

2-Phenylpropene: Detailed comments as summarized in Section A (comments on the evaluation of the available genotoxicity data) and Section B (Comments on Grouping of 2-Phenylpropene with Structurally Related Styrene, Cumene and Ethylbenzene)

Section A: Comments on the evaluation of the available genotoxicity data on 2-phenylpropene

Page 2: Pertinent and reliable information (equivalent to OECD TG 471/472, 473, 476) is provided by three unpublished study reports considered similar to internationally accepted guidelines with GLP compliance and one NTP study, covering all genotoxic endpoints (NTP, 2007; Study report mutagenicity, 1989; Study report mutagenicity, 1991a; Study report mutagenicity, 1991b; Study report mutagenicity, 1991c; Study report mutagenicity, 1997a; Study report mutagenicity, 1997b)

Comment: We agree that that the identified studies are pertinent to the assessment of 2-phenylpropene but have some reservations with respect to the claim that these are all sufficiently reliable.

We agree that there are definitive negative results in the Ames test in the form of the Japanese study for which data from all 5 OECD recommended strains with and without S9 show an absence of mutagenicity. We also agree that this key study is supported by an absence of any positive responses in other studies of lower reliability that, individually have incomplete information/data and cannot themselves be used as key studies for a definitive conclusion¹. Therefore, on the basis of this key Japanese study, combined with a weight of evidence taking into account all available Ames studies, it can be concluded with a high level of confidence that 2-phenylpropene does not induce gene mutation in bacteria.

The same level of confidence cannot be attributed, however, to the negative findings in the other vitro mutagenicity tests available for 2-phenylpropene. In the case of these studies, we recognise methodological deficiencies that limit the interpretation of these studies likely reflect their age and advancements in respective OECD guidelines. For example, the in vitro mammalian cell (CHO) hprt mutation study cannot be interpreted because of deficiencies in the study design and the in vitro mammalian cytogenetic studies did not use the appropriate measures of cytotoxicity rendering it impossible to determine whether appropriate concentrations were used². The inclusion of the SCE data is commented on below. The same reservations apply to the three in vitro mammalian cytogenetic studies that, despite being negative, i.e. not showing any evidence that 2-phenylpropene can induce chromosomal effects in vitro, suffer from methodological deficiencies that, in our view,

¹ See Appendix 1 Ames tests

² See appendix 2 In vitro mammalian studies gene mutation

prevent them from being used to draw definitive conclusions concerning the ability of 2-phenylpropene to induce chromosomal effects³.

Page 21: The results derived from standard in vitro mutagenicity tests of sufficient quality are, hence, not indicative of any mutagenic potential related to 2-phenylpropene exposure.

Comment: We agree that that there are definitive negative results in the Ames test sufficient to conclude with confidence that 2-phenylpropene does not induce gene mutation in bacteria. We also agree that there are no indications from other vitro mutagenicity tests available that indicate a positive concern for mutagenicity for 2-phenylpropene. So, we agree that based upon a thorough review of the available studies using current OECD recommendations the evidence supports the CLH report conclusion that “classification of 2-phenylpropene for mutagenicity is not recommended”. However, we find the available evidence in vitro mammalian cells to be insufficient to exclude any concern and therefore propose further in vitro testing on this endpoint.

Page 21: The report states **“As part of the genetic toxicity evaluation, a sister chromatid exchange (SCE) assay comparable to OECD TG 479 was conducted by NTP (NTP, 2007). A statistically significantly increased frequency of SCEs was seen in the presence of metabolic activation. The effect was reproducible and dose-dependent. Being an indicator test for genotoxicity, the SCE assay detects damage to the DNA to indicate putative mutagenic effects.”**

Comment: We are mindful that the Sister Chromatid Exchange (SCE) assay is no longer considered to be appropriate for the evaluation of genotoxicity. It is now recognised that this endpoint is not actually reflective of genetic damage (Wilson and Thompson, 2007; Claussin et al., 2017). Based on this current understanding, the OECD TG for in vitro SCE was deleted during the last round of TG revisions. We note that the CLH report includes a discussion as to why the SCE test is no longer appropriate, and states that **“Given the aforementioned restrictions of the SCE test system and the limited quality of the data, both studies do not provide reliable information for the purpose of classification”**, it nevertheless includes the studies and their results in the report. Accordingly, we consider it incorrect to make the claim that the SCE assay is an **“Being an indicator test for genotoxicity, the SCE assay detects damage to the DNA to indicate putative mutagenic effects”**. Furthermore, we consider it inappropriate to link this with the observation of **“statistically significantly increased frequency of SCEs was seen in the presence of metabolic activation”** to infer that 2-phenylpropene is a putative mutagen by causing damage to DNA.

³ See Appendix 3 In vitro mammalian cytogenetic studies

Furthermore, the inclusion of the studies of Norppa and Tursi, 1984 and Norppa and Vainio, 1983 and the authors conclusion of “**a reactive metabolite generated due to erythrocyte-mediated metabolic activation is presumably responsible for the genotoxic effect**” (as cited in the CLH dossier) is nothing more than speculation and a forward or lead-in to the next speculation, that of presumed epoxidation of 2-phenylpropene to form 2-phenylpropene oxide, and finally, analogy to styrene and its biotransformation gives rise to styrene-7,8-oxide.

While this linking of uninterpretable study data and speculation creates the basis for a possible concern for human relevance, it fails to recognise not only the low reliability of the evidence and that while both Rosman et al., 1986 provided clear evidence for mutagenicity of 2-phenylpropene oxide in bacteria and styrene oxide was mutagenic in bacteria⁴, 2-phenylpropene was reliably negative in bacteria, with and without external metabolism. Therefore, while plausible, there is currently no reliable evidence that 2-phenylpropene oxide formation occurs in practice and this remains purely speculative. This point is discussed in further detail in due course.

Since the SCE assay was included in the CLH report and implicated in this speculative line of thinking, a few comments are provided on the limitations of the studies⁵.

Page 21-24: The report describes the NTP (2007) MN test in mice following long-term inhalation exposure and provides a narrative and summary in table 14 of the comparison of this study design/results with the criteria defined in OECD TG 474. It then goes on to conclude “**owing to the adapted testing design (integration in a subchronic RDT study), the acceptability criteria defined within the OECD TG 474 may not be entirely fulfilled. However, due to the fact that the testing protocol is well-established and frequently used by NTP as a standard in vivo mutagenicity test, the quality of the study is acceptable** (underlining added by us). **Regarding the biological relevance of the test results, significant uncertainties exist which do not allow for a final conclusion as to whether or not the result in high-concentration females can be regarded as a clear positive result.**”

Comment: We agree with the identified deficiencies of this study but place greater significance to these than apparently the CLH reviewer does. In particular, we note that the background level for micronuclei was unusually high in this experiment. For male mice, the response in NCEs was negative, with substantial variability in the number of micronuclei in the top exposure group. In female mice only the top concentration yielded a positive response. However, the 3 lower exposure group had fewer micronuclei than the chamber control, while the second highest concentration had the same level of micronuclei as the chamber control. Thus, the positive dose response in NCEs is based solely on the increased level in the top (toxic) concentration and the statistically significant trend reflected the

⁴ [Registration Dossier - ECHA \(europa.eu\)](https://echa.europa.eu)

⁵ Appendix 4: Sister Chromatid Exchange Assays

depressed levels below control. Furthermore, PCE and not NCE scoring is recommended in the TG and these were scored in the chamber controls and top concentration for both males and females and found to be negative.

We note that the NTP investigators make an argument that the single positive results in NCEs (in females) occurred because the damage took a long time to manifest. This would be very unusual, given that the exposure was continuous for 3 months and it would be expected that PCEs would show an effect before the NCE effect could be seen. Generally, with the micronucleus test and continuous exposure, a steady state is obtained for micronucleus following a few days of treatment. That is, the number of micronuclei being generated is offset by cells leaving the system. In our opinion, therefore, the argument put forward by the NTP investigators is unlikely and consequently the relevance of this study remains unclear.

Considering all these aforementioned weaknesses, we are of the view that “... **the fact that the testing protocol is well-established and frequently used by NTP as a standard in vivo mutagenicity test**” is not sufficient justification to conclude that “... **the quality of the study is acceptable.**”

Page 24: The report describes the MN test in male mice following short-term oral exposure conducted by Rim et al. (2012). The report concludes “**Minor deviations to OECD TG 474 are noted (number of analysed PCEs and total erythrocytes less than recommended, no historical control data). Given that no evidence of bone marrow exposure is provided (ratio of PCEs among erythrocytes unaltered in all exposure groups), the result may not be considered clearly negative**”.

Comment: We agree, with the identified deficiencies in this study that prevent it from being used for definitive results. To these we add that oral exposure is not the most relevant route for 2-phenylpropene. Granted no proof of bone marrow exposure was available from this study, it is a reasonable certainty that orally administered 2-phenylpropene in mice used in this study was systemically available (and thus assuring bone marrow exposure) similar to what was reported in the rat oral ADME studies cited in the CHL Report.

Page 25: “**Hence, there is no evidence for the ability of 2-phenylpropene to induce mutations in bacterial and mammalian cell cultures. Positive findings obtained from available SCE tests are indicative of some genotoxic activity in vitro. The relevance of these results is, however, unknown. Hence, the information from available in vitro tests does not support classification.**”

Comment: We agree to this statement regarding the ability of 2-phenylpropene to induce mutations in bacterial and mammalian cell cultures and to the conclusion that the information from available in vitro tests does not support classification. However, for the reasons provided above, we disagree with the statement that positive findings obtained

from available SCE tests are indicative of some genotoxic activity in vitro as attested previously.

Page 26: “Multiple treatments over a long period of time were hypothesised to be required for these substances to reveal its mutagenic potential (Witt et al., 1999). However, as described above the biological relevance of the outcome in females subjected to the highest concentration in the NTP study is questionable.”

Comment: We acknowledge that the negative oral study by Rim is insufficient to negate the findings in the NTP repeat dose inhalation study. However, we do not concede that the findings in high-concentration females can be regarded as a clear positive result and the hypothesised requirement of multiple treatments proposed by Witt et al is as described, merely a hypothesis. So, while Witt et al were of the view that **“a clearly positive outcome in a MN test in peripheral blood following long-term exposure as an integrative part of a subchronic repeated dose toxicity study has been found to be highly predictive for rodent carcinogenicity”** in the case of 2-phenylpropene the outcome was neither clearly positive nor proven to be linked to a requirement of multiple treatments. As such the observation in the CLH report that **“The carcinogenic response in mice was, thereby, considerably stronger in females as compared to males, which somewhat coincides with the findings from the MN test”** may just be a chance observation.

Section B: Comments on Grouping of 2-Phenylpropene with Structurally Related Styrene, Cumene and Ethylbenzene

The proposed Harmonised Classification and Labelling (CLH Report) on 2-phenylpropene groups this substance with structurally related styrene, cumene and ethylbenzene with regards to its genotoxic potential. The primary focus of the comments below is to address whether such a grouping is appropriate in view of the available data on these molecules. While drawing inferences from structural analogy is a useful tool for data poor substances, such is not the case with 2-phenylpropene where the available data, albeit currently insufficient to fully exclude genotoxicity, is not enhanced by speculative analogy to other similar substances with dissimilar toxicological profiles. Comments are presented below on the relevant statements excerpted from the CLH Report.

Page 21: “With regard to the presumed biotransformation of 2-phenylpropene, an epoxidation of the vinyl group has been suggested as the initial metabolic step (cf. 9.1), giving rise to the formation of 2-phenylpropene oxide.”

Comment: While generation of an epoxide through biotransformation is a theoretical possibility for 2-phenylpropene given its metabolite profile, this putative epoxide (2-phenylpropene oxide has not been experimentally confirmed. This fact is duly acknowledged by the CLH Report on page 29. The biological activity of a reactive metabolite

depends upon its half-life. Since this intermediate was not experimentally identified, it is evident that the epoxide is readily converted to downstream metabolites (duly acknowledged by the CLH Report). It seems likely that this epoxide metabolite of 2-phenylpropene, even if formed in vivo, is rapidly converted to non-reactive downstream metabolite/conjugate virtually abolishing its potential macromolecular reactivity. This inference is based on the observation that, unlike styrene (Moore et al., 2019), 2-phenylpropene is not mutagenic in the Ames bacterial reverse mutation assays even in the presence of an externally supplied metabolic activation system (rat liver S9).

Page 21: *“Metabolic activation resulting in the formation of a genotoxic metabolite has also been described for styrene.”*

Comment: The above statement is made in the context of comparing 2-phenylpropene to styrene to generate a genotoxic metabolite. As discussed previously, even if an epoxide intermediate of 2-phenylpropene is formed, such an intermediate is rapidly converted to non-reactive downstream metabolite/conjugate, virtually abolishing its potential to react with DNA. This inference is based on the observation that, unlike styrene (Moore et al., 2019), 2-phenylpropene is not mutagenic in the Ames bacterial reverse mutation assays even in the presence of an externally supplied metabolic activation system.

Page 26: *“Taking together, the mutagenic potential of this group of aromatic hydrocarbons appears to be dependent on the formation of a reactive epoxide. Quantitative difference in metabolic activations may explain the different mutagenic responses. Metabolic activation resulting in the formation of a mutagenic intermediate (2-phenylpropene oxide) appears to be a plausible mechanism by which 2-phenylpropene might confer its genotoxic properties.”*

Comment: The CLH Report appropriately states that a classification for mutagenicity is not appropriate based on the total weight of evidence analysis. The document further commented that “..... the existence of some genotoxic potential attributed to 2-phenylpropene, or its metabolite(s) cannot be ruled out”. This assertion was based on comparison to the structural analogue styrene where epoxidation of the side chain double bond results in the formation of the reactive styrene-7,8-oxide (SO). 2-Phenylpropene was also compared to two other structural analogues, viz., cumene and ethylbenzene where the likelihood for the formation an epoxide intermediate exists during biotransformation. 2-Phenylpropene is clearly negative in multiple Ames tests even in the presence of an externally supplied metabolic activation. Similarly, cumene and ethylbenzene were also not positive in the Ames test both with and without metabolic activation (Gollapudi et al., 2021; IARC, 2000, 2012). On the other hand, styrene has been reported to be positive in the Ames test in the presence of metabolic activation (Moore et al., 2019). Thus, the divergent Ames test results suggests that grouping these substances for their potential mutagenicity simply based on the likelihood of forming an epoxide metabolic intermediate is not scientifically warranted. In addition to quantitative differences in the formation of the epoxide

intermediates, the rapidity of their elimination is likely responsible for the absence of mutagenic activity for 2-phenylpropene, cumene and ethylbenzene. Negative result in the Ames test is an excellent sentinel for the absence of DNA reactivity of a substance. Thus, reliance on experimental data from well conducted studies, rather than ascribing mutagenic potential based on the likelihood to generate an epoxide metabolic intermediate should be the preferred approach.

Page 30: “Similar to what has been observed with the close structural analogue, styrene, epoxidation as the first metabolic step has been proposed and appears to be plausible. The hypothetical first reactive metabolite in this pathway, 2-phenylpropene oxide, is mutagenic in bacteria. However, the relevance of this potential step in the biotransformation of 2-phenylpropene for in vivo mutagenicity is unknown.”

Comment: As discussed previously, while the plausibility of an epoxide formation exists during biotransformation of 2-phenylpropene, the available data strongly points to the possibility that such an intermediate is unlikely to be of significance vis-à-vis its genotoxic potential owing to a very short half-life. Unlike styrene, no epoxide intermediate of 2-phenylpropene has been detected in metabolism studies.

Page 30: “Nevertheless, the existence of some genotoxic potential attributed to 2-phenylpropene or its metabolite(s) cannot be ruled out.”

Comment: While it is recognized that the burden to establish non-genotoxicity for a substance might be high, the above statement needs to be tempered with the fact that the available data do not rule in the existence of genotoxic potential following in vivo exposure to 2-phenylpropene.

Page 41: “It is worth noting that although cumene can be metabolised to 2-phenylpropene, cumene has a broader spectrum of neoplastic responses, suggesting that 2-phenylpropene may not be the sole driver of the carcinogenic effects conferred by cumene (NTP, 2009).”

Comment: The above statement is further supported by the fact that the top tested concentration of 600 ppm 2-phenylpropene in the NTP bioassay likely resulted in a substantially higher systemic dose of 2-phenylpropene relative to that formed metabolically from cumene and the tumour profile of these two substances is quite different (especially there being no lung tumour induction with 2-phenylpropene). Thus, cumene and 2-phenylpropene should be considered independently rather than based on their structural analogy.

Page 41: “Styrene is another prominent structural similar aromatic hydrocarbon. NTP considers styrene “reasonably anticipated to be a human carcinogen” (NTP, 2011). IARC has classified styrene as “probably carcinogenic to humans” (Group 2A) (IARC, 2019). A genotoxic MoA is considered in both assessments.”

Response: The weight of evidence from multiple inhalation studies informs that styrene is not a liver carcinogen in the mouse. The evidence from the oral mouse study is weak and questionable and as such there is no similarity in tumor profile between styrene and 2-phenylpropene. While the MoA responsible for liver tumours in 2-phenylpropene exposed female mice has not been elucidated, it is likely that a non-genotoxic MoA mediated by CAR/PXR receptor activation, with little relevance to humans, was involved. CLH Report also considered this MoA was likely responsible for the marginal increase in liver tumours observed following cumene exposure. Additionally, the available data on 2-phenylpropene genotoxicity does not support a genotoxic MoA for any tumour type observed in 2-phenylpropene exposed mice or rats.

Page 41: *“Ethylbenzene is another structurally similar substance that induces (pre)neoplastic lesions similar to those observed with 2-phenylpropene.”*

Comment: The two tumour types common to 2-phenylpropene and ethylbenzene (i.e., male rat kidney and mouse liver) are species-, strain- and/or sex-specific. Ethylbenzene is not a genotoxicant, as appropriately cited in the CLH Report. There is also evidence indicating that ethylbenzene-induced rat kidney tumours are the result of treatment-induced exacerbation of chronic progressive nephropathy which is considered to have no relevance for humans (Hard, 2002). A significant difference between the two substances is that renal tumours were increased in both sexes following exposure to ethylbenzene vs. in males only with 2-phenylpropene. As detailed in the CLH Report, a conclusive MoA responsible to renal tumours in 2-phenylpropene treated rats is lacking. Similarly, the mouse strain used in both studies (i.e., B6C3F1) has a relatively high background incidence of liver tumours and is well-recognized as being susceptible to the induction of these tumours (Drinkwater et al., 1989; Cohen, 2010). A number of substances belonging to diverse chemistries have been implicated in the induction of these tumour types in the NTP carcinogenicity studies using F344 rat and B6C3F1 mouse. Thus, structural similarity of 2-phenylpropene and ethylbenzene is not necessarily the driver for the common tumour types between these substances.

Page 40: *“Generally and in relation to all neoplasms found in both species, the unresolved question on the genotoxic potential of 2-phenylpropene precludes a conclusion on whether a practical threshold can be assumed.”*

Comment: As previously stated in the commenting, the negative response in the Ames test is a good indicator that 2-phenylpropene and/or its metabolites do not have the potential to react with the DNA. Any reactive metabolites generated in vivo appear to be efficiently detoxified. There is also evidence pointing to the absence of clastogenic potential for AMS in mammalian cells based on the in vitro testing. The weight of evidence based on two erythrocyte micronucleus tests appears to suggest that 2-phenylpropene is not likely an in vivo clastogen/aneugen. The SCE response is not an indicator of DNA damage and as such the positive results reported for 2-phenylpropene for this endpoint in an in vitro assay is not

suggestive of a genotoxic response. While a plausible MoA for each tumour types in 2-phenylpropene exposed rats and mice is not fully elucidated, a genotoxic MoA can reasonably be excluded based on the available data.

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Appendix 1: Ames Tests

a) Unpublished Study report 1991:

This study was conducted by Microbiological Associates. 4 of the 5 strains used are compliant with OECD TG471. The complete data from this study are not included and therefore, this study cannot be evaluated. The study report indicates that all strains gave negative results both with and without S9.

b) NTP 2007

4 of the 5 OECD recommended strains were used, both with and without S9.

The data from this study is reported in the NTP report and all of the experiments are clearly negative. Because one of the OECD recommended strains is not included, it cannot be concluded from this study that AMS does not induce mutation in the Ames test.

c) Unpublished Study report (in Japanese) 1997

The study report is in Japanese. There is a summary of the study in the OECD 1998 document. The data tables in the study report are in English and therefore the data can be evaluated. The study used 5 of the appropriate OECD strains and was conducted both with and without S9. The results are clearly negative for all 5 strains. This data can be interpreted as evidence that AMS does not induce mutation in the Ames test.

d) Unpublished Study report 1989

Only a brief summary of this study is available and it is in German. 5 strains were used, 4 of which are the OECD TG471 recommended strains. The data are not available for evaluation and therefore, definitive conclusions cannot be drawn for the strains that were used. The summary indicates that the results were negative.

e) Ames data from Presumed Metabolite (2-phenylpropene oxide): Rossman et al. 1986

Rossman et al. evaluated a series of para-substituted AMS oxide derivatives in the Ames test. The preincubation method was used with strains TA100 and TA1535 and only the without metabolic activation condition was tested. The oxides gave only marginal responses in TA1535. 2-phenylpropene oxide was very clearly positive in TA100. The top concentration of 10 mmol/tube showed signs of toxicity but still approximately 3500 revertants/plate were seen with the concurrent

background cultures showing values of 106, 156 and 147. Because TA100 was positive, it is not necessary for all of the OCED recommended strains to be tested. It can be concluded that 2-phenylpropene oxide is mutagenic. A positive result of an AMS presumed metabolite, is not, however, sufficient evidence to conclude that AMS itself is mutagenic.

Appendix 2: In vitro mammalian studies gene mutation

a) CHO hprt unpublished study report 1991c

This study was performed by Microbiological Associates and the study report indicates that the results were negative both with and without metabolic activation. The report states that the results are presented in tables, but the tables are not available for review. The report states that spontaneous mutant frequencies normally vary from 0 to 25 per million clonable cells and the laboratory considers responses above 40 mutants per million cells as positive. In the text of the report, the background for the 2-phenylpropene experiment is indicated to be 17.1 per million for the solvent control and 3.7 per million for the untreated control (without metabolic activation). The treated cultures, which showed less than ideal levels of cytotoxicity across the dose range, had mutant frequencies between <1 to approximately 5 mutants per million cells. For the with metabolic activation treatment condition, the background was 0.9 per million for the solvent control and 7.7 per million for the untreated control. The cytotoxicity was excessive for 3 of the 5 concentrations and the mutant frequency varied from <1 to approximately 5 mutants per million. A confirmatory assay was conducted, but the problems with concentration selection and the very low levels for the mutant frequency both with and without metabolic activation were similar to the first experiment. A careful review of the number of cells used for treatment, expression and mutant selection shows that the protocol did not meet the requirements outlined in TG476 with regard to using a sufficient number of cells to provide adequate statistical power. Therefore, while the study is reported to be negative, it is not appropriate to use this data to draw a conclusion with sufficient confidence that 2-phenylpropene is negative for hprt mutation induction.

Appendix 3: In vitro mammalian cytogenetic studies

a) CHL cells unpublished study report in Japanese 1997:

While this study report is written in Japanese, the data tables are in English. The protocol did not use the appropriate measure for cytotoxicity, therefore, it is not possible to determine if any of the test concentrations were within an acceptable toxicity range. It is clear that at least the top concentrations used were excessively toxic: there were not enough cells that could be scored. The study protocol indicates that only 200 cells were scored per concentration while the current recommendation is to score 300 cells. The aberrations reported includes gaps which are not considered appropriate. While the data presented does not show any increase in the number of chromosome aberrations with increasing concentration, the study deficiencies make it inappropriate to draw any conclusions concerning whether AMS can induce chromosome aberrations.

b) CHO cells NTP 2007:

The protocol did not use the appropriate measure for cytotoxicity, therefore, it is not possible to determine if any of the test concentrations were within an acceptable toxicity range.

In addition, the treatment times used were not the normally recommended length. Without S9 treatment was for 8 hours, after which Colcemid was added and incubation continued for another 2 hours. With S9, the treatment was on 2 hours, the cells were washed and allowed another 10 hours in fresh medium with Colcemid included in the cultures for the final 2 hours. These are not the treatment times recommended in TG473. Slides were prepared and 200 cells scored per test concentration. The current recommendation is that 300 cells be scored.

Two trials both with and without S9. Doses in the second trial were increased so that a toxic dose (not scored) was seen. There is clearly no increase in chromosome aberrations, but unclear as to whether the doses were appropriate as the appropriate measure of cytotoxicity was not used. While the study was called negative by NTP, given the deficiencies in the study, it is not appropriate to use this data to make a definitive conclusion.

c) Unpublished study report 1991

This study was conducted by Microbiological Associates using CHO cells. Mitotic Index was used as the cytotoxicity measure rather the currently recommended methods. Thus, it is not possible to evaluate whether concentrations used were appropriate. Cells were treated both with and without S9. Only two (short) treatment times were used while the current TG473 recommends three. BrdU was added to the cultures which is not generally the standard practice. The protocol called for 200 cells (when possible) to be scored per concentration while the current recommendation is for 300 cells. A range finding experiment was conducted and concentrations selected for the full study. In spite of this narrowing of the concentrations tested, the top concentrations used were very toxic and there were no metaphases to score. The study report indicates that the data was presented in tables, but the tables were not available for evaluation. The results were reported to be negative both with and without S9. Study deficiencies prevent using this study for definitive conclusions.

Appendix 4: Sister Chromatid Exchange Assays

a) CHO NTP 2007

CHO cells were treated both with and without S9. Given that the TG for SCE was not revised, there no updated recommendation for the appropriate cytotoxicity measure and therefore it is not clear whether the concentrations that were used were "appropriate". Both with and without S9 the top concentrations are indicated as too toxic. The results are reported as weakly positive with S9. This is based on a culture giving 9.76 SCEs/cell with a background of 7.6 SCEs/cell. A second trial with S9 was done. Using doses between the trial 1 top usable dose and the top toxic dose. The second trial gives a more convincing positive response, but it still is not clear if the dose levels were appropriate (or too toxic) and only 25 cells scored for this 2nd trial. For these reasons this study is uninterpretable.

b) Human lymphocytes Norppa and Vainio 1983

In this study the authors evaluated styrene and 11 styrene analogues, one of which was 2-phenylpropene. Whole blood human lymphocyte cultures were used. No exogenous activation was used. It is indicated that when possible 25 cells were scored per culture (generally 50 per concentration). Data are presented graphically. While a number of the analogues gave large numbers of SCEs/cell, the response with 2-phenylpropene was very small. The results for 2-phenylpropene are reported as positive by the study authors.

c) Human lymphocytes Norppa and Tursi 1984

This publication was not available for review, but there is some information and also a data table from the CLH report. Human lymphocytes were used for this in vitro treatment which was conducted both with whole blood and isolated lymphocytes. There is no indication as to how cytotoxicity and therefore an appropriate concentration range were selected. Only two concentration levels were used. The results were reported as positive, however given that only two concentrations with unknown levels of cytotoxicity were used, the results really cannot be properly interpreted.