23, pp. 288-325 Non-GLP Published	Y	N	Not applicable	Public domain

CA 5.5/01	██████████	1988b	Two year dietary toxicity and oncogenicity study in rats ██████████ ██████████ Report No. A.7.3.9 GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.5/02	██████████	1988c	Two year dietary oncogenicity study in mice ██████████ ██████████. Report No. A.7.3.8 GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.6.1/01	██████████ ██████	1987	Two-generation reproduction study with Alar in rats (one litter per generation) ██████████. Report No. A.7.6.15 GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.6.1/02	██████████ ██████████	1994	Oral (gavage) rat two-generation reproductive toxicity study ██████████ Report No. FAL 0015 GLP Unpublished	Y	N	Not applicable	Fine Agrochemicals Limited
CA 5.6.2/01	Khra, K.S., Whalen, C., Trivett, G., Angers, G	1979	Teratologic assessment of maleic hydrazide and daminozide, and formulations of ethoxyquin, thiabendazole and Haled in rats J. Environ Sci Health (1979) Vol. B14(6), pp. 563-577	Y	N	Not applicable	Public domain
CA 5.6.2/02	██████████	1993	Daminozide oral (gavage) rat developmental toxicity (teratogenicity) ██████████. Report No. FAL 0016 GLP Unpublished	Y	N	Not applicable	Fine Agrochemicals Limited
CA 5.6.2/03	██████████ ██████	2006a	Oral Dosage-Range Developmental Toxicity Study of Daminozide Technical in Rabbits ██████████. Report No. TZE00003 GLP Unpublished	Y	Y	New data for AIR3 renewal	Fine Agrochemicals Limited

CA 5.6.2/04	██████████ ██████████ ██████████	1985	Alar-Teratology study in rabbits ██████████ ██████████ Report No. A.7.6.9 GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.6.2/05	██████████ ██████████	2006b	Oral Developmental Toxicity Study of Daminozide Technical in Rabbits ██████████. Report No. TZE00002 GLP Unpublished	Y	Y	New data for AIR3 renewal	Fine Agrochemicals Limited
CA 5.7.1/01	██████████	2012a	Daminozide: An acute neurotoxicity study in rat ██████████ Report No. 399-232 GLP Unpublished	Y	Y	New data for AIR3 renewal	EU Daminozide Task Force
CA 5.7.1/02	██████████	2012b	Daminozide: A 90-day oral (gavage) neurotoxicity study in rats ██████████ Report No. 399-233 GLP Unpublished	Y	Y	New data for AIR3 renewal	EU Daminozide Task Force
CA 5.8.1/01	██████████	1987a	13 week toxicity study in rats ██████████ ██████████. Report No. A.7.2.4 GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.8.1/02	██████████	1987b	13 week toxicity study in mice ██████████ ██████████. Report No. A.7.2.5 GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.8.1/03	██████████	1989a	Two year oncogenicity study in rats (UDMH) ██████████ ██████████ Report No. A.7.3.18 GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.

CA 5.8.1/04		1989b	Two year oncogenicity study in mice (UDMH) – low dose Report No. A.7.3.19 GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.8.1/05		1990	Two year oncogenicity study in mice (UDMH) – high dose Report No. A.7.3.21 GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.8.1/06	Stanowski, L.F.	1988	Unsymmetrical dimethylhydrazide (UDMH) CHO/HPRT mammalian cell forward gene mutation assay Pharmakon Research International, Inc. Report No. A.7.6.17 GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.8.1/07	Stanowski, L.F., Tunman, W.	1987	Unsymmetrical dimethylhydrazide (UDMH) CHO/HPRT mammalian cell forward gene mutation assay Pharmakon Research International, Inc. Report No. A.7.6.17 GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.8.1/08	Barknecht, T.R.	1986	UDMH – Rat hepatocyte primary culture/DNA repair test Pharmakon Research International, Inc. Report No. A.7.6.12 GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.
CA 5.8.1/09	San Sebastian, J.R.	1986	In Vitro chromosome aberration analysis in Chinese Hamster Ovary (CHO) cells Pharmakon Research International, Inc. Report No. A.7.6.13 GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Ltd.

CA 5.8.1/10	Cliet, I., Fournier, E., Melcion, C., Cordier, A.	1989	In vivo micronucleus test using mouse hepatocytes Mutation Research/Environmental Mutagenesis and Related Subjects, 216(6), 321-326 Non-GLP Unpublished	Y	N	Not applicable	Public domain
CA 5.8.1/11	Sagelsdorff, P., Lutz, W. K., Schlatter, C.	1988	DNA methylation in rat liver by daminozide, 1, 1- dimethylhydrazine, and dimethylnitrosamine Fundamental and Applied Toxicology, 11(4), 723- 730 Non-GLP Unpublished	Y	N	Not applicable	Public domain
CA 5.8.2/01	██████████	2011	Immunotoxicity evaluation of daminozide technical in CD-1 female mice: anti-sheep red blood cell (SBRC) response ████████████████████ Report No. BRT 20110408 GLP Unpublished	Y	Y	New data for AIR3 renewal	EU Daminozide Task Force