RESPONSE TO TMPTA GENOTOXICITY DATA CLH REVIEW

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I, the undersigned declare that the following independent report provides a true and accurate analysis of the data presented; all views and opinions are the author's own.

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Introduction

Trimethylolpropane triacrylate (TMPTA) has been reviewed as part of the classification and labelling (CLH) regulation (CLP regulation Annex VI, Part 2). TMPTA was on the ECHA Community rolling action plan (Corap) for review by France in 2014. During this review the carcinogenicity endpoint was identified as needing updating as well as clarification of concerns over mutagenicity. Additional data including an *in vivo* comet assay study were submitted in updated dossier in 2017 and the substance evaluation completed in late 2018.

The existing genotoxicity data on TMPTA including the more recent comet study was reviewed in a publication by Kirkland and Fowler in 2018 [1]. The authors conclude that the available data are sufficient to confer a lack of genotoxicity from TMPTA despite there being evidence that TMPTA is an *in vitro* clastogen.

The genotoxicity issues raised by the CLH report are addressed in this document.

This document is an independent opinion of the remarks made during the CLH review and whilst Dr. Fowler has been supported financially for this work by the PARAD consortium, all opinions and views remain the author's own and were not influenced by any other individual or agency.

CLH opinions on mutagenicity

***In vitro* genotoxicity tests**

The CLH review stated that:

"In summary, results from all *in vitro* studies showed that TMPTA induced chromosome aberrations in human lymphocytes and CHO cells and mutagenic responses (likely by a clastogenic mode of action) in L5178Y cells. The addition of metabolic activation decreases the genotoxic response suggesting an effect associated to TMPTA itself rather than its metabolites. The positive results were reported in the presence of cytotoxicity (of various degree)."

This agrees with the review of [1] where the reported Ames tests were largely negative, albeit with scattered responses in TA1535. Chromosome aberration studies were positive as were mouse lymphoma assays although predominantly for the presence of small colony mutants and therefore likely to be due to clastogenic effects.

In vivo genotoxicity tests

In vivo data consisted of two micronucleus assays in mice, one of which was performed as part of the US NTP programme and an OECD 489 [2] compliant comet assay also in mice, sampling the bone marrow and liver.

Mouse bone marrow micronucleus studies

The CLH report noted the following limitations with the bone marrow micronucleus studies:

> "The first study (Anonymous, 2006) was performed according to OECD 474 but presents some limitations: low number of animals analysable per group and mainly the fact that there was no evidence of bone marrow exposure. Indeed, even if 2 deaths were reported at the highest dose in males (reason unknown), no systemic effect was found in females. PCE/NCE

> ratio was not altered and plasma levels of the test substance were not investigated. Furthermore, no kinetics data was available in mice to estimate the distribution profile of TMPTA after oral exposure. Therefore, from this study, the negative result is questionable since there is no adequate evidence of target tissue (bone marrow) exposure. The second study (NTP, 2005) has been disregarded because it does not follow any guideline and no positive control was included to validate the protocol."

The mention of a low number of animals analysable per group in the first study [3] is incorrect. Whilst there were reduced numbers of animals in several treatment groups (4 rather than 5), both male and female animals were used, hence there were twice the number of individuals at each dose level and as such no loss of statistical significance.

The lack of measured TMPTA tissue concentration is a valid comment which is also mentioned in [1], there is no measured data suggesting that TMPTA reached the systemic circulation or the bone marrow target tissue. There is however a number of deaths (males only) at the highest dose tested suggesting that these deaths may be treatment related.

The NTP study has been disregarded in the CLH review due to lack of a concurrent positive control. Positive controls are included in studies to demonstrate adequate technical performance of the assay as well as slide scoring if performed manually. The NTP testing laboratories have a long history of performing these assays and should be considered highly competent to perform them, making a lack of positive control a minor omission rather than justification to dismiss an otherwise useful study.

Comet assay in the bone marrow and liver of mice

The CLH report noted the following limitations with the comet study:

> "First, the choice of the solvent is rather unusual. Due to its viscous properties and its anti-inflammatory properties, PEG 400 appears not a suitable solvent. The viscous properties of PEG 400 is not favourable to intravenous injection. In this context and considering the soluble properties of TMPTA in organic solvents, it is not clear why a more common solvent had not been used (ex. CMC or corn oil). In the literature, PEG 400 is reported as well tolerated in different species and several routes, including IV route (Pandey et al., 2017; Gad et al., 2016; Healing et al., 2015; Thackaberry et al., 2010). However, publications report antiinflammatory / anti-oxidant properties of PEG 400 as well as some protective effects when administered with other substances (Ackland et al., 2010; Juarez-Moreno et al., 2015, Ma et al., 2017; Hodoshima et al., 2004; Klugman et al., 1981). In particular, pegylation is used in pharmaceutical sector in order to improve the tolerability of medicine. Considering that, PEG 400 may reduce or affect the intrinsic toxicity of TMPTA. It can be hypothesized that PEG 400 may counteract the irritation induced by TMPTA that may contributed to DNA damage. In this context, it cannot be ruled out that using PEG 400 may mask/decrease the reactivity of TMPTA. Secondly, according to OECD guideline 489, "animals should be given daily treatments over a duration of 2 or more days (i.e. two or more treatments at approximately 24 hour intervals), and samples should be collected once at 2-6 h (or at the Tmax) after the last treatment". In contrast, in the study, the samples were collected 30 min after the last treatment. Even if this short interval may be justified by the IV administration, there is no adequate kinetics study to confirm the relevance of this sampling time. For

example, an immediate Tmax (< 30 minutes) can be expected after parenteral administration. In this context, the sampling time is not compliant with the OECD guideline since the Tmax was not checked. Finally, according to the authors, the increased mean tail intensity values reported in bone marrow in the second experiment remained within the historical control. However, the reported historical vehicle controls are not considered relevant since they consist only on 5 animals exposed orally to CMC (and not PEG 400 administered by IV route as in the present study). In addition, it is noted that the tail intensity mean in the bone marrow (0.18) with PEG 400 is lower than that reported with these historical control with CMC as solvent (0.24-0.72). In this context, only comparison with the concurrent vehicle data is judged appropriate. "

i. Use of PEG-400 as a vehicle

The CLH document suggests that the use of PEG-400 as a vehicle in the comet study in mice is unusual and potentially problematic because of its perceived anti-inflammatory effects as seen in a number of studies referenced in the text but not included in the full reference list at the end of the document. These are discussed below:

Ackland *et al.* [4] evaluated the anti-inflammatory effects of low and high molecular weight (MW) PEG. Whilst high MW PEG was detrimental to survival, low MW PEG reduced inflammation from induced models of sepsis via LPS treatment. The publication shows effects from both *in vitro* and *in vivo* studies however the bulk of the experimental data is performed in isolated human neutrophils. *In vivo* treatments in C57 mice and Han Wistar rats were performed to assess survival rates with low and high MW PEG and to assess haemodynamic effects, temperature and cytokine changes respectively.

For the mouse survival experiments, 25 mL/kg low MW PEG was administered via i.p. injection 2 hours post LPS injection, also delivered to the peritoneal cavity. In the comet study [5] where PEG was delivered via i.v. infusion, the total delivery was 2mL/kg (also containing TMPTA), at least 5 fold lower than delivered in the Ackland study however the route of exposure also differs (i.p. vs i.v.) making comparisons difficult.

The same conditions (25 mL/kg PEG via i.p. injection) were observed in the rat study, low MW PEG did not cause any adverse effects on stroke volume but did reduce temperatures as well as plasma levels of IL6 and TNFα induced via LPS injection.

The Juarez-Moreno publication (2015) [6] shows antioxidant effects of varying PEG species to hydrogen peroxide inactivation of proteases. No treatments of cells or animals is performed, and the study only illustrates that PEG 400 (and others) is capable of reducing the effects of hydrogen peroxide on substrates.

The Hodoshima study [7] evaluated the reduction in vancomycin hydrochloride (VH) nephrotoxicity in male Sprague Dawley rats delivered intravenously (2mL/minute) with and without the addition of D-Mannitol and PEG 400 (MEEK). The authors treated rats with either the therapeutic dose of 40 mg/kg or a nephrotoxic dose of 400 mg/kg. At the 40 mg/kg dose there were no differences in pharmacokinetics and kidney effects between VH and MEEK treatments, at 400 mg/kg MEEK significantly reduced nephrotoxicity over VH addition alone. Whilst this route of administration is at least comparable to the comet study, the amount of PEG administered was at least 8 times higher

than in the comet study and also did not show any effects until ten-fold higher doses were administered.

Other studies mentioned in the CLH document but not fully referenced include Ma *et al*. 2017 and Klugman *et al*. 1981. Neither of these references could be retrieved in PubMed searches with a combination of the author and date.

The references mentioned in the CLH document provide some evidence of what is most likely an antioxidant effect of PEG 400, none of these studies adequately demonstrate that any effects would be apparent at the levels that PEG 400 was administered in the comet assay. The blood plasma already contains significant amounts of catalase and other antioxidant materials that are not present in the peritoneal cavity. With i.v. infusions containing 2 mL/kg TMPTA in PEG 400 the additional antioxidant effects would most likely not significantly contribute to the overall antioxidant potential of the blood plasma. The justification for rejecting the comet study over the use of PEG 400 is therefore not valid.

The argument put forward in the CLH review was that the perceived antioxidant effect of PEG could potentially "counteract the irritation induced by TMPTA that may have contributed to DNA damage". One of the inherent problems with genotoxicity evaluation is interpreting the real DNA (or chromatin) interactive events without being hampered by excess toxicity which commonly results in false positives via a variety of mechanisms [8, 9]. Furthermore, the effects of reactive oxygen are far less pronounced *in vivo* where the whole organism has extensive antioxidant systems in blood plasma and tissues to mitigate the effects of exogenous and endogenous reactive oxygen species (ROS).

PEG 400 has been described as an "unusual" vehicle however it is not that uncommon and has been used as a vehicle in several comet studies submitted to ECHA in the last 12 months (i.e. EC Nºs 915-623-1 and 249-204-3). Furthermore, neither corn oil or carboxymethylcellulose are suitable for i.v. infusion as suggested in the CLH review. The i.v. route of administration has been utilised due to the low aqueous solubility of TMPTA and to reduce local irritation at the administration site.

ii. OECD 489 adherence

The CLH review quotes the OECD 489 [2] guidance around sampling times which states that "animals should be given daily treatments over a duration of 2 or more days (i.e. two or more treatments at approximately 24 hour intervals), and samples should be collected once at 2-6 h (or at the Tmax) after the last treatment".
The comet study samples 30 minutes after the second dose of TMPTA and this is mentioned as a deficiency in the CLH review.

OECD 489 also includes an extensive section on sampling time which states:

"The optimum sampling time(s) may be substance- or route specific, resulting in, for example, rapid tissue exposure with intravenous administration or inhalation exposure. Accordingly, where available, sampling times should be determined from kinetic data (e.g., the time (Tmax) at which the peak plasma or tissue concentration (Cmax) is achieved, or at the steady state for multiple administrations)"

For the comet assay there are no TK data available as part of the same study from which we can calculate the Tmax and justify the earlier sampling time of 30 minutes however there is data available from the NTP [10] where a group of five male rats were given a single bolus dose of 9.4 mg/kg [^{14}C] labelled TMPTA. Blood was collected at 0.08, 0.5, 1, 3, 6, 24 and 48 hours post administration. The tabulated data can be seen in the below table taken directly from [10]:

TABLE L8
Concentration of Trimethylolpropane Triacrylate Equivalents in the Blood of Male F344/N Rats after a Single Intravenous Injection of 9.4 mg/kg [^{14}C]-Trimethylolpropane Triacrylate[a]

Time (hours after dosing)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Mean ± Standard Deviation
0.08	15.2	24.3	39.7	27.0	33.5	27.9 ± 9.3
0.5	20.2	21.7	25.2	18.6	21.2	21.4 ± 2.4
1	12.9	22.0	16.9	10.8	19.7	16.5 ± 4.6
3	23.0	21.9	16.5	15.9	17.1	18.9 ± 3.3
6	22.1	20.7	20.0	14.9	16.6	18.9 ± 3.0
24	20.4	18.7	19.5	14.3	16.4	17.9 ± 2.5
48	17.6	17.1	17.0	12.6	15.4	15.9 ± 2.0
72	14.8	15.0	14.8	11.3	13.4	13.9 ± 1.6

[a] Data are presented as µg equivalents per gram of blood.

The level of TMPTA in the systemic circulation at 5 minutes post i.v. dosing (0.08 hours) is higher than the level at 30 minutes however it is not practically possible to take and process multiple tissues in the comet assay within 5 minutes of dosing, particularly with a slow infusion. The 30-minute sampling point therefore represents as close to Tmax as it is practical to achieve whilst still reaching a demonstrably high exposure to TMPTA.

Comment is also made in the CLH review of the fact that the historical control range in the comet study is from studies using an alternative vehicle to PEG-400 and as such is not applicable. Considering that the historical range is used to demonstrate the individual laboratory's control over the assay itself i.e. the reproducibility of negative responses and the technical aspects of performing the study the historical range is still considered as useful for interpretation of the data where PEG 400 is used as a vehicle.

Reference is made to the statistical significance noted at the lowest and mid doses in experiment 2 of the comet assay (5 and 10 mg/kg) in bone marrow cells as evidence of DNA damage albeit without a dose related effect (There were no effects noted in the livers of TMPTA treated animals). The actual values were tail intensities of 0.18 in the control group and 0.67 and 0.29 in the 5 and 10 mg/kg groups respectively. This increase is small, well within historical ranges (despite this range being from animals treated via a different route and vehicle). The CLH report also notes that the statistics (ANOVA) used in the comet study may not have been appropriate for the data and instead proposed that using a non-parametric test (kruskall-Wallis) would not result in statistical significance at 10 mg/kg.

The individual animal data for the 5mg/mL dose group in experiment 2 (see below taken directly from [5]) contains a single high outlier in the data from animal M0602 (in red text) of at least twice the value of all other individuals that may be responsible for increasing the average of the group.

The remaining statistical significance at the lowest tested dose (5 mg/kg) in the bone marrow comet assay is not biologically relevant if, at two higher doses there are no such statistical increases and no linear trend to suggest a dose response. It is also worth noting that the statistical significance may likely have arisen from a low concurrent control in the second experiment compared to that of experiment 1 (0.18 in experiment 2 compared to 0.29 in experiment 1).

Table 12.7: Bone Marrow: Animal Comet Data – Experiment 2

Group	Animal ID	Tissue	Dose Level (mg/kg/day)		Slide Code	No. Comets	Tail Intensity (%)		Hedgehogs (%)
							Mean	Standard Deviation	
6	M0501	Bone Marrow	0	Vehicle	F359-	150	0.10	0.04	0.73
6	M0502	Bone Marrow	0	Vehicle	F284-	150	0.11	0.09	0.58
6	M0503	Bone Marrow	0	Vehicle	F986-	150	0.22	0.19	0.40
6	M0504	Bone Marrow	0	Vehicle	F442-	150	0.16	0.13	0.00
6	M0505	Bone Marrow	0	Vehicle	F275-	150	0.17	0.04	0.00
6	M0506	Bone Marrow	0	Vehicle	F115-	150	0.32	0.42	0.50
7	M0601	Bone Marrow	5	TMPTA	F355-	150	0.21	0.11	1.28
7	M0602	Bone Marrow	5	TMPTA	F534-	150	1.71	2.17	0.00
7	M0603	Bone Marrow	5	TMPTA	F236-	150	0.82	0.83	0.37
7	M0604	Bone Marrow	5	TMPTA	F230-	150	0.54	0.55	0.45
7	M0605	Bone Marrow	5	TMPTA	F888-	150	0.26	0.14	0.00
7	M0606	Bone Marrow	5	TMPTA	F651-	150	0.48	0.58	0.00

It is therefore concluded that the incidences of statistical significance are not biologically relevant and that the comet assay in the bone marrow of TMPTA treated mice is negative for induction of DNA damage.

Conclusions

TMPTA has been evaluated for genotoxicity with a series of *in vitro* and *in vivo* tests. TMPTA appeared to induce a clastogenic effect *in vitro* which did not translate into similar responses *in vivo*. The CLH document presented arguments as to why these studies were unreliable or not fully predictive of the genotoxic potential of TMPTA. The studies themselves contained some deficiencies, many of which have been previously discussed by [1], predominantly around demonstrating target tissue exposure, route of exposure, suitability of vehicle and variability in biological assays. Despite minor deficiencies in individual studies, the weight of evidence of genotoxicity data for TMPTA shows a lack of DNA and chromosome damage *in vivo.* There is not strong enough evidence that justifies the CLH rejection of these studies.

References

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