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Expert statement:

Comment to ECHA targeted consultation Mutagenicity/Carcinogenicity

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Prepared for:
The Daminozide Task Force

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CLIET *et al*., 1989

The daminozide task force supports a conclusion that neither daminozide nor UDMH are genotoxic.

Cliet *et al*. 1989 describes the investigation of UDMH and NDMA genotoxicity by means of a mouse liver micronucleus (MN) test and was included in the EFSA PPPR opinion on daminozide (EFSA Journal (2004), 61, 1-27). There are significant deficiencies in this study that limit its reliability for assessment of UDMH genotoxicity. The liver MN endpoint described has been insufficiently validated, the results have not been independently replicated and a physiologically irrelevant route of administration has been used (intraperitoneal injection) that is no longer recommended for genotoxicity testing. However, the most critical deficiency is a lack of characterisation of the UDMH and NDMA used: no batch numbers or purity information are reported and UDMH was formulated in water with no indication of any measures taken to limit oxidation or pH-dependent degradation. Concerns regarding the reliability of results using "unprotected" aqueous formulations of UDMH were highlighted in the EFSA PPPR opinion. Cliet et al. shows clear evidence of confounding degradation of UDMH to NDMA. The MN frequency induced by UDMH is not dose-related and is comparable to the NDMA response, despite NDMA being tested at 10-fold lower doses. In contrast, in the daminozide task force submitted UDMH studies (where NDMA was used as the +S9 positive control) NDMA produced >6x more HPRT mutants (Stankowski 1988) and 4x more aberrations (San Sebastian 1986) than UDMH at 1/50th of the maximum UDMH concentration. The Cliet et al. reported findings for UDMH are likely to be due to the presence of a mutagenic degradation product (i.e. NDMA) rather than direct UDMH-DNA reactivity. In conclusion, Cliet *et al*. 1989 is unreliable (Klimisch reliability score 4 due to unsuitable test conditions ie instability of the tested formulation) as it provides inadequate characterisation of the UDMH batch, the UDMH formulation was not protected from oxidation/pH-dependent degradation and an irrelevant route of exposure was used. The data generated are inconsistent with results obtained in all of the daminozide task force *in vitro* studies (which included protective measures to limit UDMH oxidation prior to cell exposure).

Cliet *et al*. 1989 describes the investigation of UDMH and NDMA genotoxicity by means of a mouse liver micronucleus test and was included in the 2004 EFSA PPPR opinion on daminozide. There are several significant deficiencies in this study that limit its reliability for use in an assessment of UDMH genotoxicity. The liver micronucleus endpoint described has been insufficiently validated and the results have not been independently replicated. Additionally, a physiologically irrelevant route of administration has been used (intraperitoneal injection) that is no longer recommended for genotoxicity testing. However, the most critical deficiency is a lack of adequate characterisation of the UDMH and NDMA used; no batch numbers or purity information are reported and UDMH was formulated in water with no indication of any measures taken to limit oxidation. Concerns regarding the reliability of results using such "unprotected" aqueous formulations of UDMH were also raised in the 2004 EFSA PPPR Opinion. A water formulation of UDMH is likely to be at approximately pH 5-pH 7. In contrast, all daminozide task force 's sponsored studies used formulations of UDMH that were prepared in dilute hydrochloric acid and therefore maintained at low pH. UDMH is known to degrade to NDMA when exposed to light and air, with the degradation also reported to be pH dependant. According to Lunn *et al.*, 1991 (https://doi.org/10.1002/em.2850170109) and Lunn &

Sansone, 1994 https://doi.org/10.1016/0045-6535(94)90287-9) almost no degradation occurs at highly acidic pH. Furthermore, Sedgwick, 1992 (https://cancerres.aacrjournals.org/content/canres/52/13/3693.full.pdf) concluded that under the specific conditions that they examined, chemical oxidation of UDMH in solution resulted in the generation of mutagenic derivatives capable of inducing an adaptive response of *E. coli* to alkylation damage. It is noted that Sedgwick, 1992 did not identify the mutagenic derivatives of oxidised UDMH. With respect to Lunn *et al.*, 1991, the 2004 EFSA PPPR Opinion highlighted that the profile of mutagenic responses observed in different bacterial strains with oxidised UDMH was different to that observed with the pure NDMA sample. This led them to conclude that the results emphasised the importance of possible formation of active mutagenic derivatives on open air oxidation of UDMH, but also seem to indicate that NDMA may not be the only active derivative formed. This possibility was also acknowledged by the report authors, although they confirm that on analysis of the samples only UDMH and NDMA were identified; no other peaks were seen by gas chromatography (Lunn *et al.*, 1991).

It is therefore feasible that the positive results reported for micronuclei in hepatocytes are a consequence of either the presence of NDMA as an impurity of the UDMH batch used and/or UDMH degradation to NDMA and/or other mutagenic products prior to administration to the intraperitoneal cavity. Evidence supporting this hypothesis is apparent in the Cliet *et al*. 1989 publication. The magnitude of micronucleus frequency induced by UDMH, shows no dose-response and is very similar to the magnitude of the NDMA response, despite NDMA being tested at approximately 10-fold lower doses (Table 1). In contrast, in the daminozide task force submitted *in vitro* studies on UDMH, in which NDMA was included as the +S9 positive control, NDMA produced at least 6 times more HPRT mutants (Stankowski, 1988) and approximately 4 times more aberrations (San Sebastian, 1986) than UDMH at 1/50th of the maximum UDMH concentration (Table 2). The findings for UDMH in mouse hepatocytes are more likely to be due to the presence of a low concentration, mutagenic degradation product, i.e. NDMA, than direct UDMH-DNA reactivity.

Conclusion:

The Cliet *et al*. 1989 paper is unreliable (Klimisch 4) as it provides inadequate characterisation of the UDMH batch, the investigators did not protect the UDMH formulation from oxidation and an irrelevant route of exposure was used. The data generated is inconsistent with results obtained in all of the daminozide task force *in vitro* studies (which included protective measures to limit UDMH oxidation prior to cell exposure). The findings reported by Cliet *et al*. indicate a confounding genotoxic effect caused by UDMH degradation to NDMA prior to animal exposure. Overall, the Cliet *et al*. 1989 paper does not provide any further reliable data and should not be used in consideration of UDMH genotoxicity.

Table 1: Comparison of UDMH and NDMA micronucleus response in hepatocytes, splenocytes and spermatids

Tissue	Sample time (days)	Dose (mg/kg)	MN/1000 cells		Reference
		UDMH / NDMA	UDMH	NDMA	

Hepatocytes	5	0 / 0	3.3-3.9	3.3-3.9	Cliet *et al*., 1989
		14 / 1.5	7.1	4.0	
		28 / 3	10.5	9.1	
		56 / 6	8.4	11.0	

Table 2: Comparison of UDMH and NDMA response in HPRT and chromosome aberration tests

Assay/cell type	**Condition**	**Dose (µg/mL)**	**Mutant frequency**	**Aberration frequency**	**Reference**
HPRT / CHO	1st Expt	0	10.44		Stankowski, 1988
	5 hr +S9	UDMH 1000	17.59		
		UDMH 3750	38.39		
		UDMH 5000	7.31		
		NDMA 100	240.17		
	2nd Expt	0	11.83		
	5 hr +S9	UDMH 3750	16.66		
		UDMH 4000	15.47		
		UDMH 5000	8.92		
		NDMA 100	173.82		
Chromosome Aberration / CHO	5 hr +S9	0		0.04	San Sebastian, 1986
		UDMH 500		0.07	
		UDMH 1500		0.02	
		UDMH 5000		0.09	
		NDMA 100		0.340	

Campbell *et al*., 1978

This early (1978) study in the NTP carcinogenicity testing programme, conducted with both F344 rats and B6C3F1 mice, was part of the data assessed in the 2004 EFSA PPPR Opinion of daminozide. No GLP or OECD test guidelines were in force at the time the study was conducted.
Under the conditions of this NTP study, daminozide was not carcinogenic in male Fischer 344 rats or in female B6C3F1 mice. In the male B6C3F1 mice, the apparent distribution of hepatocellular carcinomas would appear attributable to a low incidence in controls; this control group was compromised in any case by 6/20 (30%) animals missing, leaving a group size of only 14 mice. Daminozide appeared to be carcinogenic in female Fischer 344 rats, inducing adenocarcinomas of the endometrium of the uterus and leiomyosarcomas of the uterus; however since daminozide degradation in diet was not adequately managed the tumour response in either species cannot be definitively attributed to daminozide. Uterine tumours in female F344 rats were not repeatable under more appropriate test conditions in the GLP- and guideline-compliant study reported in 1988.

Due to the unsuitability of test diet stability, a Klimisch reliability score of 4 is appropriate. Unexplained loss of 6 from 20 male control mice also suggests poor animal care and management.

The daminozide task force recommends the 2004 EFSA PPPR Opinion which declined to rely on the NTP data:
"In the more recent studies conducted according to Good Laboratory Practice (GLP) standards (author redacted, 1988), where the purity of the test material (daminozide) was known, no statistically significant increase in tumour incidences was reported. These two studies used daminozide technical material of known purity, containing typical amounts of the impurity UDMH (~30 ppm). No significant oncogenic effects were found in either Fischer 344 rats or CD-1 mice fed up to 10,000 ppm (corresponding respectively to about 500 and 1,500 mg daminozide/kg bw per day)."
"The PPR Panel concluded that these studies do not provide any evidence that daminozide induces carcinogenic effects in rats and mice." (Opinion of the Scientific Panel on Plant Health, Plant Protection Products and their Residues on a request from the Commission related to the evaluation of daminozide in the context of Council Directive 91/414/EEC, EFSA Journal (2004), 61, 1-27.)

This study, conducted with both F344 rats and B6C3F1 mice, was not previously evaluated in the Daminozide DAR (June 1999 or Addendum, June 2002) but was included in the 2004 EFSA PPPR evaluation.

No GLP or OECD test guidelines were in force at the time the study was conducted, however, there was some basic compliance with OECD 451 with at least the following significant deficiencies:

- OECD 451 guidelines suggest that at least three dose levels and a control group should be used, whereas this study used two dose groups (low dose and high dose) and a control group. The control groups contained only 20, not 50, animals per sex.

- The highest dose level should elicit evidence of toxicity, but from the preliminary sub-chronic study, no toxicity was observed at 10,000 ppm of daminozide.
- Individual animal data should be provided for all the parameters evaluated whereas the data in the current study is reported as an overall for each exposure group. Food consumption was not measured; test compound intake cannot be calculated.
- Some animals in the study were lost and unaccounted for without explanation (including 6 of 20 male control mice), so the validity and reliability of the study is questionable.

Treated diet storage and use in this study was not appropriate for the stability of the chemical, which was found by analysis of diets not to be stable under the conditions of this study. The report concludes daminozide was carcinogenic in female Fischer 344 rats, inducing adenocarcinomas of the endometrium of the uterus and leiomyosarcomas of the uterus. These differences did not achieve statistical significance. The increased incidence of uterine tumours was considered to be outside the historical control level (stated to be 2/220 adenocarcinoma and 0/220 leiomyosarcoma) but the historical control at this point in the NTP testing program was limited. In male mice, the distribution of liver tumours is cited as a significant difference ("may have been associated with the administration of test material") but all incidences were stated to be within the range of historical controls (cited as 21/216). The high background incidence and therefore reliability of liver tumour profiles in B6C3F1 mice is specifically noted in the 2017 ECHA "Guidance on the Application of the CLP Criteria". By comparison with more recent NTP HCD, the liver tumour incidence in controls appears very low for this strain, with the incidence in daminozide-treated males being within the expected range. The control group of male mice was clearly significantly flawed; since 6 of the original group size of 20 animals were lost, this control group consisted of only 14 animals.

Conclusion:

This was an early study in the NTP carcinogenicity testing programme. Under the conditions of this study, daminozide was not carcinogenic in male Fischer 344 rats or in female B6C3F1 mice. In the male B6C3F1 mice, the apparent distribution of hepatocellular carcinomas would appear attributable to a low incidence in controls; this control group compromised in any case by 6/20 (30%) animals missing. Daminozide appeared to be carcinogenic in female Fischer 344 rats, inducing adenocarcinomas of the endometrium of the uterus and leiomyosarcomas of the uterus (not statistically significant); however since daminozide degradation in diet was not adequately managed the tumour response in either species cannot be definitively attributed to daminozide. Uterine tumours in female F344 rats were not repeatable under more appropriate test conditions in the GLP- and guideline-compliant study reported in 1988.

Due to the unsuitability of test diet stability, a Klimisch reliability score of 4 is appropriate. Loss of 6 from 20 male control mice also suggests poor animal care and management.