RESPONSE TO CUMENE 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

Cumene (CAS 98-82-8) has been reviewed as part of the classification and labelling (CLH) regulation (CLP regulation Annex VI, Part 2). Cumene is used in the manufacture of various chemicals (acetone and phenol), a thinner in varnishes and paints, is present in aviation and motor fuels and a component of printer inks.

A recent CLH report submitted by the Danish EPA [1] discusses the germ cell mutagenicity of Cumene concluding that "a primarily genotoxic mode of action is unlikely" with several caveats.

The genotoxicity issues raised by the CLH report are discussed and rationalised 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 phenols and derivatives consortium, all opinions and views remain the author's own and were not influenced by any other individual or agency.

CLH opinions on mutagenicity

The CLH review stated that:

"In conclusion, there are no data on germ cell mutagenicity from cumene or metabolites. For somatic cells, the vast majority of available tests gave negative results and there are only few indications for a genotoxic potential:

- Some DNA damage in male liver or female mice may not be excluded, as evidenced by recent Comet assay analysis from NTP (2012). It is speculated that this DNA damage may be a secondary genotoxic effect, e.g., due to oxidative damage in target organs,
- The postulated metabolite α-methylstyrene oxide may be mutagenic; however, the quantitative relevance of this substance for cumene metabolism has not been assessed and the finding is not confirmed by direct observations with cumene,
- Changed profiles and increases of K-ras and p53 mutations in cumene induced lung tumours may either point to mutagenicity of cumene or secondary genotoxicity (e.g., from reactive oxygen species, resulting in genetic instability and/or impairment of repair mechanisms) or epigenetic changes (e.g., from altered histone deacetylase)."

These points are discussed individually below:

1) **NTP Comet studies in rats and mice**

There are several *in vivo* study data sets, from an NTP study of cumene [2] with a comet assay evaluating multiple tissues from male and female mice and male rats including liver, kidney, lung and blood. Cumene was concluded to have given positive responses in the liver of male rats and the lung of female mice. As part of the same study blood was also analysed for the presence of micronuclei via flow cytometry which was found to be negative.

Cumene is volatile and has been tested via inhalation in a 2-year bioassay also by the NTP [3]. The acute studies were treated via oral gavage as there is sufficient evidence available that oral dosing of Cumene results in a systemic exposure.

Male Fisher 344 rats and both sexes of B6C3F1 mice (6 animals per group) were treated with Cumene in corn oil via oral gavage once daily for 4 consecutive days. Maximum tolerated doses in rats (800 mg/kg/day) and mice (1000 mg/kg/day and 1250 mg/kg/day for female and males respectively) were determined from initial dose range finding studies.

The choice of animals, sexes and tissues analysed were those in which effects were noted from the 2-year bioassay study, namely the presence of tumours in the lungs of male B6C3F1 mice, lung and liver in female B6C3F1 mice and kidney in male F344 rats. Female rats were not included in the study as the only site of tumours noted from the 2-year bioassay was the nasal epithelium and it was not practical to test this tissue in the comet assay.

All animals were treated via oral gavage with Cumene in corn oil at a volume of 10mg/kg over 4 days. Sampling from Lung, Liver, Blood and Kidney was performed 3 hours after the final dose on day 4. Necropsy samples from all tissues excluding kidney were minced and immediately flash frozen for later slide preparation whereas kidney samples were processed immediately.

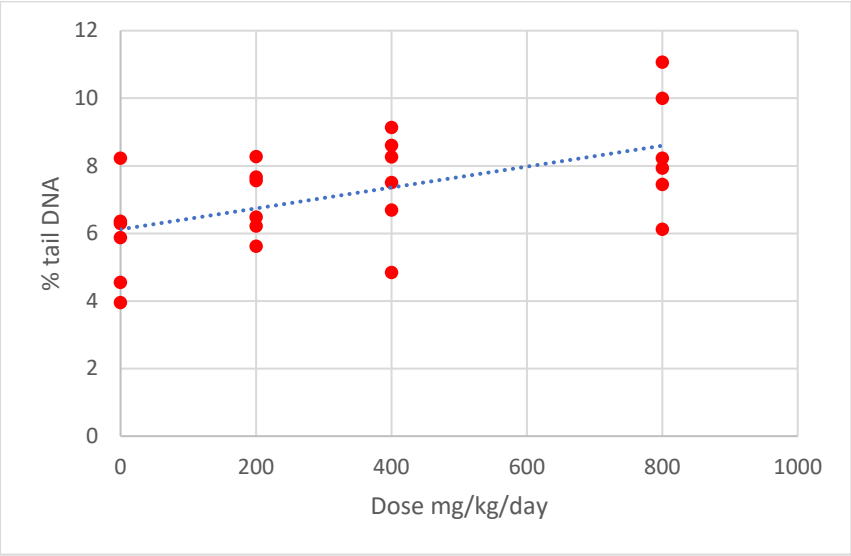
Data for the individual tissues can be seen in the following charts and tables, male mice were negative for increases in % DNA in comet tails for all tissues analysed. Female mice were statistically significant for increases in % tail DNA at the highest tested dose in the lung only. In male rats the only tissue with statistically significant increases in % tail DNA was liver. Both of these tissues tested positive in a linear trend analysis.

Table 1. % Tail DNA male F344 rats - Liver

Dose (mg/kg)	N	Mean	S.E.	p-value
0	6	5.876	0.616	
200	6	6.967	0.415	0.110
400	6	7.505	0.637	0.043
800	6	8.465	0.730	0.004

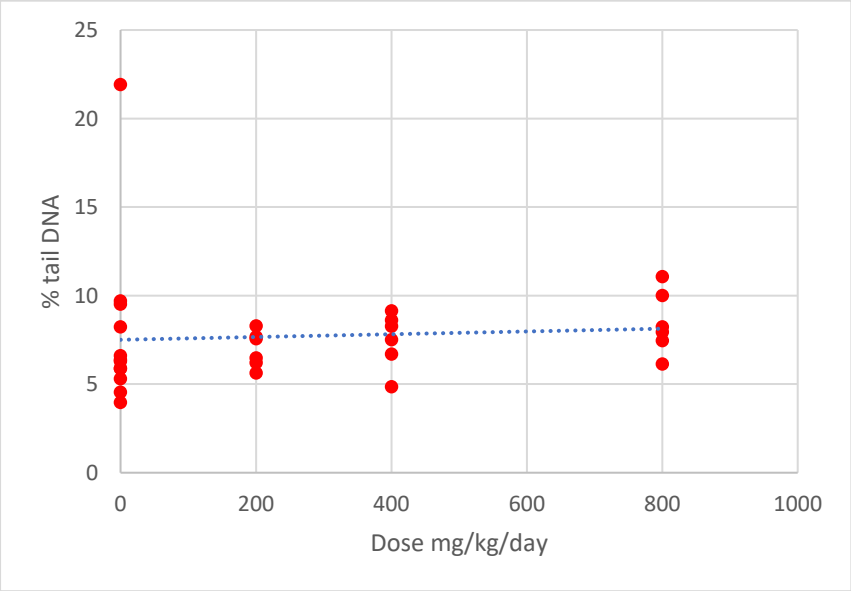
Whilst statistical analysis describes a slight dose related effect (Table 1), four of the 6 animals in the highest dose group (800 mg/kg/day) are within the range of the vehicle control, the highest two responding individuals are responsible for skewing the average and causing the statistical difference (Chart 1).

Chart 1, Individual animal data for % tail DNA in Male rat liver with trendline



NTP studies often do not use a historical (normal) range to apply biological relevance, instead relying on statistical difference to the concurrent vehicle control. This approach can lead to artefactual positive responses when the values in the vehicle control are close together giving a narrow range and as such more likelihood of a pseudo positive response. In an attempt to rationalise the data from F344 rats, other studies in the NTP comet database with a liver endpoint have been combined (Chart 2).

Chart 2. Individual animal data for % tail DNA in male rat liver with additional vehicle control group values added.



F344 rats are not commonly used for genotoxicity evaluation, in the NTP comet database there is one additional study since 2009 using F344 animals. When the vehicle control animals from this study are included as a very limited historical range, the spread is far wider (Chart 2). There is one very high value in the vehicle control of the other study, even if this is removed there is no positive linear trend for Cumene when re-running linear trend analysis including the additional vehicle control data (p= 0.368). The majority of the data in NTP comet database is from Sprague Dawley or Han Wistar rats, both of which have lower background levels of %tail DNA than F344 rats making them unsuitable as a reference for these studies.

Including the additional historical vehicle control data for F344 rats greatly reduces the difference between control and treated animal groups. There is no longer a prominent dose relationship and a single individual from the high dose group now lies outside the vehicle control range. It is therefore considered that whilst the original data showed what appeared to be a weak positive response only in the high dose group this increase is not biologically relevant and Cumene treatment in male F344 rats does not convincingly increase DNA damage at the highest tested dose of 800 mg/kg/day.

In B6C3F1 mice there is a single study in the NTP comet database, Cumene. Without any additional vehicle data, it is not possible to add biological relevance to the original statistically significant increase in % tail DNA. When the variability of the data is analysed it appears that for lung in particular, female B6C3F1 mice have a low variability in the vehicle control group which is approximately half that of the treatment groups (Table 2).

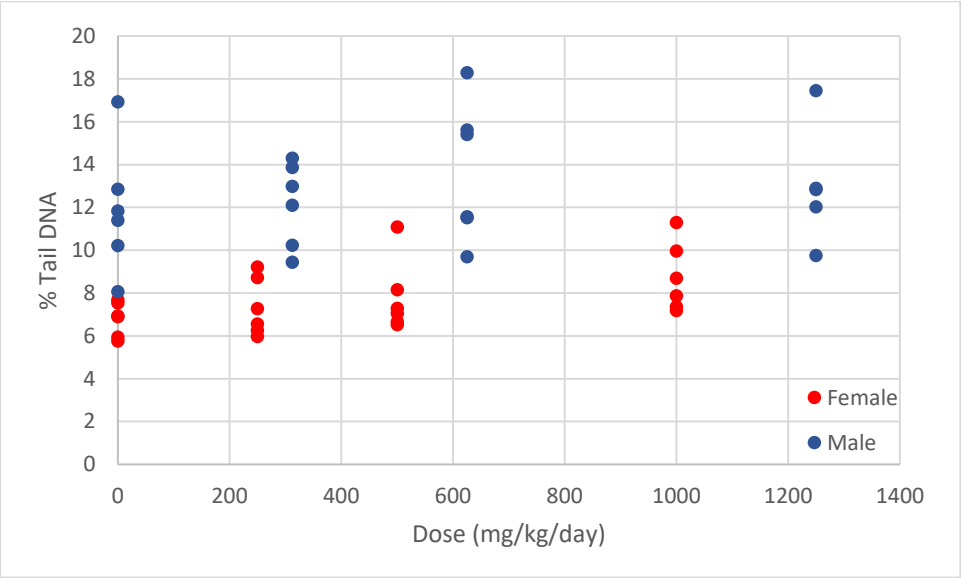
Table 2. Female B6C3F1 mice % tail DNA multiple tissues

	Female mice, Blood				Female mice, Liver				Female mice, Lung				Female mice, Kidney			
mg/kg	n	Mean	S.E.	p-value	n	Mean	S.E.	p-value	n	Mean	S.E.	p-value	n	Mean	S.E.	p-value
0	6	2.097	0.175		6	10.417	1.676		6	6.785	0.324		6	5.646	0.746	
250	6	2.362	0.357	0.468	6	11.182	1.913	0.486	6	7.328	0.551	0.257	6	4.416	0.275	0.880
500	6	1.949	0.210	0.548	6	10.993	0.958	0.566	6	7.787	0.698	0.139	6	4.406	0.436	0.933
1000	6	2.063	0.245	0.582	6	9.303	1.834	0.601	6	8.723	0.660	0.016	6	5.512	0.301	0.699
Trend p-value		0.660			0.732				0.008				0.421			

It is likely that had the variability been similar to all other treatment groups, the single statistically significant increase in % tail DNA in the highest dose group would have been less significant although it is difficult to say whether this would translate into a negative call rather than positive.

Comparing comet data in the lung of male and female B6C3F1 mice highlights the greater degree of variability in males compared to females (Chart 3). Roughly equal variance was noted across dose groups in males.

Chart 3. Individual animal data for male and female B6C3F1 mice, % tail DNA in lung tissue.



Whilst it is possible to add biological relevance to the comet assay data from male rats, it is not as clear with female mice. Considering that the variability in the vehicle controls from lung were so low as well as the lack of any positive effects from the Ames and micronucleus tests (*in vitro* and *in vivo*), It is highly unlikely that Cumene induces strand breaks in the livers of male F344 rats and the lung of B6C3F1 female mice. Using a weight of evidence approach there is no clear genotoxic potential from Cumene demonstrated by these data.

A similar opinion was reached by the German MAK commission for occupational exposure to cumene (isopropyl benzene) in 2018 when the data was re-evaluated [4]. They concluded that cumene was non genotoxic.

According to the current version of the OECD guideline for the *in vivo* alkaline comet assay (OECD 489) [5] caution is recommended for the freezing and storage of tissues, at the time of OECD guidance acceptance (2016) there was no universally accepted method. Differences in variability noted between tissues may be due to the cryopreservation methodology as well as the use of uncommon rodent strains in acute genotoxicity tests.

2) Genotoxicity of α Methylstyrene

α methylstyrene (purity 99.5%) tested negative in an Ames test with TA97, TA100, TA98 and TA1535 in the presence and absence of S-9 (up to 30% Aroclor induced rat and hamster S-9) to 3333 µg/plate, limited by toxicity [1]. It was also negative in an *in vitro* chromosome aberration test, without metabolic activation in Chinese hamster ovary (CHO) cells [1]. Positive effects were noted in sister chromatid exchange (SCE) studies although weakly, in one using acetone as a solvent where

there was less than a doubling in SCE's compared to solvent controls [6]. Positive responses were also noted in an NTP SCE test [7] where increases in SCE's were only seen in the presence of S-9. It is unclear from these studies what toxicity measures if any were used to set the limit dose. At a concentration of 166.7 µg/ml there are no scoreable cells and it is therefore likely that at the second highest tested concentration, 149.9 µg/mL there would have also been significant toxicity, the confirmatory trial in these tests also only analysed 25 cells per concentration which is of questionable statistical power to reliably detect a positive response.

There are also *in vivo* micronucleus studies published by the NTP performed on α methylstyrene [8] in male and female B6C3F1 mice integrating a micronucleus endpoint into a 13 week inhalation toxicology study. The species and sexes of animals chosen mirrored those which gave positive responses in a 2-year bioassay. The data are shown below:

Table 3. Micronucleus frequencies in peripheral blood of Male B6C3F1 mice

Tissue: Blood; Sex: Male; Number of Treatments: 65; Time interval between final treatment and cell sampling: 24 h							
	MN PCE/1000			MN NCE/1000			% PCE
Dose (ppm)	N	Mean ± SEM	p-Value	N	Mean ± SEM	p-Value	Mean ± SEM
Vehicle Control[1]	10	3.90 ± 0.66		10	5.30 ± 0.50		3.70 ± 0.17
75.0				10	5.80 ± 0.44	0.3171	
150.0				10	5.80 ± 0.63	0.3171	
300.0				10	5.00 ± 0.65	0.6165	
600.0				10	4.60 ± 0.45	0.7597	
1000.0	10	5.00 ± 0.58	0.1213	10	6.30 ± 1.02	0.1759	3.27 ± 0.17
Trend p-Value		0.1210			0.3460		
Trial Summary: Negative							

Table 4. Micronucleus frequencies in peripheral blood of Female B6C3F1 mice

Tissue: Blood; Sex: Female; Number of Treatments: 65; Time interval between final treatment and cell sampling: 24 h							
	MN PCE/1000			MN NCE/1000			% PCE
Dose (ppm)	N	Mean ± SEM	p-Value	N	Mean ± SEM	p-Value	Mean ± SEM
Vehicle Control[1]	10	4.10 ± 0.59		10	5.10 ± 0.46		3.76 ± 0.19
75.0				10	2.40 ± 0.43	0.9991	
150.0				10	2.90 ± 0.90	0.9931	
300.0				10	3.60 ± 0.48	0.9465	
600.0				10	5.30 ± 0.42	0.4221	
1000.0	8	4.75 ± 0.59	0.2561	8	9.13 ± 0.77	< 0.001 *	3.53 ± 0.29
Trend p-Value		0.2560			< 0.001 *		
Trial Summary: Positive							

The MN frequency in male B6C3F1 mice were similar to and not statistically different to concurrent controls for both micronucleated PCE and NCE. In female B6C3F1 mice however, although the frequency of micronucleated PCE's was similar to the concurrent control, there was an increase in micronucleated NCE's at the highest tested dose (1000 ppm). These data are indicative of a chromosome damage event that occurred at least 72 hours prior to sampling as there are no increases in micronuclei in immature erythrocytes but there are in mature erythrocytes which have a half life of approximately 40 days in mice.

Without a historical range to compare the data to it is difficult to determine the biological relevance of this increase [1]. There are also only 8 animals in the high treatment dose group in female rats which may be due to deaths, this is not clear from the data. With inhalation studies it is also impossible to rule out the effects of hypoxia at high exposures as lower oxygen levels has also been shown to increases micronucleus frequency in rats and mice [9] and cumene itself is an irritant inducing pulmonary inhibition at high doses [10].

Whilst there is some evidence that α methylstyrene is genotoxic, it has only shown effects at high doses and/or extremes of toxicity. Indeed, the NTP conclude that α methylstyrene oxide (a product of cumene metabolism) is the most likely cause of the liver tumours noted in the 2 year cumene bioassay study in rats and mice [11]. Metabolism of cumene results in side-chain oxidation of α methylstyrene to α methylstyrene oxide potentially causing DNA damage. α methylstyrene oxide is clearly positive for induction of point mutation when tested in the TA100 strain of *Salmonella typhimurium.*

The levels that would be required to form α methylstyrene oxide from cumene are either higher than the maximum tested concentrations of cumene *in vitro* and *in vivo* or the metabolite itself has a very short half-life, as no increases in genotoxicity were observed when testing cumene itself.

3) Increases of K-ras and p53 mutations in cumene induced lung tumours of B6C3F1 mice

Studies showing increases in K-ras and p53 mutations as well as the additional mechanistic FLARE study hint at a mode of action for cumene carcinogenesis that is based on elevated levels reactive oxygen species (ROS).

The increase in G-T transversion and A-G transition mutations (codons 12 and 61 respectively) in K-ras and G-A and C-T transition mutations in p53 (codons 155 and 133 respectively) are commonly seen from cumene induced tumours in B6C3F1 male and female mice. K-ras mutations were detected in 87% of neoplasms from cumene induced tumours compared to 14% in controls. p53 mutations were detected in 52% of cumene induced neoplasms with none found in controls [12].

Loss of heterozygosity was also noted in cumene induced tumours on chromosomes 4 and 6 (13% and 12% respectively compared to zero in controls).

Cumene induced mutation spectra have been postulated as possibly due to DNA damage or genomic instability due to the lack of these mutations in control animals and spontaneous tumours. Similar mutation spectra have been identified from indirect acting DNA damage agents such as ROS and this is postulated as the likely mode of cumene induced tumours as they are consistent with 8-OG-G adducts produced from oxidative DNA damage. Information from the FLARE experiments also add weight to the MOA being ROS related.

Both male and female B6C3F1 mice exhibited tumours in the lung in a lifetime whole body inhalation exposure bioassay, there did not seem to be any obvious sex related differences in responses to cumene exposure.

Male animals were negative in the comet assay for increases in DNA damage in the lung, but female animals were weakly positive (see Chart 3). As previously discussed, this discrepancy is most likely due to a lack of historical control range and low variability in the vehicle control group in females rather than any inherent DNA reactivity.

Cumene is irritating to the respiratory system with higher doses causing pulmonary inhibition in rats and mice [10], it is therefore equally likely that the mutation spectra in the lung are ROS related and a response to inflammation in respiratory tissues from continued cumene exposure over the lifetime of the animal.

Furthermore, the lung effects may also be explained via metabolism in lung tissue itself. The main metabolic route of alkylbenzenes in mammals goes via side chain oxidation to aryl alcohol, which is further oxidated to aryl aldehyde and then to an acid which conjugates with an endogenous acid. The aldehyde is rapidly metabolized by aldehyde dehydrogenase in the liver but not in the lungs where aldehyde dehydrogenase is deficient [10].

Conclusions

The CLH review of Cumene was pragmatic and considered a weight of evidence for the likely genotoxicity of Cumene. A deeper evaluation of the data presented suggests that:

1) Cumene is non-genotoxic *in vivo* due to a lack of variability in comet assay vehicle control animals in male rats and female mice.
2) Whilst metabolites of cumene may be genotoxic, this is likely to be via a non-direct mode of action (i.e. ROS) and as such non-linear and thresholded. The lack of positive responses in genotoxicity tests with Cumene in the presence of S-9 as well as whole animal studies do not show positive effects, indicating that the conversion of cumene to reactive metabolites are low enough to be below threshold levels.
3) Mutation spectra of lung tumours suggesting a DNA damage MOA are probably due to altered metabolism in the lung or a consequence of irritation combined with inflammation leading to ROS generation rather than any direct activity from cumene itself.

For somatic cells, the majority of genotoxicity tests on cumene gave negative results and of the few that did show effects, these were shown to be largely irrelevant once biological variability was taken into account.

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