Committee for Risk Assessment

RAC

Annex 1

Background document

to the Opinion proposing harmonised classification and labelling at EU level of

2-phenylpropene; α-methylstyrene

EC Number: 202-705-0
CAS Number: 98-83-9

CLH-O-0000007252-80-01/F

The background document is a compilation of information considered relevant by the dossier submitter or by RAC for the proposed classification. It includes the proposal of the dossier submitter and the conclusion of RAC. It is based on the official CLH report submitted to consultation. RAC has not changed the text of this CLH report but inserted text which is specifically marked as 'RAC evaluation'. Only the RAC text reflects the view of RAC.

Adopted

16 March 2023

CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2

Chemical name:

2-phenylpropene; α-methylstyrene

EC Number: **202-705-0**

CAS Number: **98-83-9**

Index Number: **601-027-00-6**

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ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON 2-PHENYLPROPENE; α-METHYLSTYRENE

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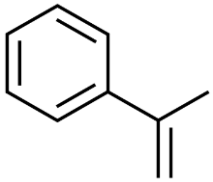
ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON 2-PHENYLPROPENE; α-METHYLSTYRENE

1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 1: Substance identity and information related to molecular and structural formula of the substance

Name(s) in the IUPAC nomenclature or other international chemical name(s)	Isopropenylbenzene
Other names (usual name, trade name, abbreviation)	2-Phenylpropene 2-phenylpropene α-Methylstyrol β-Phenylpropene (1-Methylethenyl)benzene 1-Methyl-1-phenylethylene Benzene, (1-methylethenyl)- [CAS]
ISO common name (if available and appropriate)	-
EC number (if available and appropriate)	202-705-0
EC name (if available and appropriate)	2-Phenylpropene
CAS number (if available)	98-83-9
Other identity code (if available)	-
Molecular formula	C_9H_{10}
Structural formula	
SMILES notation (if available)	CC(=C)C1=CC=CC=C1
Molecular weight or molecular weight range	118.18 g/mol
Information on optical activity and typical ratio of (stereo) isomers (if applicable and appropriate)	-
Description of the manufacturing process and identity of the source (for UVCB substances only)	-
Degree of purity (%) (if relevant for the entry in Annex VI)	≤ 100

1.2 Composition of the substance

Table 2: Constituents (non-confidential information)

Constituent (Name and numerical identifier)	Concentration range (% w/w minimum and maximum in multi-constituent substances)	Current CLH in Annex VI Table 3 (CLP)	Current self-classification and labelling (CLP)
2-Phenylpropene (CAS no. 98-83-9)	≤ 100	Flam. Liq. 3 Eye Irrit. 2 STOT SE 3 Aquatic Chronic 2	Flam. Liq. 3 Asp. Tox. 1 Skin Sens. 1B Eye Irrit. 2 STOT SE 3 Repr. 2 Aquatic Chronic 2

Table 3: Impurities (non-confidential information) if relevant for the classification of the substance

Impurity (Name and numerical identifier)	Concentration range (% w/w minimum and maximum)	Current CLH in Annex VI Table 3 (CLP)	Current self-classification and labelling (CLP)	The impurity contributes to the classification and labelling
-				

Table 4: Additives (non-confidential information) if relevant for the classification of the substance

Additive (Name and numerical identifier)	Function	Concentration range (% w/w minimum and maximum)	Current CLH in Annex VI Table 3 (CLP)	Current self-classification and labelling (CLP)	The additive contributes to the classification and labelling
-					

Table 5: Test substances (non-confidential information) (this table is optional)

Identification of test substance	Purity	Impurities and additives (identity, %, classification if available)	Other information	The study(ies) in which the test substance is used
-				

2 PROPOSED HARMONISED CLASSIFICATION AND LABELLING

2.1 Proposed harmonised classification and labelling according to the CLP criteria

Table 6:

	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATEs	Notes
					Hazard Class and Category Code(s)	**Hazard statement Code(s)**	**Pictogram, Signal Word Code(s)**	**Hazard statement Code(s)**	**Suppl. Hazard statement Code(s)**		
Current Annex VI entry	601-027-00-6	2-Phenylpropene	202-705-0	98-83-9	Flam. Liq. 3 Eye Irrit. 2 STOT SE 3 Aquatic Chronic 2	H226 H319 H335 H411	GHS02 GHS09 GHS07 Wng	H226 H319 H335 H411		STOT SE 3; H335: C ≥ 25 %	
Dossier submitters proposal					**Add** Carc. 2 Skin Sens. 1B	**Add** H351 H317	**Add** GHS08	**Add** H351 H317			**Add** D*
Resulting Annex VI entry if agreed by RAC and COM					Flam. Liq. 3 Carc. 2 STOT SE 3 Eye Irrit. 2 Skin Sens. 1B Aquatic Chronic 2	H226 H351 H335 H319 H317 H411	GHS02 GHS08 GHS07 GHS09 Wng	H226 H351 H335 H319 H317H411		STOT SE 3; H335: C ≥ 25 %	D*

*As polymerisation inhibitor 4-tert-Butylpyrocatechol is used (10 - 20 ppm). According to Annex VI Part 1 Section 1.1.3 of Regulation (EC) No. 1272/2008 the substance 2-phenylpropene must bear the note D. This note D points out that the substance is to be placed on the market in a stabilized form.
However, if the substance 2-phenylpropene does not contain the stabilizer for certain reasons, the distributor is obliged by Note D to put the statement "unstabilized" on the label of the substance.

Table 7: Reason for not proposing harmonised classification and status under consultation

Hazard class	Reason for no classification	Within the scope of consultation
Explosives	*hazard class not assessed in this dossier*	*No*
Flammable gases (including chemically unstable gases)		
Oxidising gases		
Gases under pressure		
Flammable liquids		
Flammable solids		
Self-reactive substances		
Pyrophoric liquids		
Pyrophoric solids		
Self-heating substances		
Substances which in contact with water emit flammable gases		
Oxidising liquids		
Oxidising solids		
Organic peroxides		
Corrosive to metals		
Acute toxicity via oral route		
Acute toxicity via dermal route		
Acute toxicity via inhalation route		
Skin corrosion/irritation		
Serious eye damage/eye irritation		
Respiratory sensitisation		
Skin sensitisation	**harmonised classification proposed**	**Yes**
Germ cell mutagenicity	**data conclusive but not sufficient for classification**	**Yes**
Carcinogenicity	**harmonised classification proposed**	**Yes**
Reproductive toxicity	*hazard class not assessed in this dossier*	*No*
Specific target organ toxicity-single exposure		
Specific target organ toxicity-repeated exposure	**data conclusive but not sufficient for classification**	**Yes**
Aspiration hazard	*hazard class not assessed in this dossier*	*No*
Hazardous to the aquatic environment		
Hazardous to the ozone layer		

3 HISTORY OF THE PREVIOUS CLASSIFICATION AND LABELLING

2-Phenylpropene is listed in Annex VI of the CLP Regulation (EC) No 1272/2008 of the European Parliament and of the Council (latest consolidated version: 26.07.2019).

RAC general comment

2-Phenylpropene (α-methylstyrene) is a volatile liquid (boiling point 165 °C, vapour pressure 253 Pa at 20°C). The substance is used as a co-monomer in a range of polymerisation processes, e.g. in the production of resins, paints, adhesives and plasticizers.

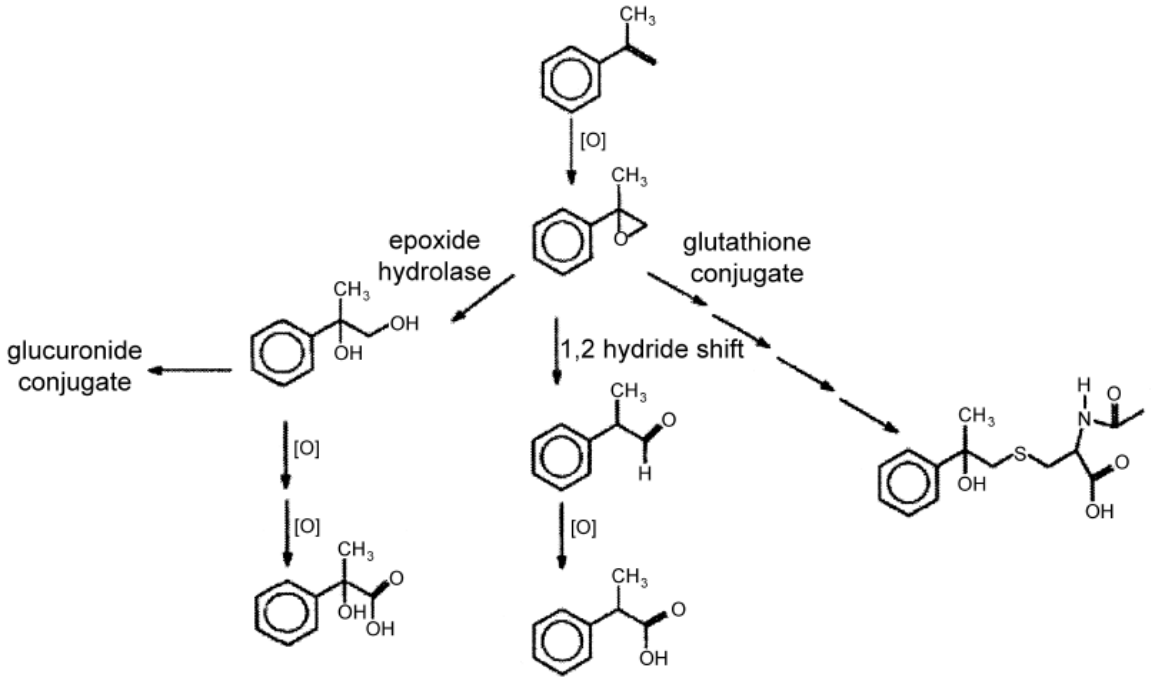
Note D

The dossier submitter (DS) proposed to add Note D because polymerisation inhibitor 4-*tert*-butylpyrocatechol is used (at 10-20 ppm) in the commercial formulations. Note D reads: *Certain substances which are susceptible to spontaneous polymerisation or decomposition are generally placed on the market in a stabilised form. It is in this form that they are listed in Part 3. However, such substances are sometimes placed on the market in a non-stabilised form. In this case, the supplier must state on the label the name of the substance followed by the words 'non-stabilised'.*

RAC agrees with the DS's proposal to **add Note D**.

Metabolism of α-methylstyrene

In an ADME study with α-methylstyrene in male F344 rats by NTP (2007), around 90 % of the radiolabel recovered after inhalation exposure was excreted in urine. The most abundant urinary metabolites were a glucuronide conjugate of 2-phenyl-1,2-propanediol, atrolactic acid (2-hydroxy-2-phenylpropanoic acid) and a mercapturate. A diagram of the proposed metabolic pathway is shown below (from NTP, 2007).



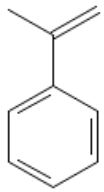
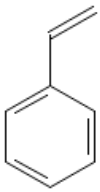
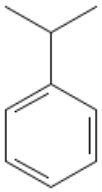
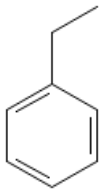
The first step in the proposed pathway is oxidation to 2-methyl-2-phenyloxirane (also known as α-methylstyrene oxide). This side-chain epoxide was not found in the urine or blood of rats administered α-methylstyrene. However, the structure of the identified metabolites and the fact that an analogous metabolite (styrene-7,8-oxide) has been detected in many studies with styrene provide sufficient confidence that α-methylstyrene is metabolised via the side-chain epoxide. In addition, a decrease in hepatic glutathione was observed in a short-term mouse inhalation study with α-methylstyrene (Morgan *et al.*, 1999; for details see 'supplemental information' in the carcinogenicity section), which is consistent with formation of a reactive intermediate in the liver.

Metabolites originating from hydrolysis and glutathione conjugation of α-methylstyrene oxide comprised about 85 % and 10 % respectively of the dose recovered from rat urine.

In an *in vitro* study in human liver slices from a single donor (De Costa *et al.*, 2001), 25 % of the radioactivity was present as 2-phenyl-1,2-propanediol, ≈1 % as atrolactic acid and ≈1 % as 2-phenylpropionic acid. The remainder of the metabolites accounted for less than 0.3 % of the radioactivity.

Structurally related substances

The DS used information from three structurally related substances, namely styrene, cumene and ethylbenzene, as supplementary information in the assessment of mutagenicity and carcinogenicity of α-methylstyrene. The structures of these substances are shown below.

α-methylstyrene	styrene	cumene	ethylbenzene
			

A brief overview of metabolism, genotoxicity, carcinogenicity and target organ toxicity of styrene, cumene and ethylbenzene can be found in the mutagenicity section under 'supplemental information'. The conclusions of the comparison can be summarised as follows.

- The main metabolic pathways of styrene are analogous to those of α-methylstyrene (side-chain epoxidation followed by hydrolysis or glutathione conjugation). Unlike α-methylstyrene, styrene is mutagenic *in vitro*, induces an increase in lung tumours in mice and causes severe hepatic necrosis in this species.
- Cumene is partly metabolised via α-methylstyrene in rats and mice and there are toxicological similarities between the two substances such as α2μ-globulin response in male rats and an increase in liver tumours in female mice. However, cumene additionally caused nasal tumours in rats and lung tumours in mice.
- The metabolism of ethylbenzene is not assumed to involve side-chain epoxidation. Despite some toxicological similarities (such as liver tumours in female mice) the dissimilar metabolism prevents the use of data on ethylbenzene in the assessment of α-methylstyrene.

In conclusion, data on styrene and cumene can be used to a limited extent in the assessment of α-methylstyrene, with due awareness of the significant toxicological differences. Use of data on ethylbenzene is not considered justified because of the dissimilarity of metabolic pathways.

4 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

[A.] There is no requirement for justification that action is needed at Community level. (carc.)

[B.] Justification that action is needed at Community level is required. (skin sens.)

Differences in self-classification

5 IDENTIFIED USES

This substance is used by consumers, in articles, by professional workers (widespread uses), in formulation or re-packing, at industrial sites and in manufacturing.

6 DATA SOURCES

Sources: PUBMED, SCOPUS, WEB OF SCIENCE, ScienceDIRECT, Wiley, ECHA dissemination site, EMBASE, IUCLID (Reg data), OECD sids, IARC, Scifinder

7 PHYSICOCHEMICAL PROPERTIES

Table 8: Summary of physicochemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
Physical state at 20°C and 101,3 kPa	liquid	REACH registration data	experimental result (expert judgement)
Melting/freezing point	249.95 K (-23.2 °C)	REACH registration data	experimental result (handbook data)
Boiling point	ca. 438.15 K at 1013 hPa (ca. 165 °C)	REACH registration data	experimental result (handbook data)
Relative density	0.91 at 20 °C	REACH registration data	experimental result (handbook data)
Vapour pressure	2.53 hPa at 20 °C	REACH registration data	experimental result (handbook data)
Surface tension	- (based on the structure surface active properties are not to be expected)	REACH registration data	-
Water solubility	100 mg/L at 25 °C (pH ca. 6)	REACH registration data	experimental result (handbook data)
Partition coefficient n-octanol/water	3.48 at 25 °C (pH ca. 6)	REACH registration data	experimental result (handbook data)
Viscosity	0.94 mPa · s (dynamic) at 20 °C	REACH registration data	experimental result (handbook data)

The information in this table marked with „REACH registration data“ is based on information taken from the REACH registration dossier and ECHA’s public registration information as accessed on 27.07.2020.

8 EVALUATION OF PHYSICAL HAZARDS

Not assessed in this dossier.

9 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Table 9: Summary table of toxicokinetic studies

Method	Results	Remarks	Reference
Toxicokinetic study, similar to OECD TG 417 Investigation of disposition, metabolism (urinary and blood profile of metabolites), distribution (tissue, blood), and excretion (urine, breath, faeces) of 2-phenylpropene Male rats (F344/N) Inhalation, intravenous, oral (gavage) 1 - 5 animals/group	**Metabolism:** *Urinary metabolites (inhalation, intravenous, oral):* • **2-phenyl-1,2-propanediol glucuronide** and **atrolactic acid** main urinary metabolites [%] of the total urinary excretion: - 2-phenyl-1,2-propanediol glucuronide (inhalation: 46 % [300 ppm] and 31 % [900 ppm]; intravenous: 50 %)	**Reliable with restrictions** **Restrictions:** Percent absorption for oral administration not specified, no GLP Proposed metabolic pathways: **1.** side-chain epoxidation to yield 2-phenylpropene oxide (CAS: 2085-88-3)	(NTP, 2007) Results are partly published in (De Costa et al., 2001)

Method	Results	Remarks	Reference
[^{14}C]2-phenylpropene doses: Inhalation (6 h exposure to 300 and 900 ppm [approx. 1450 and 4350 mg/m^3]), intravenous (single dose:10.8 mg/kg bw), oral (1000 mg/kg bw) *Administration by inhalation:* - excretion (urine, faeces, expired air; n = 4) - tissue distribution (n = 3 - 5) - urinary metabolites (n = 1) - blood concentration (n = 5) - blood metabolites - toxicokinetics *Intravenous administration:* - cumulative excretion (urine, faeces, expired air; n = 4) - tissue distribution (n = 4) - urinary metabolites (n = 1) *Oral administration:* - cumulative excretion (urine, faeces; n = 1) - urinary metabolites (n = 1) In addition: Human liver slices (one donor) were incubated with 2-phenylpropene and metabolites were determined in the media	- atrolactic acid (inhalation: 37 % [300 ppm] and 53 % [900 ppm]; intravenous: 27 %) - S-(2 hydroxy-2-phenylpropyl)-N-acetylcysteine (inhalation: 10 % [300 ppm] and 11 % [900 ppm]; intravenous: 13 %) - 2-phenyl-1,2-propanediol (inhalation: 2 % [300 ppm] and 1 % [900 ppm]; intravenous: 3 %) - 2-phenyl propionic acid (inhalation: 1 % [300 ppm] and 1 % [900 ppm]; intravenous: 1 %) - Unknown metabolite (inhalation: 2 % [300 ppm] and 2 % [900 ppm]; intravenous: 3 %) • similar urinary profile following oral administration *Blood metabolites (inhalation):* • 2-phenyl-1,2-propanediol (most abundant), atrolactic acid, 2-phenylpropionic acid, and unidentified metabolite **<u>Distribution:</u>** *Administration by inhalation:* • concentration of radioactivity in residual carcass and tissues (% recovered at 72 h): 5.9 ± 3.8 % (300 ppm), 1.6 ± 0.6 % (900 ppm) • highest concentrations of radioactivity were found in adipose tissue, bladder, liver, kidney and skin • blood concentrations dropped shortly after cessation of exposure following by a steady decrease at a slow rate (recovery: 74 ± 10 % (300 ppm), 82 ± 9 % (900 ppm)) *Intravenous administration:* • low tissue concentration	**2a.** epoxide hydrolase to yield 2-phenyl-1,2-propanediol **2a1.** glucuronidation to yield 2-phenyl-1,2-propanediol glucuronide **2a2.** oxidation to yield atrolactic acid **2b.** conjugation with glutathione to yield S-(2 hydroxy-2-phenylpropyl)-N-acetylcysteine **2c.** 1,2 hydride shift and oxidation to yield 2-phenyl propionic acid	

Method	Results	Remarks	Reference
	(% recovered at 72 h): 0.3 % **Excretion:** • mainly via urine (% of concentration) - inhalation 300 ppm: 88.2 ± 3.9 % (72 h) - inhalation 900 ppm: 92.4 ± 1.0 % (72 h) - intravenous: 86 ± 1 % (72 h) - oral: 74.6 % (48 h) • little excretion via faeces (inhalation 300 ppm: 2.2 ± 0.3 %; inhalation 900 ppm: 2.6 ± 0.2 %; intravenous: 2 %; oral: 2.96 %) and expired air (inhalation 300 ppm: 3.1 ± 0.9 %; inhalation 900 ppm: 2.5 ± 0.4 %; intravenous: 2 %) **Toxicokinetic parameters:** • $t_{1/2}$ = 4.99 ± 1.14 h (300 ppm) and 2.81 ± 0.54 h (900 ppm) • AUC_{INF} (hours × mg/L) = 26.8 ± 4.9 h (300 ppm) and 132.6 ± 33.5 h (900 ppm) • V_z(L/kg) = 38.6 ± 15.1 h (300 ppm) and 11.2 ± 4.1 h (900 ppm) • Cl (L/hours per kg)= 5.3 ± 0.9 h (300 ppm) and 2.7 ± 0.7 h (900 ppm) **Human liver metabolites:** • profile of metabolites similar to those observed in the urine of rats • 2-phenyl-1,2-propanediol was the primary metabolite		
Non-guideline human study to determine the biotransformation of 2-phenylpropene in humans Humans (number not reported) Inhalation (gas chamber) 8 h Expired air and urine were analysed for metabolites of 2-phenylpropene	Atrolactic acid was identified as a metabolite of 2-phenylpropene in the urine of exposed subjects	Study details regarding the applied methodology and presentation of the results are insufficiently reported	(Bardodej and Bardodejova, 1970)

9.1 Short summary and overall relevance of the provided toxicokinetic information on the proposed classification(s)

(NTP, 2007). Using radiolabelled 2-phenylpropene, the disposition, excretion, and metabolism were analysed in male rats for different routes of exposure (inhalation, intravenous, oral). 2-phenylpropene is readily absorbed following inhalation or oral exposure. Upon inhalation of 300 or 900 ppm for 6 hours, > 90 % of the absorbed amount of radiolabelled 2-phenylpropene was eliminated within 48 hours post initiation of exposure mainly via the urine (~90 %), with little amounts excreted with breath (~3 %), and faeces (~2 – 3 %). The elimination half-life was reported 3 – 5 hours. The overall retention in tissues and the residual carcass was rather low (~2 – 6 % at 72 h after initiation of exposure). The highest concentrations were found in adipose tissue, bladder, liver, kidney and skin. Five metabolites were identified in the urine with 2-phenyl-1,2-propanediol glucuronide and atrolactic acid being the most abundant metabolites followed by S-(2 hydroxy-2-phenylpropyl)-N-acetylcysteine, 2-phenyl-1,2-propanediol, and 2-phenyl propionic acid. Four metabolites were extracted from blood samples. The most abundant blood metabolite was 2-phenyl-1,2-propanediol. The concentration of 2-phenylpropene in the blood dropped shortly after cessation of exposure followed by a steady decrease at a slow rate. Similar to inhalation exposure, the vast majority (86 % of the initial dose) of the radiolabelled 2-phenylpropene was recovered within the urine when administered intravenously. Only 2 % were found both in faeces and breath. The amount of recovered radioactivity in tissues was very low (0.3 %, 72 h post dosing). The main urinary metabolites were 2-phenyl-1,2-propanediol glucuronide and atrolactic acid, representing 50 and 27 % of the identified metabolites, respectively. Similar results were obtained with oral administration.

All identified metabolites were chemically transformed at the vinyl group, whereas the ring was not altered. The primary step in the proposed metabolic pathway is a side-chain oxidation (non-stereoselective epoxidation of the vinyl group) yielding the epoxide, 2-phenylpropene oxide (CAS: 2085-88-3), which is similar to what has been described for the biotransformation of styrene. Subsequent conversion presumably involves different pathways. Enzymatic hydrolysis of the epoxide may form 2-phenyl-1,2-propanediol which may be further oxidised to atrolactic acid or undergo glucuronidation to yield 2-phenyl-1,2-propanediol glucuronide. Glutathione conjugation of the epoxide and subsequent cleavage to the mercapturate may yield S-(2 hydroxy-2-phenylpropyl)-N-acetylcysteine. Due to a 1,2-hydride shift (rearrangement to an aldehyde) and the transfer of oxygen, the epoxide may be metabolised to 2-phenyl propionic acid (De Costa et al., 2001; NTP, 2007).

In addition to the data obtained with experimental animals, some information on human toxicokinetics exists. In the study by De Costa, et al. (2001), human liver slices were treated with 2-phenylpropene. The measured metabolites were similar to those seen in the urine of rats. 2-phenyl-1,2-propanediol was identified as the primary metabolite (De Costa et al., 2001). In addition, a non-guideline study concerning the biotransformation of 2-phenylpropene in humans is available (Bardodej and Bardodejova, 1970). Following 8 hours of inhalation exposure, excreta (urine, expired air) of participants were analysed for the presence of metabolites. Atrolactic acid was identified as a metabolite of 2-phenylpropene in the urine of exposed subjects. The quality of the study is low and the reporting is insufficient to assess the effects of 2-phenylpropene in humans.

10 EVALUATION OF HEALTH HAZARDS

Acute toxicity

10.1 Acute toxicity - oral route

Hazard class not assessed in this dossier.

10.2 Acute toxicity - dermal route

Hazard class not assessed in this dossier.

10.3 Acute toxicity - inhalation route

Hazard class not assessed in this dossier.

10.4 Skin corrosion/irritation

Hazard class not assessed in this dossier.

10.5 Serious eye damage/eye irritation

Hazard class not assessed in this dossier.

10.6 Respiratory sensitisation

Hazard class not assessed in this dossier.

10.7 Skin sensitisation

Table 10: Summary table of animal studies on skin sensitisation

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance,	Dose levels duration of exposure	Results	Reference
Local lymph node assay (LLNA) **Key Study** According to OECD TG 429 (EU Method B.42) GLP: yes Reliability: 1 (reliable without restrictions)	Species: mice Strain: CBA/Ca (CBA/CaOlaHsd) Sex: females No/group: n = 5 / dose Treatment: 3 consecutive days (25 µl topical on the surface of the ear); injection of radioactively labelled thymidine on day 6	**2-phenyl-propene** (99.8 % purity) Vehicle: acetone/olive oil (4:1 v/v) Positive control: yes (hexyl cinnamic aldehyde; conc. 25 % v/v)	Concentrations: 25 %, 50 %, 100 % (v/v in vehicle))	**Positive** **Conc.** / **SI*** : 25 % / 2.35 (neg.); 50 % / 3.13 (pos.); 100 % / 4.50 (pos.); Pos. ctrl. / 6.08 *SI = stimulation index **EC3: 46 %** **Conc.** / **DPM*** : Ctrl. / 661.04 ± 262.06; 25 % / 1550.37 ± 630.17; 50 % / 2071.57 ± 478.40; 100 % / 2971.66 ± 288.36 *DPM = disintegrations per minute (expressed as mean DPM ± standard deviation	(Study report skin sens, 2016) ECHA dissemination page: 001 Key | Experimental results Unpublished study report Information taken as reported on ECHAs dissemination page
Non-guideline skin sensitisation study in guinea pigs Secondary source in Russian without detailed information on individual results GLP: no	Species: guinea pig Strain: not specified Sex: not specified No/group: not specified Treatment: not specified	**2-phenyl-propene** (purity not specified) Vehicle: not specified Positive control: not specified	Concentrations: not specified	**Positive**	ECHA dissemination page: 002 other | Experimental results Information taken as reported on ECHAs dissemination page

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance,	Dose levels duration of exposure	Results	Reference
Reliability: 4 (not assignable)					

Table 11: Summary table of human data on skin sensitisation

Type of data/report	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
Medical examination of exposed workers Unpublished study without detailed information on individual test design and results Reliability: 4 (not assignable)	**2-phenyl-propene** (purity not specified)	No information available	Possibly sensitising in humans	ECHA dissemination page: 003 Other | Other result type Information taken as reported on ECHAs dissemination page
Review of sensitisation data Reliability: 4 (not assignable)	**2-phenyl-propene** (purity not specified)	No information available	Positive for sensitisation in humans	(Ishii et al., 2009) ECHA dissemination page: 004 Other | Other result type Information taken as reported on ECHAs dissemination page
Medical data Secondary source without detailed information on	**2-phenyl-propene** (purity not specified)	No information available	"overexposure" may cause sensitisation in humans	ECHA dissemination page: 005 Other | No specified result type Information taken as reported on

Type of data/report	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
individual results Reliability: 4 (not assignable)				ECHAs dissemination page

10.7.1 Short summary and overall relevance of the provided information on skin sensitisation

For the purpose of classification, relevant data related to skin sensitisation properties of 2-phenylpropene are provided by one LLNA test. The study is conducted according to OECD TG 429, including GLP compliance and considered a reliable source of information (key study). The test result is positive for skin sensitisation in female mice with an EC3 value of 46 % (Study report skin sens, 2016). Another animal study available on ECHAs dissemination page does not provide sufficient information and is therefore disregarded.

10.7.2 Comparison with the CLP criteria

Hazard categories for skin sensitisers (CLP Regulation 1272/2008, Table 3.4.2)

"Category 1:

Substances shall be classified as skin sensitisers (Category 1) where data are not sufficient for sub-categorisation in accordance with the following criteria: (a) if there is evidence in humans that the substance can lead to sensitisation by skin contact in a substantial number of persons; or (b) if there are positive results from an appropriate animal test (see specific criteria in section 3.4.2.2.4.1).

Sub-Category 1A:

Substances showing a high frequency of occurrence in humans and/or a high potency in animals can be presumed to have the potential to produce significant sensitisation in humans. Severity of reaction may also be considered.

Sub-Category 1B:

Substances showing a low to moderate frequency of occurrence in humans and/or a low to moderate potency in animals can be presumed to have the potential to produce sensitisation in humans. Severity of reaction may also be considered."

As mentioned above, there is only one reliable key study in experimental animals available. The study consists of a local lymph node assay (OECD TG 429 with GLP). An EC3 value of 46 % was established. According to the CLP Regulation 1272/2008, Table 3.4.4, substances showing an EC3 value above 2 % can be allocated into Category 1B. While, for the lack of detailed information, the available human evidence is insufficient for classification purposes, the reporting of skin sensitising properties of 2-phenylpropene in humans does not oppose a classification.

10.7.3 Conclusion on classification and labelling for skin sensitisation

Based on one experimental animal study, classification of 2-phenylpropene in Category 1B is recommended.

RAC evaluation of skin sensitisation

Summary of the Dossier Submitter's proposal

The DS proposed to classify the substance as Skin Sens. 1B based on a positive LLNA giving an EC3 of 46 %.

Comments received during consultation

Two member state competent authorities (MSCAs) supported the DS's proposal.

Assessment and comparison with the classification criteria

In a GLP-compliant local lymph node assay (2016) according to OECD TG 429, female CBA/Ca mice (5/dose) were treated with α-methylstyrene in acetone/olive oil (4:1) at concentrations of 25 %, 50 % and 100 %. Hexyl cinnamic aldehyde (25 %) was used as a concurrent positive control. Stimulation indices at the individual concentrations are shown below.

Results of the LLNA (2016)	
Concentration	**Stimulation index**
0 %	1.00
25 %	2.35
50 %	3.13
100 %	4.50
Positive control (HCA 25 %)	6.08

The test is considered valid and the result is positive with an EC3 of 46 %. No other properly documented animal or human information on skin sensitisation can be found in the dossier.

With a positive reliable LLNA, the substance meets the criteria for classification. An EC3 of > 2 % corresponds to subcategory 1B. Thus, RAC agrees with the DS's proposal of **Skin Sens. 1B**.

10.8 Germ cell mutagenicity

Table 12: Summary table of mutagenicity/genotoxicity tests *in vitro*

Method, guideline, deviations if any	Test substance,	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
***In vitro* bacterial gene**	**2-phenyl-propene** (99.6 %	**Supporting study** (Reliable with restrictions)	**Negative** (all strains +/- S9) Cytotoxicity: yes (≥ 200 µg/plate	(Study report mutagenicity, 1997b)

Method, guideline, deviations if any	Test substance,	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
mutation test Equivalent or similar to OECD TG 471/472 GLP: yes Full study report is not available (limiting reporting)	purity)	Strains: - *Salmonella typhimurium* TA98, TA100, TA1535, TA1537 - *Escherichia coli* WP2 uvrA Metabolic activation: +/- S9 mix from rat liver Test concentrations: 0, 12.5, 25, 50, 100, 200, 400 µg/plate Solvent: DMSO Negative control: yes Positive control: yes	+/- S9) Confounding factors (e.g. precipitations): not specified Controls: valid negative and positive controls	ECHA dissemination page: 001 Key | Experimental results Unpublished study report Information taken as reported on ECHAs dissemination page
***In vitro* bacterial gene mutation test** Similar to OECD TG 471 GLP: not specified Deviation: - *E.coli* WP2 or *S. typhimurium* TA102 not included - individual plate counts not provided	**2-phenyl-propene** (> 99 % purity)	**Supporting study** (Reliable with restrictions) Strains: *Salmonella typhimurium* TA97, TA98, TA100, TA1535 Metabolic activation: +/- rat and hamster liver S9 (10 and 30 %) Test concentrations: five concentrations from 1 – 3333 µg/plate (concentration range) Solvent: DMSO Negative control: yes Positive control: yes Study protocol according to Zeiger et al. (1992)	**Negative** (with and without metabolic activation) Cytotoxicity: at high-dose exposure (333 – 3333 µg/plate) Confounding factors (e.g. precipitations): not specified Controls: valid negative and positive controls	(NTP, 2007) ECHA dissemination page: 004 Supporting| Experimental results
***In vitro* bacterial gene mutation test** Equivalent or similar to OECD	**2-phenyl-propene** (99 % purity)	**Supporting study** (Reliable with restrictions) Strains: *Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538	**Negative** (all strains +/- S9) Cytotoxicity: yes (≥ 100 µg/plate +/- S9) Confounding factors (e.g. precipitations): not specified	(Study report mutagenicity, 1991b) ECHA dissemination page: 005 Supporting|

Method, guideline, deviations if any	Test substance,	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
TG 471 GLP: yes Full study report is not available (limiting reporting)		Metabolic activation: +/- S9 mix from rat liver Test concentrations: - range-finding study: 0 – 5000 µg/plate (ten doses, +/- S9) - mutagenicity assay: 0 – 1000 µg/plate (five doses, +/- S9) - confirmatory assay: 0 – 1000 µg/plate (five to six doses, +/- S9) Solvent: DMSO Negative control: yes Positive control: yes	Controls: valid negative and positive controls	Experimental results Unpublished study report in Japanese Information taken as reported on ECHAs dissemination page
***In vitro* bacterial gene mutation test** Equivalent or similar to OECD TG 471 GLP: not specified Full study report is not available (limiting reporting)	**2-phenyl-propene** (purity not specified)	**Supporting study** (Reliable with restrictions) Strains: *Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538 Metabolic activation: +/- S9 mix from rat liver Test concentrations: 10 – 5000 µg/plate (no. of concentrations not specified) Solvent: DMSO Negative control: yes Positive control: yes	**Negative** (all strains +/- S9) Cytotoxicity: yes (≥ 100 µg/plate) Confounding factors (e.g. precipitations): not specified Controls: valid negative and positive controls	(Study report mutagenicity, 1989) ECHA dissemination page: 006 Supporting| Experimental results Unpublished study report Information taken as reported on ECHAs dissemination page
***In vitro* mammalian chromosome aberration test** (structural chromosome aberrations) Equivalent or similar to OECD TG 473	**2-phenyl-propene** (99.6 % purity)	**Supporting study** (Reliable with restrictions) Cell line: Chinese hamster lung cells (CHL/IU) cells Metabolic activation: +/- S9 mix from rat liver Test concentrations: - continuous treatment (- S9): 0, 40, 90, 170 µg/mL	**Negative** (+/- S9) Cytotoxicity: yes (≥ 170 µg/mL) Controls: valid negative and positive controls	(Study report mutagenicity, 1997a) ECHA dissemination page: 002 Key | Experimental results Unpublished study report in Japanese

Method, guideline, deviations if any	Test substance,	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
GLP: yes Full study report is not available (limiting reporting)		- short-term treatment (-S9): 0, 40, 90, 170 µg/mL - short-term treatment (+S9): 0, 60, 120, 230 µg/mL Treatment time: - continuous treatment: 24 h and 48 h - short-term treatment: 6 h Sampling time: not specified Solvent: DMSO Negative control: yes Positive controls: yes		Information taken as reported on ECHAs dissemination page
***In vitro* mammalian chromosome aberration test** (structural chromosome aberrations) Equivalent or similar to OECD TG 473 GLP: no Deviations: - short-term treatment for only 2 h and only with metabolic activation) - 200 instead of 300 metaphases scored	**2-phenyl-propene** (> 99 % purity)	**Supporting study** (Reliable with restrictions) Cell line: Chinese hamster ovary (CHO) cells Metabolic activation: +/- S9 mix from rat liver Test concentrations: - 1st trail: 0, 100.5, 150, 200 µg/mL - 2nd trail: 0, 33.7, 125.7, 251.3 µg/mL Treatment time: - +S9: 2 h - -S9: 10 h (continuously exposure) Sampling time: - +S9: 12 h - -S9: 10 h Solvent: DMSO Negative control: yes Positive controls: yes	**Negative** (+/- S9) Cytotoxicity: yes (highest dose 2nd trail) Controls: valid negative (solvent control) and positive controls	(NTP, 2007) ECHA dissemination page: 007 Supporting| Experimental results
***In vitro* mammalian chromosome aberration**	**2-phenyl-propene** (99 % purity)	**Supporting study** (Reliable with restrictions) Cell line: Chinese hamster	**Negative** (+/- S9) Cytotoxicity: yes (≥ 0.1 µl/mL)	(Study report mutagenicity, 1991a)

Method, guideline, deviations if any	Test substance,	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
test (structural and numerical chromosome aberrations) Equivalent or similar to OECD TG 473 GLP: yes Full study report is not available (limiting reporting)		ovary (CHO) cells Metabolic activation: +/- S9 mix from rat liver Test concentrations: - pre study: 0 – 5 µl/mL - main study: 0 – 0.15 µl/mL Treatment time: not specified Sampling time: - +S9: 20 h - -S9: 18 h Solvent: DMSO Negative control: yes Positive controls: yes	Controls: valid negative and positive controls	ECHA dissemination page: 008 Supporting| Experimental results Unpublished study report Information taken as reported on ECHAs dissemination page
***In vitro* mammalian gene mutation test** Equivalent or similar to OECD TG 476 GLP: yes Full study report is not available (limiting reporting)	**2-phenyl-propene** (99 % purity)	**Supporting study** (Reliable with restrictions) Cell line: Chinese hamster ovary (CHO) cells Test concentrations: - initial study: 0.05, 0.075, 0.1, 0.125, 0.15 µl/mL (+/- S9) - confirmatory study: 0.05, 0.075, 0.085, 0.1, 0.115 µl/mL (-S9); 0.05, 0.075, 0.1, 0.125, 0.135 (+S9) Treatment time: Not specified Sampling time: not specified Solvent: DMSO Negative control: yes Positive controls: yes	**Negative** (+/- S9) Cytotoxicity: yes (dose-dependent) Controls: valid negative and positive controls	(Study report mutagenicity, 1991c)[1] ECHA dissemination page: 009 Supporting| Experimental results Unpublished study report Information taken as reported on ECHAs dissemination page
In vitro	**2-phenyl-**	**Supporting study**	**Positive** (+S9) in cells exposed to 50,	(NTP, 2007)

[1] Amoco Corp; CHO/HGPRT Mutation Assay with Confirmation, Final Report; 08/30/91; EPA Doc No. 86-910000976; Fiche No. OTS0529953 (reference on Pubchem: https://pubchem.ncbi.nlm.nih.gov/compound/alpha-Methylstyrene)

<table>
<tr><th>Method, guideline, deviations if any</th><th>Test substance,</th><th>Relevant information about the study including rationale for dose selection (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr>
<td>mammalian sister chromatid exchange test (DNA damages)

Equivalent or similar to OECD TG 479 (deleted)

GLP: not specified</td>
<td>propene (> 99 % purity)</td>
<td>(Reliable with restrictions)

Cell line: Chinese hamster ovary (CHO) cells

Test concentrations:
- -S9: 5, 16.7, 50, 166.7 µg/mL
- +S9 (trial 1): 5, 16.7, 50, 166.7 µg/mL
- +S9 (trial 2): 50, 124.4, 149.9 µg/mL

Total cells scored: 50

Treatment time:
- -S9: 25.5 h
- +S9: 2 h

Sampling time:
- -S9: 2 h
- +S9: 25.2 – 25.5 h

Solvent: DMSO

Negative control: yes

Positive controls: yes</td>
<td>124.4, 149.9 µg/mL

(weekly positive in trial 1 at 50 µg/mL; dose-dependent, clearly positive in trial 2)

<table>
<tr><th>Concentration</th><th>SCEs/Chromosome (%#)</th></tr>
<tr><td colspan="2">Trial 1 +S9</td></tr>
<tr><td>DMSO</td><td>0.36</td></tr>
<tr><td>5 µg/mL</td><td>0.38 (5.2 %)</td></tr>
<tr><td>16.7 µg/mL</td><td>0.36 (-1.8 %)</td></tr>
<tr><td>50 µg/mL</td><td>0.46 (28.4 %)*</td></tr>
<tr><td>166.7 µg/mL</td><td>cytotoxic</td></tr>
<tr><td colspan="2">Trial 2 +S9</td></tr>
<tr><td>DMSO</td><td>0.34</td></tr>
<tr><td>50 µg/mL</td><td>0.47 (39.6 %)*</td></tr>
<tr><td>124.4 µg/mL</td><td>0.51 (49.2 %)*</td></tr>
<tr><td>149.9 µg/mL</td><td>0.62 (82.8 %)*</td></tr>
</table>
#relative change of SCEs / chromosome
*positive response (≥ 20 % of the ctrl)

Negative (- S9)

Cytotoxicity: yes (166.7 µg/mL +/-S9)

Controls: valid negative and positive controls</td>
<td>ECHA dissemination page: 003 Key| Experimental results</td>
</tr>
<tr>
<td>Non-guideline study
in vitro sister chromatid exchange test in human lymphocytes (DNA damages)

Study details are insufficiently reported</td>
<td>2-phenyl-propene (98 – 99 % purity)</td>
<td>Not reliable

Cell line: human lymphocytes (derived from a healthy male donor) in a whole blood culture

Metabolic activation: presumably erythrocyte-mediated

Test concentrations: not specified

Total cells scored: 50

Treatment time: 48 h

(first 24 h without treatment; total culture time: 72 h)

Sampling time: immediate after treatment (72 h)</td>
<td>Weakly positive

0.33 mM: statistically significantly different (week effect: less than twice as many SCEs/cell as compared to the control)

1 mM: not statistically significantly different

According to the authors of the study, the test substance is presumably converted to a reactive metabolite due to erythrocyte-mediated activation

Cytotoxicity: not specified

Controls:
- valid negative controls
- no positive control</td>
<td>(Norppa and Vainio, 1983)

ECHA dissemination page: 010 Supporting| Experimental results</td>
</tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any</th><th>Test substance,</th><th>Relevant information about the study including rationale for dose selection (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr><td></td><td></td><td>Solvent: acetone

Negative control: yes

Positive controls: no</td><td></td><td></td></tr>
<tr><td>Non-guideline study
in vitro sister chromatid exchange test in human lymphocytes (DNA damages)

Study details are insufficiently reported</td><td>2-phenyl-propene (purity not specified)</td><td>Not reliable

Cell line: human lymphocytes cultured isolated (< 1000 erythrocytes/ml) or with whole-blood (2x10^8)

Metabolic activation: erythrocyte-mediated

Test concentrations: 1 and 2 mM

Total cells scored:
- whole blood: 50
- isolated lymphocytes: 33-50

Treatment time: 48 h (first 24 h without treatment; total culture time: 72 h)

Sampling time: immediate after treatment (72 h)

Solvent: not specified

Negative control: yes

Positive controls: no</td><td>Positive (treatment-related induction of SCEs in isolated and whole-blood cultures)
<table><tr><th>Concentration</th><th>SCEs/cell ± S.E.</th></tr><tr><td colspan="2">Whole blood</td></tr><tr><td>Control</td><td>7.6 ± 0.4</td></tr><tr><td>1 mM</td><td>8.9 ± 0.4*</td></tr><tr><td>2 mM</td><td>11.4 ± 0.6***</td></tr><tr><td colspan="2">Isolated lymphocytes</td></tr><tr><td>Control</td><td>10.1 ± 0.5</td></tr><tr><td>1 mM</td><td>11.1 ± 0.6</td></tr><tr><td>2 mM</td><td>12.1 ± 0.6**</td></tr></table>*P < 0.05 **P < 0.01 ***P < 0.001</td><td>(Norppa and Tursi, 1984)

ECHA dissemination page: 010 Supporting| Experimental results</td></tr>
<tr><td colspan="5">Presumed metabolite (cf. section 9): 2-phenylpropene oxide</td></tr>
<tr><td>Non-guideline in vitro bacterial gene mutation test</td><td>2-phenyl propene oxide (purity checked but not specified)</td><td>Supporting study (reliable with restrictions)

Strain: Salmonella typhimurium TA100

Metabolic activation: no

Test concentrations: 0.01, 0.1, 0.5, 1, 2.5, 5, 10 µmol / preincubation tube (seven</td><td>Positive (dose-dependent)
<table><tr><th>Concentration (µmol/tube)</th><th>Revertants*</th></tr><tr><td>0 (DMSO)</td><td>106 ± 19
156 ± 13
147 ± 13</td></tr><tr><td>0.01</td><td>131 ± 8
152 ± 8</td></tr><tr><td>0.1</td><td>150 ± 14
203 ± 14</td></tr><tr><td>0.5</td><td>499 ± 22</td></tr><tr><td>1</td><td>665 ± 42</td></tr></table></td><td>(Rosman et al., 1986)</td></tr>
</table>

Method, guideline, deviations if any	Test substance,	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
		doses in preincubation solution) Solvent: DMSO Negative control: yes (DMSO Positive control: yes (glycidol)	<table><tr><td></td><td>824 ± 43</td></tr><tr><td>2.5</td><td>2492 ± 565</td></tr><tr><td>5</td><td>3355 ± 13</td></tr><tr><td>10</td><td>3435 ± 92# 3604 ± 1019#</td></tr></table>*mean values pre plate ± SD (n = 3 per test) #cytotoxicity observed Cytotoxicity: yes (10 µmol/tube +/- S9)	

Table 13: Summary table of mutagenicity/genotoxicity tests in mammalian somatic or germ cells *in vivo*

<table>
<tr><th>Method, guideline, deviations if any</th><th>Test substance,</th><th>Relevant information about the study (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr>
<td>Micronucleus test in peripheral blood erythrocyte integrated into the 90-day subchronic inhalation toxicity study (section 10.12, Table 26)

Similar to OECD TG 474

<u>GLP:</u> GLP (21 CFR, Part 58)

<u>Deviations:</u>
- (No positive control)[2]
- Number of analysed PCEs less than recommended (focus on NCEs)

<u>General limitations of the testing design:</u>
- No repetition possible to verify unclear results</td>
<td>2-phenyl-propene (> 99 % purity)</td>
<td>Key study (reliable with restrictions)

<u>Species:</u> B6C3F$_1$ mice

<u>No./group:</u> 10 ♂ / 8-10 ♀ per treatment group

<u>Target organ/principal endpoint:</u>
bone marrow / peripheral blood erythrocyte

<u>Administration route:</u>
whole-body inhalation

<u>Concentrations:</u> 0, 75, 150, 300, 600, and 1,000 ppm (approx. 360 – 4830 mg/m^3) in ♂/♀

<u>Duration:</u> three month (91 days)

<u>Treatment:</u>
- continuously over three month
- 6 h/d plus T90 (12 minutes)
- 5 d/w (except holidays)
- 65 treatments in total

<u>Sampling times</u> (time interval between final treatment and cell sampling): 24 h

<u>Analysis:</u>
- slide-based approach
- number of MN in NCEs[3] (10000 scored)
- number of MN in PCEs[4] (in ctrl and high-concentration animals only; 1000 scored)
- bone marrow toxicity determined by the number of PCEs in</td>
<td>Negative in male mice

<table>
<tr><th>Concentration (ppm)</th><th>Micronucleated NCEs / 1000 Cells</th></tr>
<tr><td>0 (Control)</td><td>5.30 ± 0.50</td></tr>
<tr><td>75</td><td>5.80 ± 0.44</td></tr>
<tr><td>150</td><td>5.80 ± 0.63</td></tr>
<tr><td>300</td><td>5.00 ± 0.65</td></tr>
<tr><td>600</td><td>4.60 ± 0.45</td></tr>
<tr><td>1000</td><td>6.30 ± 1.02</td></tr>
</table>

Positive in female mice

<table>
<tr><th>Concentration (ppm)</th><th>Micronucleated NCEs / 1000 Cells</th></tr>
<tr><td>0 (Control)</td><td>5.10 ± 0.46</td></tr>
<tr><td>75</td><td>2.40 ± 0.43</td></tr>
<tr><td>150</td><td>2.90 ± 0.90</td></tr>
<tr><td>300</td><td>3.60 ± 0.48</td></tr>
<tr><td>600</td><td>5.30 ± 0.42</td></tr>
<tr><td>1000</td><td>9.13 ± 0.77*</td></tr>
</table>
*p = 0.0006

- positive trend in the frequency of MN in NCEs of ♀ mice with statistical significance (non-linear concentration-response)
- increased number of MN in NCEs of ♀ mice at 1000 ppm with statistical significance (♀ ctrl.: vs. 1000 ppm: 9.13 ± 0.77)
- number of MN in PCEs not significantly different in ♂/♀

<u>Bone marrow toxicity in MN test:</u>
- not observed (percentage of PCEs among erythrocytes not altered)

<u>Toxicity during 90 days of exposure:</u>
- lethality (two ♀ died on day 3 at 1000 ppm)
- reduced final mean body weight in ♂ at ≥ 600 ppm and ♀ at 75, 300 and 1000 ppm with statistical significance
- reduced final mean body weight gains in ♂ and ♀ at ≥ 300 ppm with statistical significance</td>
<td>(NTP, 2007)

ECHA dissemination page: Key study

Testing protocol according to:

(MacGregor et al., 1990; Witt et al., 2000)</td>
</tr>
</table>

[2] positive control groups are not routinely included as test animals are obtained from the subchronic RDT study (Witt et al. 2000)

[3] NCEs: normochromatic erythrocytes

[4] PCEs: polychromatic erythrocytes

<table>
<tr><th>Method, guideline, deviations if any</th><th>Test substance,</th><th>Relevant information about the study (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr><td></td><td></td><td>erythrocytes (5000/10000 scored)

<u>Negative control:</u> chamber control (air)

<u>Positive control:</u> no

<u>Scoring controls:</u> yes</td><td>- slightly decreased erythron[5] (lower values of haemoglobin and erythrocytes counts) in ♀ at 1000 ppm with statistical significance</td><td></td></tr>
<tr><td>Micronucleus test in bone marrow cells

Similar to OECD TG 474

<u>GLP:</u> yes

<u>Deviations:</u>
- no evidence of bone marrow exposure
- number of analysed PCEs and total erythrocytes less than recommended
- no historical control data reported</td><td>2-phenyl-propene (99 % purity)</td><td>Supporting study (reliable with restrictions)

<u>Species:</u> Institute for Cancer Research (ICR) mice

<u>No./group:</u> 6 ♂

<u>Target organ/principal endpoint:</u>
bone marrow / bone marrow cells

<u>Administration route:</u> oral

<u>Dose level:</u> 0, 500, 1000, 2000 mg/kg

<u>Treatment:</u> single oral exposure

<u>Sampling times</u>: 24 h

<u>Analysis:</u>
- no. of MN determined in 2000 PCEs[6]
- bone marrow toxicity determined by the no. of PCEs in 500 erythrocytes

<u>Negative control:</u> yes

<u>Positive control:</u> yes</td><td>Negative in male mice

<table><tr><th>Dose (mg/kg)</th><th>MNPCE [%]*</th></tr><tr><td>Ctrl</td><td>0.08 ± 0.07</td></tr><tr><td>500</td><td>0.06 ± 0.05</td></tr><tr><td>1000</td><td>0.06 ± 0.05</td></tr><tr><td>2000</td><td>0.20 ± 0.15</td></tr><tr><td>Positive ctrl</td><td>0.62 ± 0.62</td></tr></table>* frequency of micronucleated polychromatic erythrocytes (MNPCE) per 2000 cells (mean ± SD)

<u>Toxicity:</u>
- not observed (ratio of PCEs within total erythrocytes)</td><td>(Rim et al., 2012)</td></tr>
</table>

[5] sum of all erythrocytes and their precursors

[6] PCEs: polychromatic erythrocytes

10.8.1 Short summary and overall relevance of the provided information on germ cell mutagenicity

As summarised in Table 12, the mutagenic potential of 2-phenylpropene has been determined in several *in vitro* test systems, obtaining mostly negative results. 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). Accordingly, no mutagenic activity with or without metabolic activation was seen in all tested strains of *S. typhimurium* (TA98, TA100, TA1535, TA1537, TA1538) and *E. coli* (WP2 uvrA) in four independent *in vitro* gene mutation studies in bacteria. The same holds true for three available *in vitro* cytogenicity / chromosome aberration studies in mammalian cells, where only negative results had been gotten with 2-phenylpropene. No gene mutations were detected in mammalian cells either. 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.

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. The results, however, do not provide direct evidence of mutagenicity and are, consequently, generally less significant as compared to those obtained in mutagenicity studies (ECHA, 2017a). For the lack of understanding regarding the underlying mechanisms causing the effect, the corresponding OECD TG 479 was deleted in 2014[7] (OECD, 2015). The biological relevance of the abovementioned findings with 2-phenylpropene is, therefore, considered unknown. Additional information concerning *in vitro* mutagenicity of 2-phenylpropene, albeit of very limited quality, comes from two openly accessible publications (Norppa and Tursi, 1984; Norppa and Vainio, 1983). Both studies comprise an SCE test in human lymphocytes cultures, showing positive effects established by a statistically significant treatment-related increase of SCEs. The authors of the study concluded that a reactive metabolite generated due to erythrocyte-mediated metabolic activation is presumably responsible for the genotoxic effect. 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.

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. The mutagenic potential of this reactive metabolite was tested in a non-guideline bacterial gene mutation study in a single *S. typhimurium* strain (Rosman et al., 1986). A marked dose-dependent increase in the number of revertants was observed, providing clear evidence for mutagenicity in bacteria. Metabolic activation resulting in the formation of a genotoxic metabolite has also been described for styrene. Hereby, the primary biotransformation gives rise to styrene-7,8-oxide, a reasonably anticipated human carcinogen (Cat. 1B)[8] (NTP, 2011).

In vivo

Data related to 2-phenylpropene-mediated mutagenicity in humans are not available. In addition to *in vitro* testing, the mutagenic potential of 2-phenylpropene had been evaluated in experimental animals. Two studies concerning the induction of micronuclei (MN) in murine peripheral blood erythrocytes or bone marrow cells following exposure by long-term inhalation or single oral administration are available. Due to the significant differences in the study protocols (long-term inhalation in both sexes vs. a single oral application exclusively in males), a comparison of the results is limited.

[7] https://www.oecd-ilibrary.org/environment/test-no-479-genetic-toxicology-in-vitro-sister-chromatid-exchange-assay-in-mammalian-cells_9789264071384-en

[8] https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/71959

MN test in mice following long-term inhalation exposure conducted by NTP (2007)

The first *in vivo* micronucleus test in peripheral blood erythrocytes of male and female B6C3F1 mice by inhalation exposure was conducted by NTP as an integrative part of the GLP compliant subchronic repeated dose toxicity study (NTP, 2007). The integrated testing design has been frequently applied by NTP in its Carcinogenesis Bioassay Program as it was noted that a clearly positive outcome in a subchronic MN test is highly predictive for rodent carcinogenicity (Witt et al., 2000). According to the authors of the study, a statistically significant trend for the frequency of micronucleated normochromatic erythrocytes (NCEs) was noted in peripheral blood samples of female but not in male mice at the end of the 90-day study with 2-phenylpropene. Compared to the control, a statistically significant increase was only seen in females at the highest concentration tested (1000 ppm). The frequency of MN in immature polychromatic erythrocytes (PCEs) was not altered in either sex. While the formation of MN in PCEs is indicative of damages induced within 72 hours of sampling, an increase occurrence of MN in NCEs can be seen as a measure for the average damage occurring during an extended period of time (Witt et al., 2000). The quality of the study is considered high at least for the part of the long-term treatment, which is considered similar to OECD TG 413 with GLP compliance. However, when comparing the design and results of the integrated MN test with the acceptability criteria of the corresponding test guideline (OECD TG 474), certain quality-related issues can be noted. In addition, despite being considered a clear positive call by the authors of the NTP study, uncertainties regarding the biological relevance of the result in female mice have emerged from the analysis of the data. They are mainly attributed to the following observations: abnormally high control values (cf. Box 1), a non-linear concentration-response relationship, a positive result only in highest concentration group of one sex accompanied by marked toxicity (including lethality) during the subchronic exposure period (cf.

Table 13). A comparison with the criteria stipulated in the OECD TG 474 is provided in Table 14.

Table 14: Comparison of the NTP MN study design/results with the criteria defined in OECD TG 474

Criteria of OECD TG 474	MN test conducted by NTP
Acceptability criteria according to OECD TG 474	
The concurrent negative control data are considered acceptable for addition to the laboratory historical control database.	As compared to control data of other studies with similar testing designs, control values for males and females in the NTP study with 2-phenylpropene appear to be abnormally high (cf. Box 1). However, it had been noted that historical control data in MN studies may not provide sufficient information to evaluate the result of a single study (Witt et al., 2000).
The concurrent positive controls or scoring controls should induce responses that are compatible with those generated in the historical positive control database and produce a statistically significant increase compared with the concurrent negative control.	No concurrent positive control group was included[9]. According to Witt et al. (2000), *"positive control slides were included in the experiments to control for staining and scoring procedures."*
The appropriate number of doses and cells has been analysed.	On account of the adapted test design (long-term exposure), determining MN in NCEs may have been a priority. However, the number of analysed PCEs is less than recommended.
The criteria for the selection of highest dose are consistent with those described in paragraphs 30-33.	Evidence of study-limiting toxicity: two deaths on day 3 and reduced final mean body weight gains (- 32 %) were noted during the 90-day NTP study in the same animals (1000 ppm females) that later exhibited an increased frequency of MN in the subsequent mutagenicity test. As lethality was observed, the maximum tolerated concentration (MTC) may have been exceeded. However, no evidence of bone marrow toxicity was seen in the MN test (percentage of PCEs unaltered in all exposure groups).
Definition of a clearly positive result according to OECD TG 474	
At least one of the treatment groups exhibits a statistically significant increase in the frequency of micronucleated immature erythrocytes[10] compared with the concurrent negative control	While no effects were seen for PCEs in either sex, 2-phenylpropene caused an increased frequency of MN in matured erythrocytes (NCEs) in females. Statistical significance was only reached for the highest concentration group (1000 ppm) accompanied by marked toxicity as described above.
This increase is dose-related at least at one sampling time when evaluated with an appropriate trend test, and	Despite reaching statistical significance in the trend test analysis, the concentration-response in females is non-linear (U-shaped; the three lowest concentrations below the control value) and lacks a clear concentration-related increase.
Any of these results are outside the distribution of the historical negative control data (e.g. Poisson-based 95% control limits).	Frequencies of MN in NCEs observed in the two highest concentration-groups (600 and 1000 ppm) in females are clearly above the reported range of controls in other subchronic MN tests (cf. Box 1).

[9] positive control groups are not routinely included as test animals are obtained from the subchronic RDT study (Witt et al. 2000)

[10] according to OECD TG 474, *"the frequency of mature erythrocytes that contain micronuclei in the peripheral blood also can be used as an endpoint in species without strong splenic selection against micronucleated cells and when animals are treated continuously for a period that exceeds the lifespan of the erythrocyte in the species used (e.g. 4 weeks or more in the mouse)."*

In conclusion, 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. 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.

MN test in male mice following short-term oral exposure conducted by Rim et al. (2012)

In the second study by Rim et al. (2012), bone marrow cells of male mice were scored for micronucleated PCEs following a single oral application of 2-phenylpropene. While an increased frequency of MN was seen in the high-dose group relative to the control, the effect was not statistically significant. The result of the test was, hence, negative. 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 (Rim et al., 2012).

10.8.2 Comparison with the CLP criteria

"This hazard class is primarily concerned with substances that may cause mutations in the germ cells of humans that can be transmitted to the progeny. However, the results from mutagenicity or genotoxicity tests in vitro and in mammalian somatic and germ cells in vivo are also considered in classifying substances and mixtures within this hazard class." (CLP Regulation 1272/2008, 3.5.2.1.)

Hazard categories for germ cell mutagens (CLP Regulation 1272/2008, Table 3.5.1)

"Category 1:
Substances known to induce heritable mutations or to be regarded as if they induce heritable mutations in the germ cells of humans.

The classification is based on:

Category 1A:

Box 1: Historical control data of MN test with subchronic exposure:

No historical control data are directly provided within the NTP report. With regards to the details of the experimental protocol, NTP refers to secondary publications (MacGregor et al., 1990; Witt et al., 2000). Based on findings from 67 subchronic studies (oral, dermal, inhalation; 45 days, 13 weeks, 6 months), the range of control values for micronucleated NCEs, reported therein, was 0.32 – 2.36[a], with 1.21[a] being the mean control value (Witt et al., 2000). Chamber control values of close structural analogues obtained for the exact same endpoint (induction of MN in NCEs in mice continuously exposure via inhalation for 90 days) were 2.40 ± 0.69[1] / 2.30 ± 0.40[b] (♂/♀ mice exposed to cumene), 1.54 ± 0.16[b] / 0.92 ± 0.11[b] (♂/♀ mice exposed to ethylbenzene), as compared to 5.30 ± 0.50[b] / 5.10 ± 0.46[b] (♂/♀ mice exposed 2-phenylpropene) (NTP, 1999; NTP, 2007; NTP, 2009).

[a] micronucleated NCEs per 1000 cells

[b] micronucleated NCEs per 1000 cells (mean ± standard error)

- *positive evidence from human epidemiological studies.*

Category 1B:

- *positive result(s) from in vivo heritable germ cell mutagenicity tests in mammals; or*
- *positive result(s) from in vivo somatic cell mutagenicity tests in mammals, in combination with some evidence that the substance has potential to cause mutations to germ cells. It is possible to derive this supporting evidence from mutagenicity/genotoxicity tests in germ cells in vivo, or by demonstrating the ability of the substance or its metabolite(s) to interact with the genetic material of germ cells; or*
- *positive results from tests showing mutagenic effects in the germ cells of humans, without demonstration of transmission to progeny; for example, an increase in the frequency of aneuploidy in sperm cells of exposed people.*

Category 2:
Substances which cause concern for humans owing to the possibility that they may induce heritable mutations in the germ cells of humans

The classification in Category 2 is based on:

- *positive evidence obtained from experiments in mammals and/or in some cases from in vitro experiments, obtained from:*
  - *somatic cell mutagenicity tests in vivo, in mammals; or*
  - *other in vivo somatic cell genotoxicity tests which are supported by positive results from in vitro mutagenicity assays."*

In the absence of evidence from human epidemiological studies, Category 1A is not applicable. Similarly, the criteria for Category 1B are not fulfilled as data derived from *in vivo* heritable germ cell mutagenicity tests are not available. There is also no evidence demonstrating the ability of the 2-phenylpropene or a related metabolite to interact with the genetic material of germ cells.

As described above, results from two *in vivo* somatic cell mutagenicity test in mammals (similar to OECD TG 474) are available, which are potentially applicable for a Category 2 classification. In brief, a statistically significant increase in the frequency of MN in peripheral (normochromatic) erythrocytes relative to control was observed in high-concentration female mice at the end of the subchronic repeated dose toxicity inhalation study. There was also a statistically significant trend, albeit non-linear, for the induction of MN in NCEs across all female exposure groups, potentially indicating mutagenic effects caused by persistent damages in somatic cells. No such effects were noted in male mice (NTP, 2007). Increased frequencies of micronucleated erythrocytes with statistical significance were not seen in male mice following a single oral administration of different 2-phenylpropene doses (Rim et al., 2012). As stated in ECHAs guidance on the application of the CLP criteria, *"if there is also negative or equivocal data, a weight of evidence approach using expert judgement has to be applied"* (ECHA, 2017b).

Weight of evidence

Given the complexity of the endpoint, contradicting results have to be evaluated for their individual significance considering each genotoxic endpoint separately. According to ECHAs guidance on IR&CSA, Chapter R.7a, *"It is not unusual for positive evidence of mutagenicity to be found in just one test type or for only one endpoint. In such cases, the positive and negative results for different endpoints are not conflicting, but illustrate the advantage of using test methods for a variety of genetic alterations to increase the probability of identifying substances with mutagenic potential"* (ECHA, 2017a). Results obtained with 2-phenylpropene in several valid *in vitro* mutagenicity tests covering gene mutations in bacteria and mammalian cells as well as structural chromosome aberrations in mammalian cells are all negative. 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.

In addition, two *in vivo* mammalian somatic cell mutagenicity tests are available. Although the same genotoxic endpoint (*in vivo* structural and numerical chromosome aberrations identified by MN formation) is covered, the applied testing protocols are very different (short-term oral exposure (Rim et al., 2012) vs. long-term inhalation exposure (NTP, 2007)). Therefore, a direct comparison between the results may not be possible and the negative result obtained in the oral MN test does not generally overrule the reported positive findings in the long-term inhalation study. Similar cases where positive findings in MN tests following subchronic (13 weeks) exposure were seen while short-term exposure yielded negative results have been described for other chemicals. 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.
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. A weak response in only one sex, though, is not predictive (Witt et al., 2000). Nevertheless, 2-phenylpropene causes tumour formation in rats and mice (cf. section 10.9). 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.

As summarized in Table 15, the mutagenic effects of structural analogues have been extensively evaluated. A harmonised classification for mutagenicity is not in place for none of these substances and *in vitro* mutagenicity data for 2-phenylpropene are all negative. Therefore, the following information cannot be used to justify the assignment of 2-phenylpropene into Category 2. However, the data may provide some insights concerning a potential operating mutagenic mechanism. For styrene, strong evidence for mutagenicity has been identified. Metabolic activation, forming the electrophilic metabolite styrene-7,8-oxide, is required for styrene in order to induce genotoxicity. Styrene-7,8-oxide covalently interacts with the genetic material of the cells to form DNA adducts and is considered a genotoxic human carcinogen (IARC, 2019). A similar step has been proposed in the biotransformation of 2-phenylpropene with 2-phenylpropene oxide being the potential driver of subsequent mutagenicity (Chen et al., 2011; De Costa et al., 2001; NTP, 2007). In the case of cumene, conversion to 2-phenylpropene and 2-phenylpropene oxide has been implicated in the week genotoxic evidence (NTP, 2016). Although a few positive *in vitro* results exist, ethylbenzene is not considered genotoxic (IARC, 2000; NTP, 1999). While the formation of an epoxide is possible, the primary metabolic step is an α-hydroxylation to 1-phenylethanol (IARC, 2000; NTP, 1999). 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.

Table 15: Mutagenic effects of close structural analogues

Substance	Conclusion on mutagenic properties as reported by others	Metabolites
Cumene (EC: 202-704-5)	• *"For somatic cells, the vast majority of available tests gave negative results and there are only few indications for a genotoxic potential"* (CLH dossier 601-024-00.-X)[11] • *"…some evidence exists for a genotoxic mechanism of action for cumene (presumably via its conversion to α-methylstyrene or to other metabolites)"* (NTP, 2016)	Identified metabolite: **2-phenylpropene** Proposed metabolite: **2-phenylpropene oxide** (CAS: 2085-88-3) (Chen et al., 2011; IARC, 2012b; NTP, 2016)

[11] https://echa.europa.eu/documents/10162/10e6c6a2-7321-a5e4-3ac3-ed5439c7c4a1

Substance	Conclusion on mutagenic properties as reported by others	Metabolites
Styrene (EC: 202-851-5)	• *"There is strong evidence that both styrene and styrene-7,8-oxide are genotoxic, and this mechanism can also operate in humans."* (IARC, 2019) • *"…a concern for genotoxicity associated with oral exposure to styrene cannot be excluded"* (EFSA, 2020) • *"Detection of styrene-7,8-oxide–DNA adducts at base-pairing sites and chromosomal aberrations in lymphocytes of styrene-exposed workers supports the potential human cancer hazard from styrene through a genotoxic mode of action."* (NTP, 2011)	Identified primary metabolite: **styrene-7,8-oxide** (IARC, 2019)
Ethylbenzene (EC: 202-849-4)	• *"Ethylbenzene gave little indication of mutagenicity, in vitro or in vivo."* (NTP, 1999) • *"No increases in the frequency of micronucleated erythrocytes were observed in vivo in peripheral blood samples from male and female mice exposed to ethylbenzene for 13 weeks"* (NTP, 1999)	Identified primary metabolite: **1-phenylethanol** (epoxide intermediate possible) (IARC, 2000; NTP, 1999)

Conclusion on the Comparison with the CLP criteria

To arrive at a conclusion as to whether or not 2-phenylpropene should be classified for mutagenicity, arguments supporting or opposing the existence of a genotoxic potential of 2-phenylpropene are listed in . As mentioned above, some of these arguments are insufficient for classification purpose but may nevertheless be used as part of the weight of evidence approach to conclude on mutagenicity conferred by 2-phenylpropene.

Table 16: Arguments supporting or opposing the existence of a genotoxic potential of 2-phenylpropene

Arguments supporting a genotoxic potential of 2-phenylpropene
• positive data obtained in an in vivo mutagenicity test in somatic cells with 2-phenylpropene: o increased frequency of MN in one group (high-concentration females) with statistical significance relative to the concurrent negative control and above the range of control values from other similar studies o positive trend with statistical significance in female mice
• plausible mechanisms based on the hypothetical formation of a mutagenic intermediate (2-phenylpropene oxide) during the first step of biotransformation o proposed metabolic activation yielding 2-phenylpropene oxide (Chen et al., 2011; De Costa et al., 2001; NTP, 2007) o positive data obtained in an *in vitro* mutagenicity test with the proposed mutagenic intermediate 2-phenylpropene oxide exist (Rosman et al., 1986) o erythrocyte-mediated metabolic activation may generate a genotoxic metabolite in human whole-blood lymphocyte cultures (Norppa and Tursi, 1984; Norppa and Vainio, 1983) o mechanism appears to be similar to what has been described for the close structural analogue, styrene (epoxide formation); however, the carcinogenic profile of styrene differs as compared to 2-phenylpropene (cf. section 10.9.2)
• supporting evidence for the induction of DNA damages provided by at least one valid *in vitro* indicator test (SCE assay) conducted by NTP
• positive data on mutagenicity in peripheral erythrocytes is associated with hepatocellular carcinogenicity in female mice
Arguments opposing a genotoxic potential of 2-phenylpropene
• uncertainty regarding the biological relevance of the positive findings in the MN test in the NTP study o positive effects only in females at the highest concentration accompanied by systemic toxicity: two deaths on day 3, marked [-32] reduction in final mean body weight gains, reduction in final mean body weight [-11 %], slightly decreased erythron → note: high-concentration males also exhibited signs of systemic toxicity (reduced final mean body weight gain [-43 %], reduced final mean body weight [-17 %], no deaths), however, without showing statistically significantly increased frequencies of MN. o values of the concurrent negative control are abnormally high as compared to control data from other similar studies o non-linear concentration-response relationship (U-shaped): no clear concentration-related increase; values for all exposure groups are below or equal to the concurrent control with the exception of the highest concentration group
• contradicting findings in two independent *in vivo* MN test → however, very different protocols
• no supporting evidence from valid *in vitro* mutagenicity tests (guideline studies according to GLP)
• proposed primary metabolite, 2-phenylpropene oxide, not experimentally confirmed

The positive result in female mice obtained following subchronic inhalation exposure in the NTP study might be indicative of persistent genotoxic damages that cannot be detected upon short-term treatment. Classification of 2-phenylpropene in Category 2 might, hence, be applicable. Significant uncertainties concerning the biological relevance of this finding, however, prevent a final conclusion and render the data insufficient for classification. Negative results obtained in cell culture systems suggest that 2-phenylpropene is not mutagenic under the conditions of *in vitro* testing. 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. Therefore, based on the evidence discussed in the overall weight of evidence analysis, a classification may not be appropriate due to inconclusive data. Nevertheless, the existence of some genotoxic potential attributed to 2-phenylpropene or its metabolite(s) cannot be ruled out.

10.8.3 Conclusion on classification and labelling for germ cell mutagenicity

Classification of 2-phenylpropene for mutagenicity is not recommended.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

A standard *in vitro* battery consisting of several Ames tests, chromosomal aberration assays and an HPRT assay was negative. *In vitro* sister chromatid exchange (SCE) assays gave positive results. The presumed metabolic intermediate α-methylstyrene oxide was positive in an Ames test.

Two *in vivo* studies are available. A single dose oral bone marrow micronucleus (MN) assay in male mice (Rim *et al.*, 2012) was negative, whereas a peripheral blood MN assay integrated into the 3-month mouse inhalation study by NTP (2007) was positive in females.

The DS mentioned a number of factors to be considered in the classification, such as:

- The negative *in vitro* mutagenicity assays.
- The problematic interpretation of SCE results in relation to mutagenicity classification.
- The markedly differing protocols of the two *in vivo* MN assays. As a result, the negative oral MN test does not overrule the positive result of the inhalation study.
- The high general toxicity (including mortality) in the group of female mice showing increased MN frequencies.
- The unknown *in vivo* relevance of the presumed genotoxic metabolite.

According to the DS, the positive result in female mice in the inhalation study might warrant classification in Category 2. Still, in view of the significant uncertainties they preferred no classification due to inconclusive data.

Comments received during consultation

Comments were received from 2 MSCAs and 1 industry organisation.

The commenting MSCAs generally supported the DS's analysis and proposal for no classification, however, they also considered there is data available that might support Cat 2 classification, considering e.g. positive result found in female mice (but only in one study and one sex, the absence of a positive control and the high number of NCE also in the negative control). The other MN assay cannot be used to dismiss the positive results since the protocols are not similar with respect of duration of exposure, route of exposure and sex used i.e. male mice, that seem less sensitive than females. Further, in vitro SCE assays in the presence of S9, even of various qualities, are consistently positive and one of them with a clear dose-response, although in vitro mutagenicity assays negative. Also the toxicokinetics considerations point to the formation of an epoxide, with epoxides being highly reactive substances.

Particularly, one of the MSCAs requested QSAR analysis of the parent and metabolites, considering that metabolites are very similar to styrene glycol and styrene glycol glucuronide, which could in their view together with existing data possibly support a Cat. 2 classification. The DS confirmed that formation of α-methylstyrene oxide as the first metabolic step is predicted to occur in humans according to Meteor (Lhasa Limited). Still, the DS reiterated that relevance of this Ames-positive metabolite for *in vivo* mutagenicity of α-methylstyrene remains obscure.

The industry commenter supported no classification for mutagenicity and informed about their intention to conduct a new *in vitro* MN test in human whole blood. According to industry, this study is needed because the existing *in vitro* chromosomal aberration tests do not comply with the current OECD guidelines. Industry further proposed that, subject to a negative outcome of the *in vitro* MN test, a new *in vivo* study be performed to clarify the positive finding in the *in vivo* MN test by NTP (2007).

Industry further commented on some specific points of the DS's assessment. Regarding the *in vivo* MN test by NTP, they questioned biological plausibility of the observed increase in micronucleated normochromatic erythrocytes (MN-NCE) without a concomitant increase in micronucleated polychromatic erythrocytes (MN-PCE). Industry also challenged the use of data from styrene, cumene and ethylbenzene in the assessment of α-methylstyrene, pointing out the toxicological dissimilarities (e.g. different spectrum of neoplastic responses, positive Ames tests with styrene). They claimed that although α-methylstyrene oxide is likely to be formed *in vivo*, the negative Ames tests with α-methylstyrene together with the fact that the epoxide was not detected in the ADME study indicate that it is rapidly converted to non-reactive downstream metabolites or conjugates.

During the third-party consultation, Industry informed about their intention to conduct a new *in vitro* micronucleus test in human whole blood. The experimental part of this study started in November 2022 and the audited draft report was received by ECHA in January 2023. The study is summarised below.

In vitro micronucleus assay in human lymphocytes (Gilby, 2023)

The study was conducted according to OECD TG 487 (2016) and under GLP. Alpha-methylstyrene (purity 99.83%) was tested in human lymphocyte cultures prepared from the pooled blood of two adult donors.

Details of the method:

- Vehicle: DMSO

- Metabolic activation: S9 from livers of β-naphthoflavone/phenobarbital-induced male Sprague Dawley rats
- Mitogen stimulation: phytohaemagglutiinin, 48 hours
- Short treatment ±S9: "3+17", i.e. a 3-hour treatment followed by removal of test article and addition of cytochalasin B (cytoB); sampling at 20 hours after the beginning of treatment
- Extended treatment –S9: "20+0", i.e. exposure to the test chemical and cytoB for 20 hours, followed by sampling
- Replicates: duplicate cultures for each treatment level and positive control, quadruplicate cultures for vehicle controls
- Positive controls: Mitomycin C as a clastogenic control –S9, Colchicine as an aneugenic control –S9, Cyclophosphamide as a clastogenic control +S9
- Basis for top concentration selection: cytotoxicity
- Cytotoxicity parameter: cytokinesis-block proliferation index (CBPI)
- Number of analysed concentrations: 3 concentrations per treatment schedule selected for micronucleus analysis. The highest concentrations selected for analysis were those at which 50-60% cytotoxicity was achieved.
- Number of cells analysed for micronuclei: 2000 binucleated cells per concentration and 4000 cells for the vehicle control
- Method of analysis: fluorescence microscopy

All acceptability criteria (as specified in paragraph 56 of the OECD TG) were met. The results are summarised in the following table.

***In vitro* micronucleus test in human lymphocytes (Gilby, 2023)**					
Treatment	**Concentration (µg/ml)**	**Cytotoxicity (%, based on CBPI)**	**Mean MN cell frequency (%)**	**Historical control range (95th percentile)**	**Statistical significance**
3+17 –S9	Vehicle	–	0.38	0.20–1.00	–
	37.2	3	0.60		NS
	102	17	0.35		NS
	126	51	0.10		NS
	MMC, 0.3	33	2.00	1.50–6.08	p≤0.001
	COL, 0.07	21	1.95	1.48–3.70	p≤0.001
3+17 +S9	Vehicle	–	0.65	0.20–1.10	–
	60	2	0.75		NS
	135	29	0.50		NS
	150	52	0.65		NS
	CPA, 10.0	54	1.90	1.21–2.59	p≤0.01
20+0 –S9	Vehicle	–	0.48	0.20–1.00	–
	18.6	0	0.40		NS
	82.7	25	0.50		NS
	113	52	0.45		NS
	MMC, 0.1	20	2.70	1.35–3.65	p≤0.001
	COL, 0.02	12	1.70	1.20–2.36	p≤0.001

MMC = Mitomycin C; COL = Colchicine; CPA = Cyclophosphamide; NS = no significant increase

It is concluded that alpha-methylstyrene did not induce micronuclei in cultured human peripheral blood lymphocytes when tested up to the cytotoxicity limit prescribed by the respective OECD test guideline, in both the absence and presence of metabolic activation.

Assessment and comparison with the classification criteria

In vitro assays

The available *in vitro* assays with α-methylstyrene are summarised in the table below. Detailed descriptions of the *in vitro* mutagenicity assays were not available to RAC except the studies by NTP (2007) and the new micronucleus test (Gilby, 2023).

***In vitro* genotoxicity assays with α-methylstyrene**			
Study type; year / reference	**Method**	**Result**	**Remarks**
Ames test 1997	Plate incorporation method Rat liver S9 Top concentration 400 µg/plate Solvent DMSO	Negative ±S9 Cytotoxicity from 200 µg/plate	
Ames test NTP, 2007	Pre-incubation method Rat and hamster liver S9 Top concentration 100 to 3333 µg/plate	Negative ±S9 Cytotoxicity from 333−3333 µg/plate (depending on strain and metabolic activation)	TA102 or *E.coli* WP2 not tested
Ames test 1991	Rat liver S9 Top concentration 1000 µg/plate Solvent DMSO	Negative ±S9 Cytotoxicity from 100 µg/plate	TA102 or *E.coli* WP2 not tested
Ames test 1989	Rat liver S9 Top concentration 1000 µg/plate Solvent acetone	Negative ±S9 Cytotoxicity from 100 µg/plate	TA102 or *E.coli* WP2 not tested
Chromosomal aberrations 1997	Chinese hamster lung cells Rat liver S9 Top concentration 170 to 230 µg/ml Solvent DMSO	Negative ±S9 Cytotoxicity from 170 µg/ml	
Chromosomal aberrations NTP, 2007	Chinese hamster ovary cells Rat liver S9 Top concentration 200 µg/ml Solvent DMSO	Negative ±S9 Cytoxicity at 250 µg/ml	Relatively short exposure (+S9 2 h) and sampling time (10-12 h)

Chromosomal aberrations 1991	Chinese hamster ovary cells Rat liver S9 Top concentration 0.15 µl/ml Solvent DMSO	Negative ±S9 Cytotoxicity from 0.1 µl/ml	
Micronucleus Gilby, 2023	Human lymphocytes Rat liver S9 Highest analysed concentration 113 to 150 µg/ml Solvent DMSO	Negative ±S9 Cytotoxicity: CBPI reduction by >50% at the highest analysed concentration in each experiment	
HPRT test 1991	Chinese hamster ovary cells Rat liver S9 Top concentration 0.15 µl/ml Solvent DMSO	Negative ±S9 Excessive cytotoxicity above 0.1 µl/ml	
Sister chromatid exchange NTP, 2007	Chinese hamster ovary cells Rat liver S9 Top concentration 50 to 150 µg/ml Solvent DMSO	Positive +S9 with a dose response Cytotoxicity at 167 µg/ml	
Sister chromatid exchange Norppa and Vainio, 1983	Human lymphocytes (whole blood) No external metabolic activation Solvent acetone	Weakly positive	12 substances tested Results in graphical form, confusing presentation of data The authors assumed that styrene and related compounds were converted to a reactive metabolite by erythrocytes present in the *in vitro* system
Sister chromatid exchange Norppa and Tursi, 1984	Human lymphocytes (whole blood) No external metabolic activation Top concentration 2 mM (236 µg/ml)	Positive	

The DS further presented an Ames test with α-methylstyrene oxide by Rosman *et al.* (1986). The authors investigated mutagenicity of styrene oxide, α-methylstyrene oxide and related compounds in *Salmonella* strains TA1535 and TA100 using the pre-incubation method. A strong and reproducible dose-related increase in revertants was observed for α-methylstyrene oxide in TA100 at non-cytotoxic concentrations, whereas only a marginal activity was seen in TA1535. Styrene oxide tested positive in TA100.

Ames tests with α-methylstyrene were negative. The lack of investigations in TA102 or *E.coli* WP2 in some of the studies is not considered critical given that the metabolite α-

methylstyrene oxide and the structurally related substance styrene tested positive in TA100 and/or TA1535.

In vitro chromosomal aberration assays were negative but some deviations from the current version of OECD TG 473 are noted, such as short harvest time and lower number of scored cells. Such deviations generally decrease assay sensitivity.

The recently submitted *in vitro* micronucleus test in human lymphocytes, conducted according to OECD TG 487 (2016) without deviations, is negative.

A gene mutation test in mammalian cells (HPRT test) was negative. A detailed description of the study is not available to RAC.

A positive result was obtained with metabolic activation in the SCE assay by NTP (2007). However, SCE tests are generally given lower weight than mutagenicity tests in the classification.

To sum up, α-methylstyrene was negative in a battery of standard *in vitro* mutagenicity tests. The substance tested positive for sister chromatid exchange.

In vivo assays

The two available *in vivo* tests with α-methylstyrene are summarised in the following table.

***In vivo* genotoxicity assays with α-methylstyrene**			
Study type; year / reference	**Method**	**Result**	**Remarks**
Micronucleus, mouse, peripheral blood, integrated into a 3-month inhalation study NTP, 2007	B6C3F1 mice 10/sex/group Inhalation (whole body), 14 weeks (6 hours/day, 5 days/week) Concentrations: 0, 75, 150, 300, 600, 1000 ppm 1000 NCEs and 1000 PCEs per animal No concurrent positive control	NCEs: males negative, females positive at 1000 ppm PCEs: negative in both sexes	Concurrent negative controls above HCD range No bone marrow toxicity General toxicity at 1000 ppm included mortality (2 females before exposure on day 3), clinical signs of toxicity (moderate to severe sedation in males, ataxia in both sexes) and target organ effects (liver, nose)
Micronucleus, mouse, bone marrow, oral (gavage) Rim *et al.*, 2012	ICR mice 6 males/group Doses: 0, 500, 1000, 2000 mg/kg bw Vehicle olive oil Single exposure Sampling at 24 h 2000 PCEs per animal	Negative in males (females not tested)	No bone marrow toxicity No clinical signs of toxicity

NCE = normochromatic erythrocyte; PCE = polychromatic erythrocyte

The results of the MN test by NTP, integrated into a 3-month study, are shown in detail in the table and the graph below. HCD and individual data have been obtained from the NTP online database (NTP, 2022a).

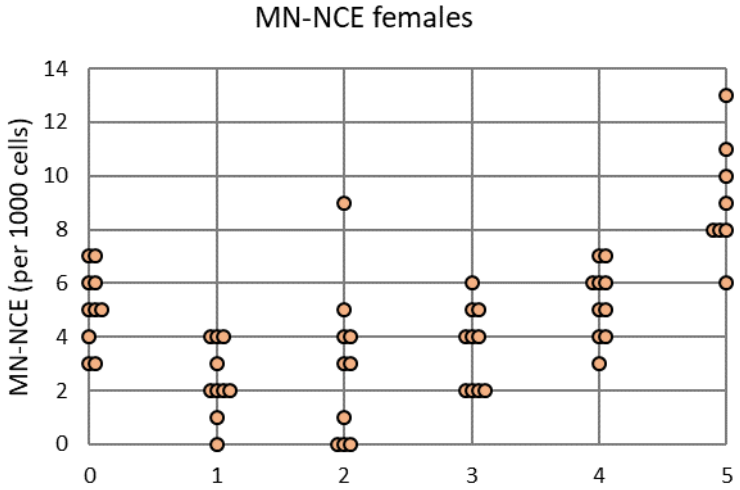
Mouse micronucleus test in the 3-month inhalation study (NTP 2007)				
Concentration (ppm)	**Micronucleated cells/1000 cells**			
	Males		**Females**	
	PCEs	**NCEs**	**PCEs**	**NCEs**
0	3.9	5.3	4.1	5.1
75		5.8		2.4
150		5.8		2.9
300		5.0		3.6
600		4.6		5.3
1000	5.0	6.3	4.8	9.1*
HCD inhalation[a]		mean 1.6 SD 0.9 max. 3.4		mean 1.4 SD 1.0 max. 4.0
HCD all routes[b]			mean 2.4 SD 1.1 max. 4.5	mean 1.3 SD 0.9 max. 4.2

NCE = normochromatic erythrocyte; PCE = polychromatic erythrocyte; SD = standard deviation

* Statistically significant difference on pairwise comparison, $p \leq 0.025$

[a] 17 three-month NTP inhalation studies in B6C3F1 mice (peripheral blood, slide scoring) conducted between 1996 and 2005 (current study 2000); the maximum values represent group mean (not individual data)

[b] MN-NCE: 69 three-month studies in female B6C3F1 mice conducted between 1996 and 2005; MN-PCE: 14 three-month studies in female B6C3F1 mice conducted between 1996 and 2005



Numbering of groups: 0 = control, 1 = 75 ppm, 2 = 150 ppm, 3 = 300 ppm, 4 = 600 ppm, 5 = 1000 ppm

The study did not strictly follow OECD TG 474. One of the deviations is that the test design does not include positive control. Nevertheless, the protocol has been shown to be able to

generate positive results (e.g. the in the 3-month gavage study with butanal oxime conducted in 1996; NTP, 2004), so the lack of positive control is not considered critical in this case. Another deviation is that the concurrent control values are outside (above) the historical control distribution. This introduces some uncertainty. Lastly, the top concentration induced high general toxicity in both sexes: 2 out of 10 females died before exposure on day 3, moderate to severe sedation was observed in males.

It is noted that there was no significant increase in micronucleated polychromatic (immature) erythrocytes (MN-PCE) in top concentration females ($p = 0.26$).

Overall, the NTP MN test is considered positive in females in the presence of excessive toxicity. The positive result is associated with uncertainties such as abnormally high concurrent control value and lack of a significant increase in micronucleated immature erythrocytes.

The negative oral single-dose bone marrow micronucleus test in male ICR mice by Rim *et al.* (2012) appears well-conducted. Still, the design shows some deviations from the OECD TG. Generally, repeated administration is preferable to single treatment and when a single treatment is used, bone marrow samples should be taken at least twice (e.g. at 24 and 48h) in order not to miss the peak MN frequency. Further, testing of females should have been considered given the higher sensitivity of this sex in earlier studies (NTP, 2007; Morgan *et al.*, 1999).

Thus, there is an *in vivo* micronucleus test positive in one sex (females) above the maximum tolerated concentration. The other test is negative but the more susceptible sex (females) was not tested and the study has deficiencies potentially reducing its sensitivity.

Genotoxicity of structurally related substances

Styrene:

- *In vitro:* The majority of Ames tests were negative but some positive results have been reported in the presence of metabolic activation. *In vitro* aberration tests (having limitations) were positive, an HPRT assay was negative.
- *In vivo:* Reliable chromosomal aberration and micronucleus assays via physiological routes (oral, inhalation) were negative, via i.p. route negative (2 studies) or positive (1 study, positive only at a dose causing high mortality). A standard comet assay via inhalation in lymphocytes was negative, a positive result was obtained in the same study when Fpg was employed (Fpg modification of comet assay enhances detection of certain types of DNA damage such as base oxidation).
- Human data: Various genotoxicity endpoints have been investigated in workers exposed to styrene, some positive associations have been reported.
- RAC opinion: Mutagenicity of styrene has not been evaluated by RAC.

Cumene:

- *In vitro:* The substance was not mutagenic in bacteria. An *in vitro* chromosomal aberration assay was negative but had deficiencies. Inconclusive results were obtained for gene mutation in mammalian cells due to study limitations.
- *In vivo:* MN tests via physiological routes (oral, inhalation) were negative, via the i.p. route positive. A comet assay in rats and mice via oral route investigating the

liver, lungs, kidney and blood leukocytes was negative in all tissues except male rat liver and female mouse lung.

- RAC opinion (2020): RAC concluded that although a weak genotoxic potential of cumene cannot be excluded, the criteria for classification in Category 2 are not fulfilled.

Conclusion

In the absence of evidence of germ cell mutagenicity in humans, any positive *in vivo* germ cell mutagenicity test(s) in animals or evidence that the substance or its metabolite interact with the genetic material of germ cells, the criteria for classification in Category 1 are not met.

Classification in Category 2 is based on positive evidence obtained from experiments in mammals and/or in some cases from *in vitro* experiments, obtained from (a) *in vivo* somatic cell mutagenicity tests in mammals, or (b) other *in vivo* somatic cell genotoxicity tests which are supported by positive results from *in vitro* mutagenicity assays. Where a single well-conducted test is used for classification, it shall provide clear and unambiguously positive results (CLP, Annex I, 3.5.2.3.9).

In this case the *in vitro* mutagenicity database is negative. *In vitro* sister chromatid exchange assays are positive but these are given lower weight than mutagenicity assays and are not mentioned in the classification criteria nor in the examples in CLP, Annex I, 3.5.2.3. The *in vivo* database consists of two micronucleus tests. One is negative but has deficiencies potentially decreasing its sensitivity. The other one is positive in females (only in normochromatic erythrocytes) and negative in males. The positive result in females is associated with uncertainties such as excessive general toxicity and absence of a concomitant increase in micronucleated polychromatic erythrocytes.

The available evidence indicates that α-methylstyrene is metabolised via an Ames-positive epoxide. Nevertheless, the classification criteria are mainly based on evidence for the parent substance. The *in vivo* levels of the reactive intermediate might be low depending on the rate of formation and detoxification.

Given the negative *in vitro* mutagenicity database and the uncertainties related to the single positive *in vivo* result, RAC agrees with the DS that the available evidence **does not meet the criteria and recommends no classification for germ cell mutagenicity**.

Supplemental information - In depth analyses by RAC

Metabolism of structurally related substances

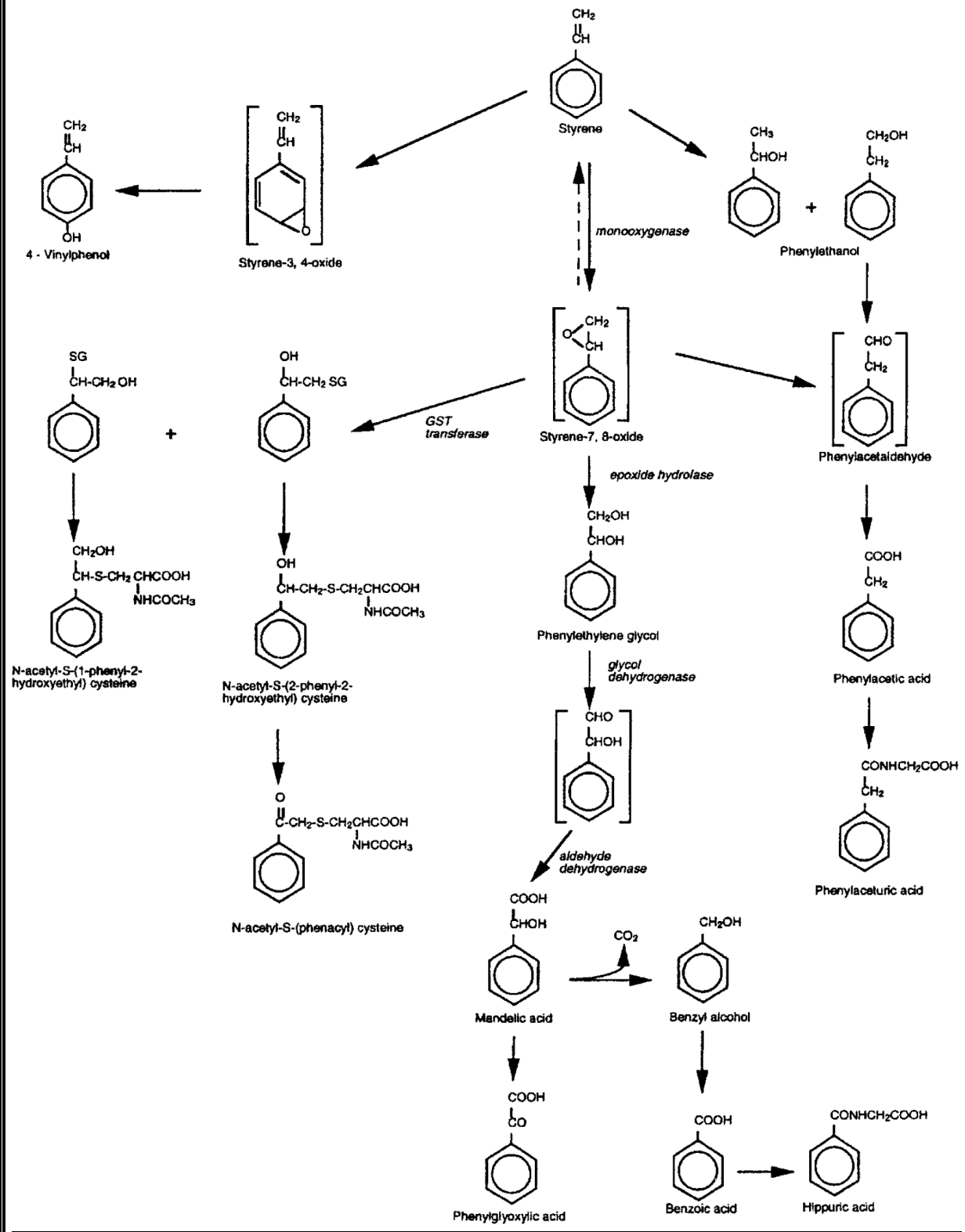
Styrene

Metabolism of styrene is outlined below (from Johanson *et al.*, 2000; a more detailed scheme can be found in IARC, 2019). The main biotransformation pathway begins with epoxidation of the vinyl double bond. The resulting styrene-7,8-oxide can bind covalently to proteins and DNA. Detoxification of styrene-7,8-oxide occurs via hydrolysis (by epoxide hydrolase) or via glutathione conjugation. Another metabolic pathway leads to formation of phenylaceturic acid and is thought to involve phenylacetaldehyde as an intermediate.

Based on urinary metabolites, humans metabolize styrene almost exclusively via the epoxide hydrolase pathway (≈95%), whereas only ≈70% and ≈50% of styrene is

metabolised via this pathway by rats and mice respectively after inhalation exposure (Cruzan *et al.*, 2002). Gluathione conjugation of styrene-7,8-oxide plays a major role in rats and mice (ca. 20-35%) but not in humans. Less than 5% is metabolised via the phenylaceturic acid pathway in rats and humans compared to ≈20% in mice.

Formation of a side-chain epoxide is proposed to be the first step in the main metabolic pathways of both styrene and α-methylstyrene. Formation of styrene-7,8-oxide from styrene has been experimentally verified in several studies. This increases the confidence that an analogous side-chain epoxide (2-methyl-2-phenyloxirane) is also formed from α-methylstyrene. Formation of small amounts of arene oxides has been reported for styrene but not for α-methylstyrene (although it is acknowledged the ADME database of α-methylstyrene is relatively small compared to styrene).

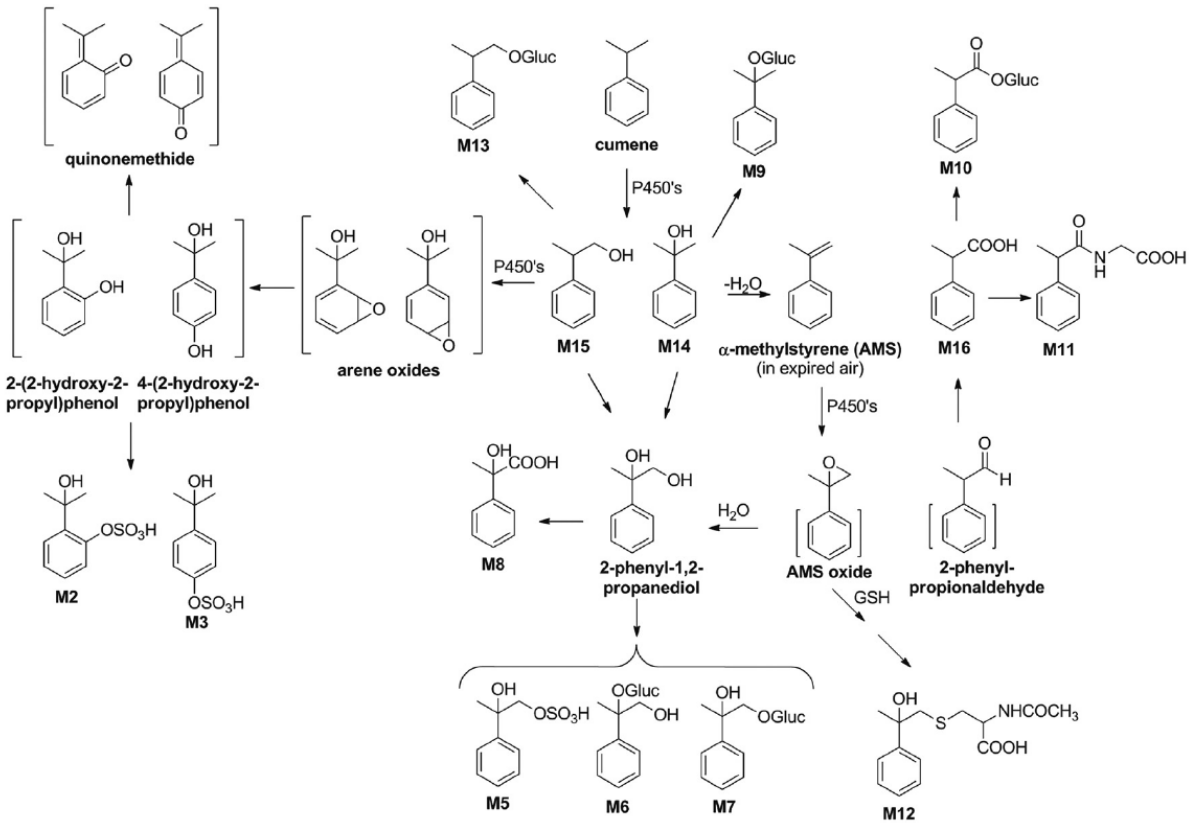


Cumene

Metabolic pathways of cumene in rats and mice after oral exposure according to Chen *et al.* (2011) are outlined in the diagram below. α-Methylstyrene has been detected in expired

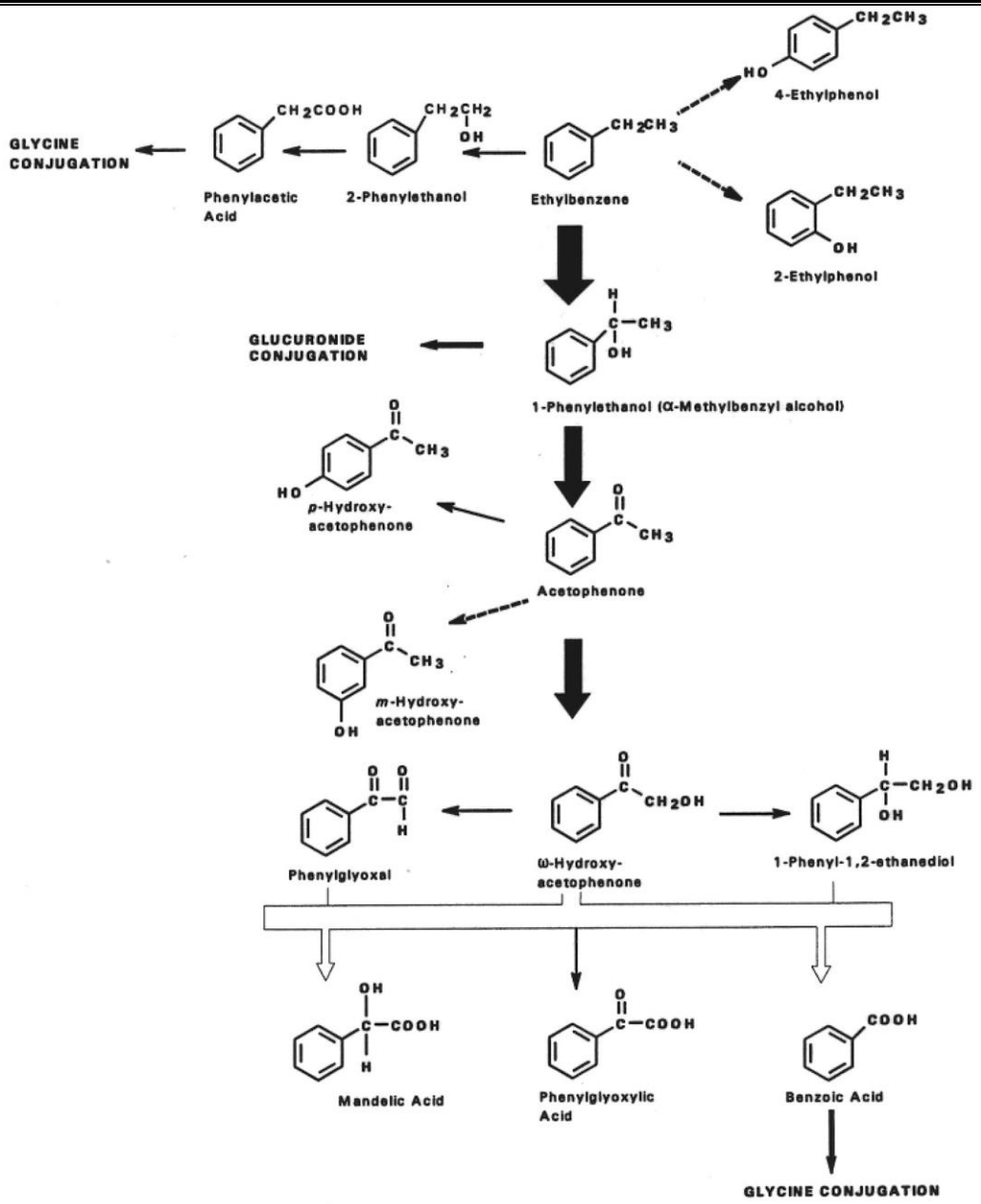
air and a substantial portion (up to tens %) of urinary metabolites might have originated from α-methylstyrene oxide. Another significant pathway, although not a major one in terms of quantity (several %) is ring oxidation, leading to several reactive molecules. The rest (ca. 30-50%) is mainly glucuronide of 2-phenyl-2-propanol (M9).

Given that α-methylstyrene is a metabolite of cumene potentially formed in significant amounts, the information on cumene may be relevant for the assessment of α-methylstyrene. As in the case of styrene, several reactive metabolites related to ring oxidation have been proposed for cumene but no corresponding metabolites were identified in the ADME study with α-methylstyrene in rats.



Ethylbenzene

The metabolism proceeds mainly via side chain oxidation in both rats and humans (NTP, 1999; Engström, 1984; Cossec *et al.*, 2010), see the figure below (from NTP, 1999). There is no indication of side chain epoxidation (in contrast to α-methylstyrene). The role of ring oxidation and glutathione conjugation is very limited.



Genotoxicity, carcinogenicity and target organ toxicity of structurally related subtances

Genotoxicity

Genotoxicity data on the four substances are briefly summarized in the following table. Only selected types of tests (i.e. those highly relevant for regulatory hazard assessment) are presented.

Structurally related substances: genotoxicity profile				
	α-methylstyrene	**styrene**	**cumene**	**ethylbenzene**
In vitro				
Ames test	Negative	Negative and positive studies (positive +S9 in TA1535 and/or TA 100)	Negative	Negative
Micronuclei / chromosomal aberrations	Negative	Positive (studies of limited reliability)	Negative (in a study with limitations)	Negative and positive studies
Gene mutations in mammalian cells	Negative	Negative and equivocal	Inconclusive	Negative and positive studies
***In vivo* (animals)**				
Micronuclei / chromosomal aberrations	Oral, inhalation: 1x negative, 1x positive	Oral, inhalation: negative (several reliable studies) I.p.: negative or equivocal	Oral, inhalation: negative I.p.: 1x positive, 1x negative	Inhalation, i.p.: negative
Comet assay (without modifications for detection of oxidized bases)	No data	Inhalation: negative I.p.: positive	Oral: equivocal	No data

Styrene-7,8-oxide, the major reactive metabolite of styrene, was consistently positive *in vitro* (Ames, HPRT, aberrations, micronuclei, mouse lymphoma assay) without metabolic activation. An *in vivo* chromosomal aberration assay via oral route was positive, whereas an *in vivo* micronucleus test via inhalation was negative (Loprieno *et al.*, 1978; Gaté *et al.*, 2012; references can be found in IARC, 2019). Studies via i.p. route were positive.

Carcinogenicity

The following table summarizes results of carcinogenicity studies with α-methylstyrene, styrene, cumene and ethylbenzene. The studies with α-methylstyrene, cumene and ethylbenzene are NTP inhalation studies in F344 rats and B6C3F1 mice (NTP, 2007; NTP, 2009; NTP, 1999). Only tumour findings providing 'some' or 'clear' evidence of carcinogenicity according to the NTP reports are included in the table. The relevant studies with styrene are inhalation studies in CD(SD) rats and CD-1 mice reported by Cruzan *et al.* (1998, 2001).

Structurally related substances: organs with neoplastic findings in carcinogenicity studies (findings providing 'equivocal' evidence of carcinogenic activity not included)					
		α-methylstyrene	**styrene**	**cumene**	**ethylbenzene**
Rat	Male	Kidney		Nose Kidney	Kidney
	Female			Nose	Kidney
Mouse	Male		Lung	Lung	Lung
	Female	Liver	Lung	Lung	Liver

Target organs

The following table lists the main target organ effects in the available studies (mostly inhalation studies).

Structurally related substances: target organ effects				
Target organ	**Effects**			
	α-methylstyrene	**styrene**	**cumene**	**ethylbenzene**
Liver	Increased weight (rat, mouse) Hypertrophy (mouse)	Severe necrosis (mouse)	Increased weight (rat, mouse) Slight inflammation and necrosis (mouse)	Increased weight, hypertrophy (rat, mouse) Mild necrosis (mouse)
Kidney	Increased weight, increased urinary markers of kidney toxicity (rat), α2μ-globulin response (male rat)		Increased weight (rat), hyaline droplets, increased α2μ-globulin, mineralization (male rat)	Increased weight, hyaline droplets (male rat), increased nephropathy (male and female rat)
Nose	Degeneration of olfactory epithelium and other lesions (mouse, rat)	Degeneration of olfactory epithelium and other lesions (mouse, rat)	Atrophy of olfactory epithelium and other lesions (mouse) Hyperplasia of respiratory epithelium (rat)	
Lung		Damage to lung epithelium including fibrosis (mouse)	Hyperplasia and metaplasia (mouse)	Increased weight, inflammation (rat) Metaplasia (mouse)
Ear	Hair cell loss in the cochlea (rat)	Hair cell loss in the cochlea, increased hearing threshold (rat)		Hair cell loss in the cochlea, increased hearing threshold (rat)

10.9 Carcinogenicity

Table 17: Summary table of animal studies on carcinogenicity

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr>
<td>2-years Carcino-genicity study

Similar to OECD TG 451 (NTP standards)

<u>GLP:</u> yes (21 CFR, Part 58)

Key study

Whole-body inhalation

<u>Species:</u> rats

<u>Strain:</u> F344/N

<u>No/group:</u> n = 50 / sex / group</td>
<td>2-phenyl-propene (> 99 % purity)

<u>Conc.:</u> ♂/♀: 0, 100, 300, 1000 ppm (approx. 0.48, 1.45, 4.83 mg/l)

<u>Duration of exposure:</u> 2 years (105 weeks)

6 h/d plus T_{90} (12 minutes)

5 d/w (except holidays)</td>
<td><u>General effects:</u>

- no treatment-related effect on survival rates
- reduced body weight in ♂ and ♀ rats at 1000 ppm (5 – 10 %) during the 2nd year of the study

<u>Neoplastic effects:</u>

Male rats positive (kidney, mononuclear cell leukaemia, testis)

Female rats negative

In ♂ rats:
<table>
<tr><th>Neoplastic effects:</th><th>No. of animals with lesions / no. of animals in the group</th></tr>
<tr><td>Kidney (renal tubule adenoma or carcinoma [combined])#

HCI†: 4/399 (1.0 % ± 1.1 %; range 0 % - 2 %)</td><td>Ctrl: 1/50 (2 %)
100 ppm: 2/50 (4 %)
300 ppm: 3/50 (6 %)
1000 ppm: 7/50* (14 %)
Trend test: p = 0.006</td></tr>
<tr><td>Kidney (renal tubule adenoma [includes multiple])#

HCI†: 3/399 (0.8 % ± 1.0 %; range 0 % - 2 %)</td><td>Ctrl: 1/50 (2 %)
100 ppm: 2/50 (4 %)
300 ppm: 2/50 (4 %)
1000 ppm: 5/50 (10 %)</td></tr>
<tr><td>Kidney (renal tubule carcinoma)

HCI†: 1/399 (0.3 % ± 0.7 %; range 0 % - 2 %)</td><td>Ctrl: 0/50 (0 %)
100 ppm: 0/50 (0 %)
300 ppm: 1/50 (2 %)
1000 ppm: 2/50 (4 %)</td></tr>
<tr><td>Kidney metastases (renal carcinoma metastasised to the lung; secondary neoplasms)</td><td>1000 ppm: 2/50 (4 %)</td></tr>
<tr><td>Mononuclear cell leukaemia (MNCL)

HCI†: 188/399 (47.1 % ± 10.3 %; range 32 % - 66 %)</td><td>Ctrl: 26/50 (52 %)
100 ppm: 32/50 (64 %)
300 ppm: 29/50 (58 %)
1000 ppm: 38/50* (76 %)
Trend test: p = 0.018</td></tr>
<tr><td>Testis (interstitial cell adenoma)

HCI†: 316/399 (79.2 % ± 3.9 %; range 72 % - 84 %)</td><td>Ctrl: 33/50 (66 %)
100 ppm: 44/50* (88 %)
300 ppm: 41/50 (82 %)
1000 ppm: 44/50* (88 %)
Trend test: p = 0.007</td></tr>
</table>
* p ≤ 0.05
# data of the standard and extended evaluations together (single and step section combined)
† HCI: historical control incidence (inhalation studies) as reported within the NTP report (data as of January 28th, 2005) (NTP, 2007)</td>
<td>(NTP, 2007)

ECHA dissemination page: 001 Key | Experimental results</td>
</tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td></td><td></td><td>

Pre-/Non-neoplastic effects:

In ♂ rats:

<table>
<tr><th>Pre-/non-neoplastic effects:</th><th>No. of animals with lesions / no. of animals in the group (0, 100, 300, 1000 ppm):</th></tr>
<tr><td>Kidney (papilla, linear mineralisation†)</td><td>12/50 (24 %), 16/50 (32 %), 10/50 (20 %), 33/50** (66 %)</td></tr>
<tr><td>Kidney (hyperplasia)#</td><td>1/50 (2 %), 0/50, 1/50 (2 %), 4/50 (8 %)</td></tr>
<tr><td>Nose (olfactory epithelium, hyperplasia, basal cell)</td><td>0/50 (0 %), 17/50** (34 %), 18/50** (36 %), 43/49** (88 %)</td></tr>
<tr><td>Nose (olfactory epithelium, degeneration)</td><td>1/50 (2 %), 3/50 (6 %), 3/50 (6 %), 16/49** (33 %)</td></tr>
</table>

* p ≤ 0.05 ** p ≤ 0.01
† severity: minimal (cf. Annex I 2.3.1.1)
# data of the standard and extended evaluations together (single and step section combined)

In ♀ rats:

<table>
<tr><th>Pre-/ non-neoplastic effects:</th><th>No. of animals with lesions / no. of animals in the group (0, 100, 300, 1000 ppm):</th></tr>
<tr><td>Kidney (papilla, mineralisation#)</td><td>1/49 (2 %), 6/50 (12 %), 8/50* (16 %), 7/50* (14 %)</td></tr>
<tr><td>Nose (olfactory epithelium, hyperplasia, basal cell)</td><td>0/49 (0 %), 14/49** (29 %), 30/50** (60 %), 49/50** (98 %)</td></tr>
<tr><td>Nose (olfactory epithelium, degeneration)</td><td>1/49 (2 %), 1/49 (2 %), 7/50* (14 %), 24/50** (48 %)</td></tr>
</table>

* p ≤ 0.05 ** p ≤ 0.01
# characterised by laminated concretions / severity: minimal (cf. Annex I 2.3.1.1)

</td><td></td></tr>
<tr><td>2-years Carcino-genicity study

Similar to OECD TG 451 (NTP standards)

GLP: yes (21 CFR, Part 58)

Key study</td><td>2-phenyl-propene (> 99 % purity)

Conc.: ♂/♀: 0, 100, 300, 600 ppm (approx. 0.48, 1.45, 2.9 mg/l)

Duration</td><td>

General effects:

- no treatment-related effect on survival rates
- reduced body weight in ♂ and ♀ mice at 600 ppm (final mean body weight: - 8 % in ♂ and - 9 % in ♀; final mean body weight gains: - 12 % in ♂ and - 20 % in ♀)

Neoplastic effects:

Male mice **positive** (liver)

Female mice **positive** (liver)

In ♂ mice:

</td><td>(NTP, 2007)

ECHA dissemination page: 002 Supporting | Experimental results</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td>Whole-body inhalation

Species: mice

Strain: B6C3F1

No/group: n = 50 / sex / treatment group</td><td>of exposure: 2 years (105 weeks)

6 h/d plus T_{90} (12 minutes)

5 d/w (except holidays)</td><td>
<table>
<tr><th>Neoplastic effects:</th><th>No. of animals with lesions / no. of animals in the group</th></tr>
<tr><td>Liver (hepatocellular adenoma or carcinoma [combined])

HCI†: 196/350 (56.0 % ± 6.2 %; range 50 % – 68 %)</td><td>Ctrl: 28/50 (56 %)
100 ppm: 36/50* (72 %)
300 ppm: 33/50 (66 %)
600 ppm: 37/50* (74 %)
Trend test: p = 0.093</td></tr>
<tr><td>Liver (hepatocellular adenoma [includes multiple])

HCI†: 134/350 (38.3 % ± 6.3 %; range 30 % – 46 %)</td><td>Ctrl: 24/50 (48 %)
100 ppm: 27/50 (54 %)
300 ppm: 27/50 (54 %)
600 ppm: 25/50 (50 %)
Trend test: p = 0.453</td></tr>
<tr><td>Liver (hepatocellular carcinoma [includes multiple])

HCI†: 85/350 (24.3 % ± 4.8 %; range 18 % – 32 %)</td><td>Ctrl: 10/50 (20 %)
100 ppm: 12/50 (24 %)
300 ppm: 11/50 (22 %)
600 ppm: 17/50 (34 %)
Trend test: p = 0.081</td></tr>
</table>
* p ≤ 0.05
† HCI: historical control incidence (inhalation studies) as reported within the NTP report (data as of January 28th, 2005) (NTP, 2007)

In ♀ mice:

<table>
<tr><th>Neoplastic effects:</th><th>No. of animals with lesions / no. of animals in the group</th></tr>
<tr><td>Liver (hepatocellular adenoma or carcinoma [combined])

HCI†: 108/347 (31.1 % ± 6.8 %; range 22 % – 39 %)</td><td>Ctrl: 13/50 (26 %)
100 ppm: 26/50** (52 %)
300 ppm: 24/50* (48 %)
600 ppm: 33/50** (66 %)
Trend test: p < 0.001</td></tr>
<tr><td>Liver (hepatocellular adenoma [includes multiple])

HCI†: 78/347 (22.5 % ± 8.1 %; range 12 % – 35 %)</td><td>Ctrl: 10/50 (20 %)
100 ppm: 20/50* (40 %)
300 ppm: 21/50** (42 %)
600 ppm: 23/50** (46 %)
Trend test: p = 0.014</td></tr>
<tr><td>Liver (hepatocellular carcinoma [includes multiple])

HCI†: 37/347 (10.7 % ± 1.8 %; range 8 % – 12 %)</td><td>Ctrl: 3/50 (6 %)
100 ppm: 9/50 (18 %)
300 ppm: 6/50 (12 %)
600 ppm: 18/50** (36 %)
Trend test: p < 0.001</td></tr>
<tr><td>Liver metastases (hepatocellular carcinoma metastasised to the lung; secondary neoplasms)</td><td>Ctrl: 1/50 (2 %)
100 ppm: 5/50 (10 %)
300 ppm: 3/50 (6 %)
600 ppm: 13/50 (26 %)</td></tr>
</table>
* p ≤ 0.05 ** p ≤ 0.01
† HCI: historical control incidence (inhalation studies) as reported within the NTP report (data as of January 28th, 2005) (NTP, 2007)

<u>Pre-/Non-neoplastic effects:</u>
</td><td></td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td></td><td></td><td>
In ♂ mice:
<table>
<tr><th>Pre-/non-neoplastic effects:</th><th>No. of animals with lesions / group (0, 100, 300, 600 ppm):</th></tr>
<tr><td>Nose (olfactory epithelium, metaplasia)</td><td>6/50 (12 %), 47/50** (94 %), 49/50** (98 %), 49/50** (98 %)</td></tr>
<tr><td>Nose (olfactory epithelium, glands, hyperplasia)</td><td>4/50 (8 %), 50/50** (100 %), 50/50** (100 %), 50/50** (100 %)</td></tr>
<tr><td>Nose (olfactory epithelium, atrophy)</td><td>0/50 (0 %), 2/50 (4 %), 8/50** (16 %), 12/50** (24 %)</td></tr>
<tr><td>Forestomach (hyperplasia of the epithelium)</td><td>1/50 (2 %), 4/49 (8 %), 7/49* (14 %), 11/48** (23 %)</td></tr>
<tr><td>Forestomach (inflammation)</td><td>0/50 (0 %), 2/49 (4 %), 1/49 (2 %), 5/48* (10 %)</td></tr>
</table>
* p ≤ 0.05 ** p ≤ 0.01

In ♀ mice:
<table>
<tr><th>Pre-/non-neoplastic effects:</th><th>No. of animals with lesions / group (0, 100, 300, 600 ppm):</th></tr>
<tr><td>Nose (olfactory epithelium, metaplasia)</td><td>2/49 (4 %), 49/49** (100 %), 47/50** (94 %), 50/50** (100 %)</td></tr>
<tr><td>Nose (olfactory epithelium, glands, hyperplasia)</td><td>3/49 (6 %), 49/49** (100 %), 50/50** (100 %), 50/50** (100 %)</td></tr>
<tr><td>Liver (eosinophilic foci)</td><td>2/50 (4 %), 5/50 (10 %), 7/50 (14 %), 12/50** (24 %)</td></tr>
<tr><td>Kidney (nephropathy#)</td><td>16/50 (32 %), 21/49 (43 %), 12/50 (24 %), 26/50* (52 %)</td></tr>
</table>
* p ≤ 0.05 ** p ≤ 0.01
# increased incidence and severity (cf. Annex I 2.3.1.2)
</td><td></td></tr>
</table>

10.9.1 Short summary and overall relevance of the provided information on carcinogenicity

According to Morgan *et al.* (1999), occupational use is the primary scenario where human exposure to the volatile hydrocarbon 2-phenylpropene occurs. Thereby, inhalation, next to dermal contact, has been described as a relevant route of human exposure (Morgan et al., 1999; NTP, 2007). Carcinogenicity data attributed to 2-phenylpropene exposure in humans are not available. However, the substance was tested under the National Toxicology Program (NTP) in experimental animals. In a 2-years bioassay, carcinogenic effects related to 2-phenylpropene exposure by inhalation were studied in rats and mice. The study was performed in accordance with accepted scientific principles (similar to OECD TG 451 with GLP) and is considered a reliable source of information. As listed in Table 17, neoplastic effects were found both in rats and mice following exposure to 2-phenylpropene by inhalation.

Rats (F344/N) - ♂ rats some evidence / ♀ rats no evidence

In rats, **renal tubule adenoma and/or carcinoma** were observed in males but not in females. When considering the data for renal tubule adenoma or carcinoma (combined) from the standard and extended evaluation, the incidence was statistically significantly greater in the high-concentration male group as compared to the control. A statistically significant trend was also noted for the male group. Two renal carcinomas metastasised to the lung in the high-concentration group. Higher incidences of non-neoplastic lesions such as linear mineralisation of the renal papilla were statistically significant in male rats at 1000 ppm and female rats at 300 and 1000 ppm. According to the authors of the study, the results may be related to a carcinogenic mechanism involving alpha-2 urinary (α2u) globulin-mediated nephropathy- a mode of action (MoA) with precluded relevance to humans (ECHA, 2017b; IARC, 1999; NTP, 2007). Findings of repeated dose toxicity studies are partly in support of this mechanistic hypothesis (cf. section 0) (Morgan et al., 1999; NTP, 2007; Study report RDT, 1997). The authors of the carcinogenicity NTP study concluded that based on neoplastic and non-neoplastic lesions of the kidney, 2-phenylpropene exhibits *some evidence of carcinogenic activity*[12] in male rats (NTP, 2007). The predictivity of these findings concerning human carcinogenicity will be further discussed in section 10.9.2 (a and k).

In addition, the incidence of **mononuclear cell leukaemia** (MNCL) was found to be higher at 1000 ppm in male rats with statistical significance. Together with the note that this tumour is very common particularly in F344/N rats, the authors of the NTP study consider these results as *equivocal findings*[13] (NTP, 2007). The biological relevance to humans has been called into question (Caldwell, 1999; Scheepmaker et al., 2005) and is further discussed in section10.9.2 (a).

Interstitial cell adenoma in testis, another common tumour in rats, were increasingly found at 100 and 1000 ppm. The results were considered incidental by the authors of the NTP study.

No neoplastic effects were observed in female rats.

Mice (B6C3F$_1$) - ♂ mice equivocal evidence / ♀ mice clear evidence

In mice, the incidences of **hepatocellular adenoma or carcinoma** (combined) in male mice at 100 and 600 ppm and female mice in all treatment groups were increased with statistical significance. When analysing individually, the incidences of hepatocellular adenoma and carcinoma alone were statistically significantly increased only in females. Hepatocellular carcinomas metastasised to the lung, especially in high-concentration female mice. Non-neoplastic lesions such as eosinophilic foci were noted in female but not in male mice. Hence, the carcinogenic response in the liver was considerably stronger in female as compared to male mice. The authors of the study, consequently, concluded that based on neoplastic and non-neoplastic lesions of the liver, 2-phenylpropene exhibits *clear evidence of carcinogenic activity*[14] in female mice and *equivocal evidence of carcinogenic activity*[15] in male mice. The predictivity of these findings concerning human carcinogenicity appears to be dependent on the underlying MoA, which will be further discussed in section 10.9.2 (a and k).

12 **some evidence of carcinogenic activity** is demonstrated by studies that are interpreted as showing a chemical-related increased incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence (NTP, 2007).

13 **equivocal evidence of carcinogenic activity** is demonstrated by studies that are interpreted as showing a marginal increase of neoplasms that may be chemical related (NTP, 2007).

14 c**lear evidence of carcinogenic activity** is demonstrated by studies that are interpreted as showing a dose-related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms, or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumours to progress to malignancy (NTP, 2007).

15 See previous page

10.9.2 Comparison with the CLP criteria

"Carcinogen means a substance or a mixture of substances which induce cancer or increase its incidence (CLP Regulation 1272/2008, 3.6.1.1.). For the purpose of classification for carcinogenicity, substances are allocated to one of two categories based on strength of evidence and additional considerations (weight of evidence)." (CLP Regulation 1272/2008, 3.6.2.1.)

Hazard categories for carcinogens (CLP Regulation 1272/2008, Table 3.6.1)

Category 1:

"Known or presumed human carcinogens

A substance is classified in Category 1 for carcinogenicity on the basis of epidemiological and/or animal data. A substance may be further distinguished as:

> ***Category 1A:*** *Category 1A, known to have carcinogenic potential for humans, classification is largely based on human evidence, or*
>
> ***Category 1B:*** *Category 1B, presumed to have carcinogenic potential for humans, classification is largely based on animal evidence."*

Category 2:

"Suspected human carcinogens

The placing of a substance in Category 2 is done on the basis of evidence obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category 1A or 1B, based on strength of evidence together with additional considerations (see section 3.6.2.2). Such evidence may be derived either from limited evidence of carcinogenicity in human studies or from limited evidence of carcinogenicity in animal studies."

Strength of evidence (CLP Regulation 1272/2008, 3.6.2.2.3.):

"Strength of evidence involves the enumeration of tumours in human and animal studies and determination of their level of statistical significance.

*Evidence of carcinogenicity can be considered **sufficient** if a causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or (b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols. An increased incidence of tumours in both sexes of a single species in a well-conducted study, ideally conducted under Good Laboratory Practices, can also provide sufficient evidence. A single study in one species and sex might be considered to provide sufficient evidence of carcinogenicity when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites;*

*Evidence of carcinogenicity can be considered **limited** if the data suggest a carcinogenic effect but are limited for making a definitive evaluation because, e.g. (a) the evidence of carcinogenicity is restricted to a single experiment; (b) there are unresolved questions regarding the adequacy of the design, conduct or interpretation of the studies; (c) the agent increases the incidence only of benign neoplasms or lesions of uncertain neoplastic potential; or (d) the evidence of carcinogenicity is restricted to studies that demonstrate only promoting activity in a narrow range of tissues or organs."*

While there are no human carcinogenicity data available, 2-phenylpropene was tested by NTP in chronic bioassays using experimental animals. Increased tumour incidences, observed with statistical significance, were noted in male rats and mice of both sexes. The data generated by NTP are considered adequate information similar to internationally accepted guideline studies including GLP compliance. Compared to the criteria laid out in section 3.6.2.2.3. of the CLP regulation (determination of the strength of evidence for carcinogenicity), the information may be considered as sufficient evidence of carcinogenicity as *"a causal relationship has been established between the agent and an increased incidence […] of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or […] an increased incidence of tumours in both sexes of a single species in a well-conducted study, ideally conducted under Good Laboratory Practices".*

Additional considerations / Weight of evidence (CLP Regulation 1272/2008, 3.6.2.2.4. - 3.6.2.2.6.):

"Beyond the determination of the strength of evidence for carcinogenicity, a number of other factors need to be considered that influence the overall likelihood that a substance poses a carcinogenic hazard in humans.

Some important factors which may be taken into consideration, when assessing the overall level of concern are (CLP Regulation 1272/2008, 3.6.2.2.6.):"

(a) tumour type and background incidence

Rats (F344/N)

The 2-year NTP study in rats revealed a statistically significantly increased incidence of **renal tubule adenoma and carcinoma (combined)** when taking the standard and extended evaluation into consideration (ctrl: 1/50 (2 %), 1000 ppm: 7/50 (14 %) p = 0.026). The historical incidence[16] of renal tubule adenoma and carcinoma (combined) reported by the authors of the NTP study is very low (4/399 [1.0 % ± 1.1 %; range 0 % – 2 %]) and, thus, consistent with the observed incidence of the control group in the study. The incidence seen in the high-concentration male group was well above the historical incidence and outside the range. A tumour type is generally relevant to humans unless there is clear evidence suggesting that the underlying MoA does not occur in humans. There is some evidence suggesting a treatment-related link between α2u-globulin-dependent nephropathy and the occurrence of the renal tumours (Morgan et al., 1999; NTP, 2007; Study report RDT, 1997). Potential involvement of this rat-specific MoA is further discussed in section 10.9.2 (k).

An increased incidence of **MNCL** was also noted in the high-concentration male group (ctrl: 26/50 (52 %), 1000 ppm: 38/50 (76 %) p = 0.016). Despite showing a statistically significant trend, only the incidence in the highest concentration group exceeded the historical control incidence (188/399 [47.1 % ± 10.3 %; range 32 % – 66 %]) as reported in the 2-year NTP study (NTP, 2007). The authors of the NTP study considered the findings as equivocal, potentially related to 2-phenylpropene exposure (NTP, 2007). MNCL, in general, is commonly seen specifically in F344/N rats (as compared to other strains such as SD rats) with a high background incidence that has been noted to rise over time (Dinse et al., 2010; ECHA, 2017b). The relevance of chemical-induced increases of MNCL in F344 rats for human carcinogenicity has been discussed by the Dutch authorities (RIVM) (Scheepmaker et al., 2005). For the following reasons, the tumour type in F344 rats was considered not relevant: spontaneous occurrence in aged F344 rats with variable and high incidence, species-specific characteristics, mechanistic considerations and reproducibility issues. On account of this general assessment and the manifestation of a statistically significant increased incidence only at the highest concentration level in the 2-year NTP study with 2-phenylpropene, the tumour type is considered negligible for the purpose of classification.

The incidence of **interstitial cell adenoma in testis** were found to be higher at 100 and 1000 ppm (ctrl: 33/50 (66 %), 100 ppm: 44/50 (88 %) p = 0.017, 1000 ppm: 38/50 (88 %) p = 0.016), exceeding the reported historical

[16] Historical control data as reported within the NTP study relate to carcinogenicity studies with NTP-2000 diet with exposure via inhalation

control incidence range only slightly (316/399 [79.2 % ± 3.9 %; range 72 % – 84 %]). The authors of the NTP study did not consider this tumour type as treatment-related and, hence, as relevant carcinogenic finding (NTP, 2007). Therefore, the tumour type is disregarded for the purpose of classification.

Mice ($B6C3F_1$)

Hepatocellular adenoma and carcinoma were found to occur more frequent following exposure to 2-phenylpropene in mice. The incidence of hepatocellular adenoma or carcinoma (combined) was higher in male mice at 100 and 600 ppm (ctrl: 28/50 (56 %), 100 ppm: 36/50 (72 %) p = 0.031, 600 ppm: 37/50 (74 %) p = 0.035) and female mice in all treatment groups (ctrl: 13/50 (26 %), 100 ppm: 26/50 (52 %) p = 0.004, 300 ppm: 24/50 (48 %) p = 0.012, 1000 ppm: 33/50 (66 %) p < 0.001) with statistical significance. When regarded separately, hepatocellular adenoma (ctrl: 10/50 (20 %), 100 ppm: 20/50 (40 %) p = 0.018, 300 ppm: 21/50 (42 %) p = 0.007, 1000 ppm: 23/50 (46 %) p = 0.005) or carcinoma (ctrl: 3/50 (6 %), 1000 ppm: 18/50 (36 %) p < 0.001) were only statistically significantly increased in female but not in male mice. The incidence in females were clearly outside the range of the historical control incidences (hepatocellular adenoma: 78/347 [22.5 % ± 8.1 %; range 12 % – 35 %]; hepatocellular carcinoma: 37/347 [10.7 % ± 1.8 %; range 8 % – 12 %]; combined females: 108/347 [31.1 % ± 6.8 %; range 22 % - 39 %]). Preneoplastic lesions (eosinophilic foci) were noted with statistical significance in high-concentration females only. The authors of the NTP study considered the findings in female mice as clear evidence of carcinogenic action. The increased incidence in male mice, however, were only considered as equivocal evidence because the numbers were only slightly higher than the range of the historical control data (combined male: 196/350 [56.0 % ± 6.2 %; range 50 % – 68 %]). Characterised by high spontaneous incidences, liver adenomas and carcinomas are frequently observed in B6C3F1 mice following chemical treatment (Laube et al., 2019). The species is hence considered sensitive for this tumour type.

(b) multi-site responses

According to the evidence presented in Table 17, exposure to 2-phenylpropene leads to higher incidences of tumours in multiple sites. A multi-site response generally increases the concern.

(c) progression of lesions to malignancy

A continuum associated with the progression of lesions to malignancy was particularly obvious in female mice (hepatocellular foci, benign hepatocellular adenoma, malignant hepatocellular carcinoma) but also noted in male rats (renal tubule hyperplasia, benign renal tubule adenoma, malignant renal tubule carcinoma) (NTP, 2007). The frequently observed metastases in the lung that originated from the hepatocellular carcinoma especially in high-concentration female mice (13/50; 26 %) underscore the malignant potential. A reported progression of lesions to malignancy generally increases the concern.

(d) reduced tumour latency

As compared to chamber control animals, a concentration-dependent reduction of the time point where the first tumour incidence occurred (tumour-associated mortality) was noted for renal tubule adenoma or carcinoma (combined) in male rats (ctrl, 100 ppm, 300 ppm, 1000 ppm (days): 729, 723, 716, 653). Given that the observed reduction was rather weak, the level of concern is considered unaffected.

(e) whether responses are in single or both sexes

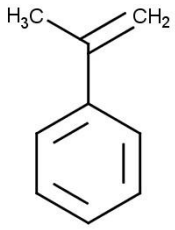
Tumour formation in rats was only seen in males. In mice, carcinogenic effects were found in both sexes, albeit the response was stronger in female mice. A response in both sexes generally increases the concern.

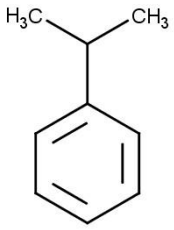
(f) whether responses are in a single species or several species

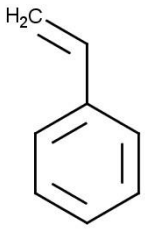
According to the evidence presented in Table 17, exposure to 2-phenylpropene leads to higher incidences of tumours in multiple species. A response in several species generally increases the concern.

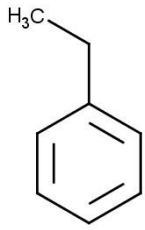
(g) structural similarity to a substance(s) for which there is good evidence of carcinogenicity

2-phenylpropene *cumene* *styrene* *ethylbenzene*









As depicted above[17], 2-phenylpropene is structurally very similar to other aromatic hydrocarbons such as cumene, styrene, and ethylbenzene; all of which have already been scrutinised within the CLH process. Corresponding RAC opinions are available for styrene (STOT RE, Repr.)[18], cumene (Carc. 1B)[19], and ethylbenzene (Asp. Tox, STOT RE/ototoxicity)[20].

cumene (EC: 202-704-5)

2-phenylpropene has been identified as a metabolite in rodents exposed to cumene (Chen et al., 2011; NTP, 2009). Cumene is also a precursor in the industrial synthesis of 2-phenylpropene (IARC, 2012a). Table 18 summarises the tumour types in rats and mice, which had been listed and discussed in the corresponding CLH dossier (601-024-00.-X)[21]. IARC has classified cumene as "*possibly carcinogenic to humans"* (Group 2B) (IARC, 2012b).

Table 18: Cumene-related carcinogenic effects (as listed in the CLH dossier)

Species and strain	Route of exposure	single or both sexes	Tumour type and background incidence
Rats / F344/N	Inhalation	♂/♀	Nose (adenoma respiratory epithelium)
		♂	Kidney (renal tubule adenoma or carcinoma [combined])
		♂	Testes (interstitial cell adenoma)
Mice / B6C3F1	Inhalation	♂/♀	Lung (alveolar/bronchiolar adenoma or carcinoma [combined])
		♀	Liver (hepatocellular adenoma or carcinoma [combined])
		♂	Hemangiosarcoma (spleen/all organs)
		♂	Thyroid gland (follicular cell adenoma)

17 structures taken from ECHAs dissemination page

18 https://echa.europa.eu/documents/10162/366be849-10df-1833-d6df-71fbe5ff860e

19 https://echa.europa.eu/documents/10162/bfc2a1b4-528b-17a1-a321-ed037f09c740

20 https://echa.europa.eu/documents/10162/d01123d0-a4d5-3bd3-6c33-9cdcc1cdf8ce

21 https://echa.europa.eu/documents/10162/10e6c6a2-7321-a5e4-3ac3-ed5439c7c4a1

Although the carcinogenic profiles of cumene and 2-phenylpropene differ, especially regarding the marked effect in the lungs, some tumour types are commonly observed in experimental animals. Incidences of neoplasm in the kidney (renal tubule adenoma or carcinoma) of male F344/N rats and the liver (hepatocellular adenoma or carcinoma) of female B6C3F1 mice were increased following exposure via inhalation with 2-phenylpropene and cumene with statistically significance. For both tumour types, human relevance was considered uncertain but not excludable by the dossier submitter due to uncertainties regarding the underlying MoA. Classification into Category 2 was proposed for cumene. RAC later concluded that the overall weight of evidence even warrants Category 1B, mostly on the basis of the observed lung tumours in mice supported by nasal and kidney tumours in rats. The liver tumours in mice were not considered "a strong indication for carcinogenicity" by RAC due to their uncertain human relevance (CAR/PXR MoA possible but not fully conclusive) and the weak response when compared to the remarkable high background incidence. When comparing the concurrent control incidences of cumene and 2-phenylpropene, a considerable difference can be noted. With respect to hepatocellular adenoma or carcinoma [combined] in female mice, the reported control incidence for cumene is 25/50 (50 %) as compared to 13/50 (26 %) for 2-phenylpropene (NTP, 2007; NTP, 2009). Based on these numbers, the effect on liver tumour formation appears to be notably stronger in the 2-phenylpropene study. For the purpose of comparison, Table 19 depicts HCI data of inhalation NTP studies (as of 2007) as provided in the NTP study report for cumene (NTP, 2009). Of the nine studies included in the HCI database, the highest HCI values were observed in the study with cumene, exceeding the upper bound of the 95 % confidence interval. The HCI database provided for 2-phenylpropene (data as of 2005, cf. Table 17 did not include these high values seen in the study with cumene (NTP, 2007).

Table 19: NTP historical control incidence database on hepatocellular neoplasms in control female B6C3F1 mice (as reported in the NTP study with cumene; data as of March 2, 2007), adapted according to NTP (2009)

Inhalation Study	Adenoma	Carcinoma	Adenoma or Carcinoma
Cumene	**18/50 (36 %)**	**10/50 (20 %)**	**25/50 (50 %)**
Decalin	7/49 (14.3 %)	4/49 (8.2 %)	11/49 (22.4 %)
Divinylbenzene	17/49 (34.7 %)	5/49 (10.2 %)	19/49 (38.8 %)
Methyl isobutyl ketone	13/50 (26 %)	6/50 (12 %)	17/50 (34 %)
α-Methylstyrene	**10/50 (20 %)**	**3/50 (6 %)**	**13/50 (26 %)**
Propargyl alcohol	15/50 (30 %)	4/50 (8 %)	17/50 (34 %)
Propylene glycol mono-t-butyl ether	14/49 (28.6 %)	4/49 (8.2 %)	18/49 (36.7 %)
Stoddard solvent IIC	9/50 (18 %)	6/50 (12 %)	13/50 (26 %)
Vanadium pentoxide	6/50 (12 %)	6/50 (12 %)	12/50 (24 %)
Overall Historical Incidence			
Total (%)	109/447 (24.4 %)	48/447 (10.7 %)	145/447 (32.4 %)
Mean ± standard deviation	24.4 % ± 8.7 %	10.7 % ± 4.1 %	32.4 % ± 8.8 %
CI (95 %) - lower bound	17.7 %	7.6 %	25.6 %
CI (95 %) – upper bound	31.1 %	13.9 %	39.2 %
Range	12 % - 36 %	6 % - 20 %	22 % - 50 %

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).

styrene (EC: 202-851-5)

Styrene is another prominent structural similar aromatic hydrocarbon. Table 18 summarises the tumour types in mice, which had been listed and discussed in corresponding CLH dossier (601-026-00-0)[22]. The corresponding carcinogenicity section of the CLH dossier contains an assessment based on the EU RAR (2008)[23].

Table 20: Styrene-related carcinogenic effects (as listed in the CLH dossier)

Species and strain	Route of exposure	single or both sexes	Tumour type and background incidence
Mice / CD1	Inhalation	♂/♀	Lung (bronchioalveolar adenomas)
		♀	Lung (bronchioalveolar carcinoma)
Mice / B6C3F1	Oral	♂	Lung (alveolar/bronchiolar adenomas or carcinomas)
		♀	Liver (hepatocellular adenomas); statistical significance only in the trend test, no statistical significance in any pairwise comparison

Only the slightly increased incidences of benign tumours in the liver of female mice resemble the effects observed with 2-phenylpropene. With respect to the hepatic response, styrene appears to be of lower potency. The MoA leading to tumour formation in the lungs of mice (Clara cell toxicity induced by cytotoxic metabolites) was considered unlikely to be relevant for humans. There was no clear evidence for carcinogenic effects in rats. No classification for carcinogenicity was proposed/adopted during the CLH process based on the EU RAR (2008) assessment. However, 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.

ethylbenzene (EC: 202-849-4)

Ethylbenzene is another structurally similar substance that induces (pre)neoplastic lesions similar to those observed with 2-phenylpropene. In rats, a higher incidence of renal tubule adenoma or carcinoma (combined) was noted in males, whereas renal tubule adenomas were more frequently observed in both sexes. In the liver of female mice, eosinophilic foci and hepatocellular adenoma/carcinoma were reported following exposure by inhalation (NTP, 1999). The increased incidence of alveolar/bronchiolar adenoma or carcinoma (combined) in male mice is consistent with effects seen with cumene and styrene. No such tumours were observed with 2-phenylpropene. During the CLH process[24], carcinogenicity was not evaluated. IARC has classified ethylbenzene as "*possibly carcinogenic to humans"* (Group 2B) (IARC, 2000).

[22] https://echa.europa.eu/documents/10162/e7c7d1a1-b42a-410b-8afe-62b8ada88ca5

[23] EUROPEAN UNION RISK ASSESSMENT REPORT. Styrene. CAS No: 100-42-5. EINECS No 202-851-5. Draft for publication, June 2008. United Kingdom.

[24] https://echa.europa.eu/documents/10162/afea3382-8cf4-4613-a199-07c6cb73b541

Table 21: Ethylbenzene-related carcinogenic effects (NTP, 1999)

Species and strain	Route of exposure	single or both sexes	Tumour type and background incidence
Rats / F344	Inhalation	♂	Kidney (renal tubule adenoma or carcinoma [combined])
		♂/♀	Kidney (renal tubule adenoma)
Mice / B6C3F1	Inhalation	♂	Lung (alveolar/bronchiolar adenoma or carcinoma [combined])
		♀	Liver (hepatocellular adenoma or carcinoma[combined])

The existence of structurally similar substances for which there is good evidence of carcinogenicity generally increases the concern.

(h) routes of exposure

2-phenylpropene related data on carcinogenicity in experimental animals are exclusively available for inhalation; a physiological route of exposure considered important for human exposure (Morgan et al., 1999; NTP, 2007). With regard to the route of exposure, the data are therefore relevant for humans.

(i) comparison of absorption, distribution, metabolism and excretion between test animals and humans

As described in section 9 of the CLH dossier and section 1 of Annex I, studies in rats have shown that 2-phenylpropene is readily metabolised and excreted mainly via the urine (~90 %, inhalation study) with very little tissue accumulation (~1 – 2 %, inhalation study). Five metabolites were identified in the urine of rats. The most abundant urinary metabolites were 2-phenyl-1,2-propanediol glucuronide and atrolactic acid regardless of the route of exposure (De Costa et al., 2001; NTP, 2007). Following exposure of human liver slices with 2-phenylpropene, the same profile of metabolites as in the urine of rats was seen, with 2-phenyl-1,2-propanediol identified as the primary metabolite (De Costa et al., 2001). In an older study of low quality, atrolactic acid was found in the urine of human subjects exposed to 2-phenylpropene (Bardodej and Bardodejova, 1970). There is, hence, no evidence suggesting a significant difference between experimental animals and humans.

(j) the possibility of a confounding effect of excessive toxicity at test doses

In the 2-year NTP study in rats, no indications of excessive toxicity potentially associated with a carcinogenic response were seen. In mice, a reduction of mean body weight gain greater than 10 % was observed (♂: - 12 %, ♀: - 20 %) at the highest concentration level (600 ppm) in the 2-year NTP study and 90-day NTP at ≥ 300 ppm. However, increased incidences of hepatocellular adenoma or carcinoma (combined) had already been seen in the absence of potentially confounding general toxicity at 100 ppm, suggesting a direct treatment-related effect.

(k) mode of action and its relevance for humans

Rats (F344/N)

As described in section 0 and Annex I 2.4.1.3, abnormal hyaline droplet accumulation, indicative of hyaline droplet nephropathy, was seen in the kidneys of male F344 rats in a subacute repeated dose toxicity study (Morgan et al., 1999). No such effect was observed in female F344 rats and male NBR rats, both of which do not produce the male-rate-specific low molecular weight protein α2u-globulin (Morgan et al., 1999). Increased

hyaline droplets in the renal tubular epithelium accompanied by basophilic alterations were also noted in a subacute oral study (Study report RDT, 1997). Consistently, the histopathological examination at the end of the 90-day NTP subchronic study revealed mild to moderately altered morphology of hyaline droplets (larger and more variable in shape), increased renal cell proliferation, and concentration-dependent elevated α2u-globulin concentrations in the kidney exclusively in male rats. The 2-year NTP carcinogenicity revealed pre-neoplastic lesions such as hyperplasia of the renal tubules in male rats (without statistical significance) and mineralisation of the renal papilla in male and female rats. In the same study, the incidence of renal tubule adenoma and carcinoma (combined) was statistically significantly increased in high-concentration males. The authors of the NTP study concluded that the kidney tumours may be related to a male-specific α2u-globulin-dependent mechanism. In comparison to other chemicals (e.g. decalin, d-limonene), the α2u-globulin-associated nonneoplastic effects were considered weak. They also stressed that other mechanisms may be involved given that renal toxicity had also been seen in female rats and mice (NTP, 2007).

Chemical-induced accumulation of α2u-globulin in the renal tubules is frequently associated with hyaline droplet alteration and degeneration, necrosis, and regeneration of the renal tubule epithelium, eventually culminating in tumour formation (IARC, 1999; NTP, 2007). A prerequisite for this mechanism to operate is the binding of a chemical to α2u-globulin, preventing its degradation and leading to cytotoxic accumulation with subsequent reparative cell proliferation and tumour development. Given that α2u-globulin is exclusively produced by male rats together with the observation that human urinary proteins do not exert such binding properties and the fact that the protein content of the human urine is generally much lower (100 – 1000 times lower), the related MoA has been considered species-specific and, hence, not relevant for humans (ECHA, 2017b; IARC, 1999). Nephropathy related to α2u-globulin is thereby different from the chronic progressive nephropathy (CPN), commonly seen in aged male rats (EPA, 1991). If the available evidence for 2-phenylpropene would convincingly support an α2u-globulin-related MoA, the observed kidney tumours may be discounted. To this end, criteria have been defined to determine the role of α2u-globulin in renal tumour formation (IARC, 1999; Swenberg, 1999). It is worth noting, though, that only a weak association between findings in prechronic studies (α2u-globulin concentrations, cell proliferation, histopathological findings related α2u-globulin nephropathy) and the formation of renal neoplasms was uncovered in a re-evaluation of NTP study results. While considering α2u-globulin nephropathy a possible contributor, the authors of the study suggested the involvement of other important factors in the renal carcinogenic response in male rats (Doi et al., 2007).

Table 22: Evaluation criteria according to (IARC, 1999; Swenberg, 1999)

Criteria	Applied to 2-phenylpropene data
essential evidence	
renal tumours occur only in male rats	**applicable** (renal tubule adenoma and carcinoma (combined) in high-concentration (1000 ppm) ♂ F344 rats in the 2-year NTP study (NTP, 2007))
acute exposure exacerbates hyaline droplet formation	**applicable** (increased severity (mild to moderate) of hyaline droplet accumulation in ♂ F344 rats following 12 days of exposure (Morgan et al., 1999); minimal to mild alteration of hyaline droplets in the 90-day NTP study (NTP, 2007); increased hyaline droplets in the renal tubular epithelium of ♂ Crj: CD(SD) rats upon oral subacute exposure (Study report RDT, 1997))
α2u-globulin accumulates in hyaline droplets	**applicable** (elevated α2u-globulin concentrations[25] in the kidney of ♂ rats in the 90-

[25] α2u-globulin concentration was deterimened in the supernatant of whole kidney homogenate using a competitive indirect enzyme-linked immunosorbent assay (ELISA)

Criteria	Applied to 2-phenylpropene data
	day NTP study (NTP, 2007))
subchronic histopathological changes including granular cast formation and linear papillary mineralisation	**somewhat applicable** (no evidence of granular casts in the renal tubules of ♂ F344 rats in subacute/subchronic RDT studies (Morgan et al., 1999; NTP, 2007); however, linear mineralisation of the renal papilla was seen in the 2-years NTP study (NTP, 2007))
absence of hyaline droplets and characteristic histopathological changes in female rats and mice	**not applicable** (no hyaline droplet accumulation in α2u-globulin deficient rats such as ♀ F344 or ♂ NBR subacute/subchronic RDT* studies (Morgan et al., 1999; NTP, 2007); minimal mineralisation of the renal papilla (characterized by laminated concretions) in ♀ F344 rats in the 2-year NTP study; other signs of renal toxicity (kidney weight, urinary parameters indicative of renal tubular epithelium toxicity) in ♀ F344 rats the 90-day NTP study; treatment-related increased nephropathy in ♀ mice in the 2-years NTP study (NTP, 2007))
negative for genotoxicity in a battery of tests	**inconclusive** (cf. section 10.8)
additional supporting evidence	
reversible binding of the chemical (or metabolites) to α2u-globulin	**not applicable** (no data)
increased and sustained cell proliferation in P2 segment of the proximal tubule in male rat kidneys	**applicable** (increased renal cell proliferation in ♂ F344 rats in the 90-day study (NTP, 2007))
similarities in the dose-response relationship between renal tumour incidence and characteristic histopathological changes (hyaline droplet severity, α2u-globulin accumulation, and renal cell proliferation)	**inconclusive** (tumour formation correlates at best with the severity of hyaline droplets; α2u-globulin accumulation and renal cell proliferation do not correlate: - renal tumour formation: 1000 ppm - slightly increased severity of hyaline droplets: ≥ 600 ppm - α2u-globulin accumulation: ≥ 150 ppm - renal cell proliferation: ≥ 150 ppm (NTP, 2007))

As summarized in Table 22, not all of the listed criteria can be applied to 2-phenylpropene. Although some renal effects seen in male rats are clearly indicative of α2u-globulin-mediated nephropathy, female rats and mice also exhibited some signs of renal toxicity. Therefore, a MoA independently of α2u-globulin may be possible. Moreover, the absence of granular casts (a marker for necrotic epithelial cells) and other related hallmarks, and the mild to moderate severity of hyaline droplet accumulation suggest only a mild manifestation of α2u-globulin nephropathy. No strong correlation can be established when comparing the dose-response relationship of tumour formation with those of the characteristic histopathological changes. As discussed in section 10.8, a genotoxic mechanism cannot be excluded. The available evidence regarding the MoA leading to renal tumour formation is, hence, inconclusive.

The carcinogenic effects in the kidney of male rats observed upon exposure with 2-phenylpropene are similar to those reported for cumene and ethylbenzene (NTP, 1999; NTP, 2009). Renal tubular hyperplasia and adenoma or carcinoma (combined) were seen at higher incidences in male rats with statistical significance (cf.

Table 18) (NTP, 2009). Due to partial non-compliance of the data with the strict scientific criteria listed in Table 22, α2u-globulin-associated renal toxicity was disregarded as the MoA by IARC (IARC, 2012a). The data questioning compliance with the criteria related to some evidence of genotoxicity, renal effects in female rats, and the lack of cell proliferation data. While a MoA attributed to α2u-globulin nephropathy was considered likely by NTP in its 14th report on carcinogens, other mechanisms (e.g. genotoxicity) contributing to the renal lesions could not be ruled out. The relevance to human cancer was concluded uncertain and considered supportive regarding the overall sufficiency of carcinogenic evidence of cumene (NTP, 2016).
In a 2-year inhalation study by NTP, increasing incidences of renal tubule hyperplasia, adenoma, and adenoma or carcinoma (combined) was seen in male rats at 750 ppm ethylbenzene (cf. Table 21). Interestingly, and in contrast to 2-phenylpropene and cumene, renal tubule hyperplasia and adenoma were also observed in female rats at 750 ppm with statistical significance. Increased severity of nephropathy was seen in high-concentration males and in all exposure groups in female rats. As no clear evidence for hyaline droplet accumulation was found, a mechanism involving α2u-globulin was considered less likely. Instead, a treatment-related exacerbation of age-associated CPN was suggested (NTP, 1999). This conclusion was later confirmed in a re-evaluation of the histopathological findings (Hard, 2002). Increased hyaline droplet and α2u-globulin accumulation, elevated S-phase DNA synthesis, and increasing regenerative cell proliferation were, consistent with an acceleration of CPN, seen in a subacute study (Stott et al., 2003)

In conclusion, there is evidence pointing to a mechanism involving α2u-globulin in the formation of renal tubule tumours in male rats following exposure to 2-phenylpropene via inhalation. However, not all of the IARC criteria listed in Table 22 are unambiguously applicable, either for a lack of adequate/conclusive information or because contradictive findings exist. Similarly inconsistent data have been obtained with close structural analogues. Thus, for a lack of mechanistic understanding as to whether or not α2u-globulin-dependent nephropathy is the sole mechanism, the predictive value of 2-phenylpropene-induced renal tumour formation for human carcinogenicity remains to be elucidated. In the absence of strong evidence conclusively demonstrating a species-specific MoA, these neoplastic findings cannot be disregarded and the relevance for humans cannot be excluded. The renal findings in male rats, therefore, support a classification of 2-phenylpropene a carcinogen.

Mice (B6C3F$_1$)

The 2-year NTP study in mice, (pre)carcinogenic effects consistent with the progression of lesions to malignancy (cf. section c) were especially seen in the liver of female mice following exposure with 2-phenylpropene. Statistical significance was established for the incidence of hepatocellular adenoma and carcinoma (combined, 600 ppm) in males and the incidence of eosinophilic foci (600 ppm), hepatocellular adenoma (≥ 100 ppm) and carcinoma (600 ppm) in females (increased incidences of hepatocellular adenoma or carcinoma (combined) from ≥ 100 ppm with statistical significance) (NTP, 2007). As compared to other mouse strains, B6C3F1 have high background tumour rates in the liver (hepatocellular adenoma: ♂ 38.3 %, ♀ 22.5 %; carcinoma: ♂ 24.3 %, ♀ 10.7 %[26]) (King-Herbert and Thayer, 2006). There is no information on the mechanism of liver tumour formation provided by the authors of the 2-year NTP study.

The predictive value of mouse liver tumours for human carcinogenicity is a matter of controversy. Several MoAs have been discussed for their relevance to humans. If the ability to interact with the DNA can be established, the substance is generally considered carcinogenic in humans (ECHA, 2017b). As discussed in section 10.8, the available data on the genotoxic potential of 2-phenylpropene are not conclusive and insufficient for the purpose of classification. Hence, there is no strong evidence in support of a genotoxic mode of action. However, on the basis of the available data, genotoxicity can also not be completely ruled out. Non-genotoxic promotion of hepatocarcinogenesis due to chronic cytotoxicity and inflammation that leads to regenerative proliferation is also considered a relevant MoA (Felter et al., 2018; Köhle et al., 2008). Conversely, hepatic tumour formation due to sustained activation of PPARα, for instance, is an established MoA that is unlikely to operate in humans (Klaunig et al., 2003). ECHAs guidance on the application of the

[26] as reported by NTP (2007) for inhalation studies

CLP criteria states that *"liver tumours in rodents conclusively linked to peroxisome proliferation"* is considered a mechanism of tumour formation not relevant for humans (ECHA, 2017b). Other nuclear hormone receptor-mediated mechanisms involving the activation of the aryl hydrocarbon receptor (AhR) and the constitutive androstane receptor (CAR) followed by transcriptional changes leading to suppression of apoptosis and hepatocellular proliferation were also considered less likely to contribute to human liver carcinogenicity (Felter et al., 2018; Köhle et al., 2008). A CAR-related MoA was suggested for the induction of liver carcinogenicity related to ethylbenzene exposure in female mice based on a comparative analysis of the underlying key events (Sweeney et al., 2015). Based on data obtained with phenobarbital, the following key events for the formation of liver tumours involving CAR have been postulated: (1) activation of CAR, (2) altered gene expression specific to CAR activation, (3) increased cell proliferation, (4) clonal expansion leading to altered hepatic foci, and (5) liver adenoma/carcinoma in addition to associative events such as altered epigenetic changes specific to CAR activation, CYP2B induction, liver hypertrophy, and inhibition of apoptosis (Elcombe et al., 2014; Felter et al., 2018).

The following nonneoplastic effects have been observed in subacute, subchronic, and chronic studies in mice following exposure to 2-phenylpropene via inhalation (cf. Table 26).

- increased absolute **liver weight** in ♀ at ≥ 600 ppm with statistical significance and in ♂ at 1000 ppm without statistical significance (90-day NTP study)
- increased relative **liver weight** in ♂ at 600 ppm after five days of exposure and at ≥ 800 ppm after one, five, and twelve days with statistical significance; in ♀ at ≥ 600 ppm after one, five, and twelve days with statistical significance (except for ♀ at 600 ppm after short-term, one day, treatment) (sub-study 2, Morgan et al. (1999))
- minimal to mild centrilobular **hypertrophy** in the liver of ♂ and ♀ at ≥ 600 ppm which contributed to the increased liver weight (90-day NTP study)
- **decreased hepatic glutathione** in ♀ at ≥ 600 ppm after one or five days and in ♂ at ≥ 600 ppm after one day of exposure and ≥ 800 ppm after five days of exposure with statistical significance (the depletion was concentration-depended after day five in both sexes) (sub-study 2, Morgan et al. (1999))
- increased incidence of **eosinophilic foci** in ♀ mice at 600 ppm with statistical significance (2-year NTP study)

None of these non-neoplastic effects is obviously indicative of clear evidence of cytotoxicity such as necrosis or apoptosis. Data demonstration a direct activation of nuclear hormone receptors such as PPARα or CAR through 2-phenylpropene are not available. However, some of the effects mentioned above are consistent with a mechanism involving CAR activation, including liver hypertrophy/increased liver weight (associative event) and increased incidence of eosinophilic foci (key event [4]). The latter, however, was only observed in female mice.

In conclusion, hepatocarcinogenic lesions have been seen in female mice and to a lesser extent in males of the same species. The background rates of these tumours are high in B6C3F1 mice (especially in males). Non-neoplastic effects could partly be linked to associated/key events related to CAR activation. Yet, they are far from being fully consistent and conclusive. While, for instance, the associative event liver hypertrophy was visible in male and female mice, treatment-related eosinophilic foci (key event [4]) were only seen in females. Thus, in the absence of adequate evidence related to the underlying MoA, a species-specific MoA cannot be assumed with confidence. Consequently, no *"strong evidence that the mechanism of tumour formation is not relevant for humans"* (cf. CLP Regulation, section 3.6.1.1.) is available. Therefore, the neoplastic findings cannot be disregarded and the relevance for humans cannot be excluded. A similar conclusion had been made by the authors of the CLH dossier for the structural analogue cumene: "*In summary, relevance for humans of the observed liver tumours in mice from cumene exposure may be low or not existing. However, the MoA is still largely unknown and has been insufficiently examined. Therefore, these data support the conclusion that cumene's induction of liver tumours in female mice is uncertain with respect to the relevance for humans."* (CLH dossier 601-024-00.-X[27])

[27] https://echa.europa.eu/documents/10162/10e6c6a2-7321-a5e4-3ac3-ed5439c7c4a1

Table 23: Summary of factors to be taken into consideration in the hazard assessment

Species and strain	**Tumour type and background incidence** (cf. 10.9.2 [a])	**Progression of lesions to malignancy** (cf. 10.9.2 [c])	**Reduced tumour latency** (cf. **10.9.2** [d])	**Responses in single or both sexes** (cf. 10.9.2 [e])	**Confounding effect by excessive toxicity?** (cf. 10.9.2 [j])	**Route of exposure** (cf. 10.9.2 [h])	**MoA and relevance to humans** (cf. 10.9.2 [k])
Rats F344/N	**Kidney -** renal tubule adenoma and carcinoma (combined) Background incidence: 1 % ± 1.1 % Study incidences: Ctrl: 2 % 100 ppm: 4 % 300 ppm: 6 % 1000 ppm: 14 %	Yes	Yes (cf. 10.9.2 [d]) First incidence (ppm/days): 0/729 100/723 300/716 1000/653	♂	Not identified	Inhalation (comparable to human exposure scenarios)	MoA possibly species-specific (α2u-globulin nephropathy) but not fully consistent Relevance to humans: **assumed** for a lack of a plausible MoA
	MNCL Background incidence: 47.1 % ± 10.3 % Study incidences: Ctrl: 52 % 100 ppm: 64 % 300 ppm: 58 % 1000 ppm: 76 %	Yes	Inconclusive First incidence (ppm/days): 0/495 100/562 300/558 1000/401	♂	Not identified	Inhalation (comparable to human exposure scenarios)	MoA largely unknown (highly spontaneous in F344 rats) Relevance to humans: **uncertain** but not fully excludable (insufficient for classification)
	Testis - interstitial cell adenoma Background incidence: 79.2 % ± 3.9 % Study incidences: Ctrl: 66 % 100 ppm: 88 % 300 ppm: 82 % 1000 ppm: 88 %	No	Inconclusive First incidence (ppm/days): 0/548 100/562 300/560 1000/519	♂	Not identified	Inhalation (comparable to human exposure scenarios)	Incidental finding Relevance to humans: **unlikely** (insufficient for classification)
Mice B6C3F1	**Liver -** hepatocellular adenoma or carcinoma (combined)	Yes	Inconclusive First incidence (ppm/days): ♂	♂/♀	Not identified	Inhalation (comparable to human exposure scenarios)	MoA unknown (highly spontaneous) Relevance to

Species and strain	**Tumour type and background incidence** (cf. 10.9.2 [a])	**Progression of lesions to malignancy** (cf. 10.9.2 [c])	**Reduced tumour latency** (cf. **10.9.2** [d])	**Responses in single or both sexes** (cf. 10.9.2 [e])	**Confounding effect by excessive toxicity?** (cf. 10.9.2 [j])	**Route of exposure** (cf. 10.9.2 [h])	**MoA and relevance to humans** (cf. 10.9.2 [k])
	<u>Background incidence ♂:</u> 56 % ± 6.2 % <u>Study incidences ♂:</u> Ctrl: 56 % 100 ppm: 72 % 300 ppm: 66 % 600 ppm: 74 % <u>Background incidence ♀:</u> 31.1 % ± 6.8 % <u>Study incidences ♀:</u> Ctrl: 26 % 100 ppm: 52 % 300 ppm: 48 % 600 ppm: 66 %		0/486 100/453 300/383 1000/429 ♀ 0/634 100/537 300/416 1000/464				<u>humans:</u> **assumed** for a lack of a plausible MoA
	Liver - hepatocellular adenoma <u>Background incidence:</u> 22.5 % ± 8.1 % <u>Study incidences:</u> Ctrl: 20 % 100 ppm: 40 % 300 ppm: 42 % 600 ppm: 46 %	No	Inconclusive First incidence (ppm/days): 0/725 100/640 300/731 1000/464	♀			
	Liver - hepatocellular carcinoma <u>Background incidence:</u> 10.7 % ± 1.8 % <u>Study incidences:</u> Ctrl: 6 % 100 ppm: 18 % 300 ppm: 12 % 600 ppm: 36 %	Yes	Inconclusive First incidence (ppm/days): 0/634 100/537 300/416 1000/612	♀			

[#] historical control incidence vs. treatment group with statistically significantly increased incidence

Conclusion on the Comparison with the CLP criteria

In the absence of human data, 2-phenylpropene has been shown to increase the incidence of several tumour types in a well-conducted and highly reliable study using experimental animals. In rats, elevated incidences of renal tubule adenoma and carcinoma (combined), MNCL, interstitial cell adenoma in the testis were found exclusively in males. As the latter tumourigenic finding is believed to be incidental and the predictive value of high-concentration chemical-induced MNCL in F344 rats for human carcinogenicity is highly questionable, both tumour types are considered insufficient for classification. In contrast, the increased incidence of renal neoplasms has to be taken into account for the purpose of classification as a species-specific MoA cannot be conclusively established. Consequently, strong evidence contradicting the relevance for humans does not exist. In mice, hepatocellular adenoma and carcinoma were seen at higher rates in female and to a lesser extent in male mice. Although such tumours are commonly seen in B6C3F1 mice, the observed incidences were outside the range of the historical control data, providing reliable evidence of treatment-related carcinogenicity. With respect to the presumed MoA, the available evidence cannot be considered strong to exclude human relevance. 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.

Hence, consistent with the strength of evidence criteria, sufficient evidence of carcinogenicity may exist. A causal relationship between the agent and a treatment-related increased incidence of an appropriate combination of benign and malignant neoplasms in multiple sites in two species has been established with statistical significance. To assess the overall level of concern, a summary of factors that have been considered are listed below in Table 24. While a number of factors are in support of increased concern, considerations particularly related to the MoA and its relevance for humans have uncovered significant uncertainties. For both tumour types (kidney in male rats and liver in mice), some indices for a species-specific MoA have been unveiled. However, the evidence is insufficient to conclusively establish rodent-specificity. Therefore, on the basis of these data, relevance for human needs to be assumed. Following the criteria, substance-related increased tumour rates in two species may justify a Category 1B classification. On account of the uncertainties identified, the evidence of 2-phenylpropene-related carcinogenicity appears to be limited rather than sufficient. Downgrading the proposed classification from Category 1B (known or presumed human carcinogen) to Category 2 (suspected human carcinogen) appears appropriate. The proposed classification is in agreement with IARCs evaluation wherein 2-phenylpropene had been categorised as possibly carcinogenic to humans (group 2B) (IARC, 2012a).

Table 24: Assessment of the overall level of concern for the purpose of classification

Factor	Conclusion
Tumour type and background incidence	Sufficient evidence in animals: Category 1B Tumour type with increased incidence observed in a susceptible species (kidney/male rat; liver/mice): decreased concern
Multi-site responses	Increases concern
Progression of lesions to malignancy	Increases concern
Reduced tumour latency	Neither increases nor decreases the concern
Whether responses are in single sex or both	Neither increases nor decreases the concern
Whether responses are in a single species or several	Increased concern
Structural similarity to a substance(s) for which there is good evidence of carcinogenicity	Increased concern

Factor	Conclusion
Routes of exposure	Neither increases nor decreases the concern
Comparison of ADME between test animals and humans	Neither increases nor decreases the concern
The possibility of a confounding effect of excessive toxicity at test doses	No confounding effect identified
Mode of action and its relevance for humans	Significantly decreases the concern: downgrade to Category 2

10.9.3 Conclusion on classification and labelling for carcinogenicity

Based on experimental animal studies, classification of 2-phenylpropene in Category 2 is recommended.

Setting of specific concentration limit for carcinogenicity

Article 10.1 of the CLP regulation allows the use of specific concentration limits (SCL) based on the potency of carcinogens. The EU has adopted the T25[28] concept for carcinogenicity to assist in establishing SCLs for carcinogens.

The SCL considerations in this section are based on the respective EC guidance document *"Guidelines for setting specific concentration limits for carcinogens"* (European Commission, 1999) and the T25 concept as described in Dybing et al. (1997). The calculated T25 value is shown in Table 25.

Table 25: T25 values derived from the key carcinogenicity study

Study	Methodological details	Calculation of T25	Resulting T25
Carcinogenicity study in male and female rats and mice by NTP (2007)	Route: inhalation Duration: 105 weeks Observation period: no Start of treatment: at 6 weeks of age Inhalation: 5 days/week	<u>Lowest concentration with statistically significantly increased tumour incidence:</u> (1) hepatocellular adenoma or carcinoma (combined) in ♀ mice at 100 ppm - ctrl: 13/50 (26 %) - 100 ppm: 26/50 (52 %) - Net %: **35.1 %**[29] (2) hepatocellular adenoma in ♀ mice at 100 ppm - ctrl: 10/50 (20 %)	*Mouse:* **(1) 59.3 mg/kg bw/d** **(2) 83.4 mg/kg bw/d** **Carcinogens of medium potency** (1 mg/kg bw/d < T25 value ≤ 100 mg/kg bw/d) *Rat:* **(3) 692.7 mg/kg bw/d** **Carcinogens of low potency**

[28] The daily dose (in mg/kg bw/d) inducing a tumour incidence of 25 % upon lifetime exposure (ECHA, 2017b)

[29] (26 x (100 / 50) - 13 x (100 / 50) / (100 - 13 x (100 / 50)) = 0,35 x 100 = 35.1 % (calculated according to Dybing et al.1997)

Study	Methodological details	Calculation of T25	Resulting T25
		- 100 ppm: 20/50 (40 %) - Net %: **25 %** (3) renal tubule adenoma or carcinoma (combined) in ♂ rats at 1000 ppm - ctrl: 1/50 (2 %) - 1000 ppm: 7/50 (14 %) - Net %: **12.2 %** <u>Daily dose per mouse/rat during the exposure period:</u> 100 ppm = 3.7 mg/mouse/day[30] 1000 ppm = 124.3 mg/rat/day <u>Daily dose per kg body weight during the exposure period:</u> Mouse (100 ppm): 83.4 mg/kg bw/day[31] Rat (1000 ppm): 339.3 mg/kg bw/day <u>T25 after 24 months:</u> *Mouse:* (1) 59.3 mg/kg bw/d[32] (2) 83.4 mg/kg bw/d *Rat:* (3) 692.7 mg/kg bw/d	(T25 value > 100 mg/kg bw/d)

[30] 6 x 1.8 x 100 x 118.18 / 24.45 x 1 / 1000 x 5 / 7 = 3.7 (calculated according to Dybing et al.1997)

[31] 1000 / 44.7 (mean weight) x 3.7 = 83.4 (weight: mean of reported weights [30.2, 49.5, 54.5]) (calculated according to Dybing et al.1997)

[32] 25/35 x 83.4= 59.3 (calculated according to Dybing et al.1997)

According to the lowest calculated T25 values which are based on the carcinogenic effects in female mice, 2-phenylpropene falls into the medium potency category. Additional considerations in arriving at a potency category are as followed:

- *Dose-response relationship:* high frequency already at the lowest dose tested
- *Site/species/strain/gender activity:* increased incidences observed at multiple tissue sites (liver and kidney) and in two species (♂/♀ mice and ♂ rats); the effects in male rats are in support of the low potency category
- *Mechanism including genotoxicity:* genotoxicity cannot be fully ruled out; the data is, however, insufficient for classification
- *Mechanistic relevance to humans:* data do not conclusively support a species-specific mechanism; human relevance is therefore assumed, albeit with uncertainties
- *Toxicokinetics:* assumed to be similar in animals and humans
- *Other elements*: structural similarity to substances for which there is good evidence of carcinogenicity (cf. 10.9.2 [g])
    - *cumene* – C&L: harmonised classification into carcinogenicity Category 2 proposed; IARC: possibly carcinogenic to humans (Group 2B)
    - *styrene* – IARC: probably carcinogenic to humans (Group 2A)
    - *ethylbenzene* – IARC: possibly carcinogenic to humans (Group 2B)

According to the EC guidance document, *"Category 3*[33] *carcinogens that are assigned to the medium potency range, will have a general concentration limit of 1%"* (European Commission, 1999). The calculated T25 value for 2-phenylpropene is not close to upper or lower potency borders and is, therefore, clearly indicative of medium potency. The abovementioned considerations do not substantiate any deviation from this potency glass. Consequently, a GCL of 1 % is recommended.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

Two carcinogenicity studies via inhalation are available, one in rats and one in mice (NTP, 2007). The DS proposed classification in Category 2 based on kidney tumours in male rats and liver tumours in female and male mice. According to the DS, the evidence is not sufficient for a 1B classification because the kidney tumours in male rats appear to be partly related to α2μ-globulin accumulation (a species-specific phenomenon), B6C3F1 mice have a high background incidence of liver tumours, there is no strong evidence of genotoxicity, and the pattern of liver effects (increased weight, centrilobular hypertrophy, eosinophilic foci, no cytotoxicity) suggests CAR activation. The DS did not propose a specific concentration limit as the T25 values correspond to medium or low potency.

[33] Category 3: *"Substances which cause concern for man owing to possible carcinogenic effects but in respect of which the available information is not adequate for making a satisfactory assessment. There is some evidence from appropriate animal studies, but this is insufficient to place the substance in Category 2."* (European Commission, 1999)

Comments received during consultation

Comments were received from 2 MSCAs and 1 industry organisation.

While industry supported the DS's proposal of Category 2, the commenting MSCAs preferred Category 1B, putting forward the following arguments:

- Clear carcinogenic effects are present in both sexes, in two species and also multi-site responses
- There is no strong evidence for a rodent-specific mode of action (MoA) of the liver tumours observed in mice, therefore human relevance has to be assumed.
- The increase in liver tumours in male mice, although less pronounced than in females, is significant and has to be considered in the classification.
- Increases in mononuclear cell leukemia and testicular tumours / adenomas above HCD observed in male rats are treatment-related and relevant for classification.
- Neoplastic effects in the liver and kidney may be related to local formation of a reactive metabolite.
- Structurally related substances are carcinogenic: cumene has a harmonized classification as Carc. 1B, styrene has been classified by IARC in group 2A, which is considered equivalent to Carc. 1B.

In response to these comments the DS pointed out the following:

- The increase in interstitial cell adenomas was considered unrelated to treatment by the study authors because the incidences only slightly exceeded the historical control range, there was no clear concentration dependency, the concurrent control was below the historical control range and testicular adenomas are very common in aged F344 rats.
- Human relevance of mononuclear cell leukemia has been questioned due to spontaneous occurrence in aged F344 rats with variable and high incidence, species-specific characteristics, mechanistic considerations and reproducibility issues.
- As to the liver tumours in male mice, statistically significant effects were only seen when incidences of adenomas and carcinomas were combined, there was no clear concentration dependence and the incidences only slightly exceeded the historical control range.
- In contrast to cumene, lung tumours in mice and nasal tumours in rats have not been observed with α-methylstyrene.

Assessment and comparison with the classification criteria

2-year carcinogenicity studies in rats and mice (NTP, 2007): general information

F344 rats (50/sex/group) were exposed to α-methylstyrene vapours (whole body, 6 h/day, 5 days/week) at 0, 100, 300 and 1000 ppm. The top concentration selection was based on the 3-month study where some but not excessive toxicity was observed at 1000 ppm. Generation of concentrations above 1000 ppm was reported to result in production of unwanted aerosols (Morgan *et al.*, 1999). General toxicity in the 2-year rat study included a mild body weight reduction (<10%) and nasal lesions such as olfactory epithelium degeneration of minimal severity. Survival was not affected. The following tumour types showed a statistically significant increase compared to concurrent control in males: renal

tubular adenoma and carcinoma (combined), mononuclear cell leukaemia, and testicular adenoma. No neoplastic effects were detected in females.

B6C3F1 mice (50/sex/group) were exposed to α-methylstyrene vapours at 0, 100, 300 and 600 ppm. The top concentration selection was based on the 3-month study where excessive toxicity including mortality occurred at 1000 ppm. General toxicity at the top concentration in the 2-year study included body weight reduction (by ca. 10-15%) and nasal lesions (olfactory epithelium metaplasia and atrophy). Survival was not affected. Increased incidence of hepatocellular adenomas and carcinomas was observed in females, a marginal increase in carcinomas also occurred in males.

The NTP report contains historical control data comprising inhalation studies beginning within six years before the current studies (1995-2000, the 2-year studies with α-methylstyrene started in July/August 2001). To make the best use of the available information, RAC compiled historical control data from the NTP online database (NTP, 2022b), including also studies conducted after the current study. The usual rules for HCD have been followed (±5 years of the current study, the same strain, source of animals, laboratory, study duration, exposure route and diet). Further details can be found under 'supplemental information'.

Kidney tumours in male rats

Neoplastic and non-neoplastic kidney findings in male and female rats from the 2-year inhalation study (NTP, 2007) are presented in the table below. Initially, a single hematoxylin and eosin-stained section of each kidney was prepared. Because of the neoplastic and non-neoplastic renal findings and a possible mode of action (MoA) via α2μ-globulin accumulation, additional kidney step sections (three to four from each kidney) were prepared from the remaining formalin-fixed tissues at 1 mm intervals for each male. This led to identification of additional males with focal hyperplasia or adenoma.

2-year inhalation study in rats: kidney histopathology					
Concentration (ppm)	**0**	**100**	**300**	**1000**	**HCD[a]**
Males					
No. of males examined	50	50	50	50	
Papilla, mineralization; incidence (mean severity)	12 (1.1)	16 (1.0)	10 (1.0)	33** (1.4)	
Nephropathy (mean severity)	41 (2.2)	46 (2.3)	46 (2.4)	45 (2.4)	
Renal tubule hyperplasia (single sections)	0	0	0	0	
Renal tubule adenoma (single sections)	0	0	1 (2%)	0	Mean±SD 0.8%±1.0% Range 0-2%
Renal tubule carcinoma (single sections)	0	0	1 (2%)	2 (4%)	Mean±SD 0.3%±0.8% Range 0-2%
Renal tubule adenoma or carcinoma (single sections)	0	0	2 (4%)	2 (4%)	Mean±SD 1.2%±1.3% Range 0-4%

Renal tubule hyperplasia (single and step sections)	1	0	1	4	
Renal tubule adenoma (single and step sections)	1	2	2	5	
Renal tubule carcinoma (single and step sections)	0	0	1	2	
Renal tubule adenoma or carcinoma (single and step sections)	1 (2%)	2 (4%)	3 (6%)	7* (14%)	
Renal tubule adenoma or carcinoma first incidence days	729	723	716	653	
Females					
No. of females examined	49	50	50	50	
Papilla, mineralization (mean severity)	1 (1.0)	6 (1.0)	8* (1.0)	7* (1.0)	
Pelvis, transitional epithelium, mineralization (mean severity)	31 (1.5)	26 (1.0)	31 (1.1)	16** (1.0)	
Nephropathy (mean severity)	34 (1.6)	27 (1.3)	35 (1.5)	31 (1.8)	

Statistically significant difference from control (Poly-3 test): *, $p \leq 0.05$; **, $p \leq 0.01$

Severity scores: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked

[a] 12 two-year studies via inhalation starting between 1997 and 2005 (the current study started in 2001); the same laboratory, source of animals and diet

There was a dose-dependent increase in renal tubule neoplasms in males, reaching statistical significance on pairwise comparison at 1000 ppm for adenomas and carcinomas combined (single and step sections). Historical control data is only available for standard (single section) histopathological evaluation. Kidney was a target organ of α-methylstyrene toxicity also in studies of shorter duration (Morgan *et al.*, 1999; 3-month study by NTP, 2007; reproductive toxicity screening, 1997; for details see 'supplemental information'). The increase in renal tubular tumours is considered treatment-related.

The authors of the NTP report (2007) discussed the kidney findings in studies with α-methylstyrene in relation to the criteria established by IARC (1999) and US EPA (1991) for male rat kidney carcinogenicity through an α2μ-globulin -associated response. This mode of action is considered not relevant for humans (see the Guidance on the application of the CLP criteria, section 3.9.2.5.3, and a review by RIVM (RIVM, 2002).

α2μ-Globulin is a protein synthesized in the liver of male rats. It is filtered through the renal glomerulus, reabsorbed in the tubules and degraded by lysosomes of tubular cells. The sequence of events leading to tumours via α2μ-globulin -associated nephropathy can be summarised as follows: A chemical or its metabolite binds reversibly to α2μ-globulin. The protein-chemical complex is resistant to breakdown, leading to accumulation of the complex in lysosomes and cell death. Dead cells are sloughed into the lumen and contribute to the development of granular casts. Cell death and degeneration causes a compensatory cell proliferation in the cortex. Sustained cell proliferation leads to promotion of spontaneously initiated cells and formation of renal adenoma and carcinoma.

IARC (1999) developed the following set of criteria for concluding that an agent causes kidney tumours through an α2μ-globulin -associated response:

1. Lack of genotoxic activity (agent and/or metabolite) based on an overall evaluation of *in vitro* and *in vivo* data
2. Male rat specificity for nephropathy and renal tumorigenicity
3. Induction of the characteristic sequence of histopathological changes in shorter-term studies, of which protein droplet accumulation is obligatory
4. Identification of the protein accumulating in tubule cells as α2μ-globulin
5. Reversible binding of the chemical or metabolite to α2μ-globulin
6. Induction of sustained increased cell proliferation in the renal cortex
7. Similarities in dose-response relationship of the tumour outcome with the histopathological endpoints (protein droplets, α2μ-globulin accumulation, cell proliferation)

The characteristic sequence of histopathological changes referred to in point 3 includes excessive accumulation of hyaline droplets, single-cell necrosis of the tubule epithelium, regenerative cell tubule proliferation, development of intraluminal granular casts from sloughed cell debris (associated with tubule dilation and papillary mineralization), foci of tubule hyperplasia in the convoluted proximal tubules and renal tubule tumours (US EPA, 1991).

Information on α-methylstyrene related to the individual criteria can be summarised as follows:

1. Genotoxicity: The available data do not meet the criteria for mutagenicity classification. α-Methylstyrene is metabolized via a DNA-reactive intermediate (α-methylstyrene oxide) but its contribution to *in vivo* carcinogenicity is unknown since detoxification mechanisms such as enzymatic hydrolysis are likely to considerably reduce its levels in tissues.
2. Male rat-specific effect: An increase in renal neoplasms was only observed in male rats. However, evidence of renal toxicity was also seen in female rats, namely increased kidney weight and increased urinary markers of kidney toxicity in the 3-month study (for details see 'supplemental information'). In addition, an increase in nephropathy was observed in female mice in the 2-year study (incidence 26/50 vs 16/50 in control, mean severity 1.6 vs 1.1).
3. Characteristic sequence of histopathological changes: The 3-month rat study reported a slight increase in hyaline droplet accumulation from 600 ppm. An increase in pelvic mineralization was observed in the 2-year study at 1000 ppm. On the other hand, the 2-year rat study did not show a significant increase in nephropathy or hyperplasia in male animals.
4. Identification of the protein as α2μ-globulin: The amount of α2μ-globulin was determined in kidney homogenates of male rats in the 3-month study. A significant increase in α2μ-globulin per μg of soluble protein was observed from 150 ppm.
5. Binding of the substance or metabolites to α2μ-globulin: not investigated
6. Increased cell proliferation: Increased proliferation (starting from 150 ppm) was detected in male rats of the 3-month study using a labelling technique.
7. Similarity in dose-response response for neoplastic and non-neoplastic findings: A statistically significant increase in renal tumours (adenomas and carcinomas combined) was observed at 1000 ppm. An increase in severity of hyaline droplets

started around 600 ppm, papillary mineralization was increased at 1000 ppm. There was no increase in the severity or incidence of nephropathy up to the top concentration.

In line with the authors of the NTP report (2007) and the DS, RAC concludes that α-methylstyrene meets some, but not all, the criteria for male rat kidney carcinogenicity through an α2μ-globulin -associated response. The uncertainties are related to lack of investigations (binding of the substance and metabolites to α2μ-globulin, cell proliferation in female rats), inconsistency of some observations with the MoA (e.g. no increase in nephropathy in male rats) or indications of other possible MoAs (evidence of renal toxicity in female rats). Human relevance of the renal tumours can therefore not be completely excluded. Nevertheless, the concern is reduced by partial involvement of α2μ-globulin -related MoA and absence of renal neoplasms in females.

Mononuclear cell leukaemia in male rats

F344/N rats used in NTP studies had a high and gradually increasing spontaneous incidence of mononuclear cell leukaemia. Nevertheless, the statistically significant increase (Poly-3 p = 0.016) in top concentration males exceeds the relevant HCD. No significant increase was observed in females.

The latency may be slightly reduced at the top concentration compared to concurrent control. Spontaneous cases in control F344 rats generally begin to appear after 70 weeks of age (Caldwell, 1999). In this study the first case in top concentration males was observed in week 64 of age compared to week 77 in the control.

Mononuclear cell leukaemia in F344 rats has been reviewed by RIVM (Scheepmaker *et al.*, 2005). They noted that other rat strains and other species including humans have a much lower incidence, increased incidence in F344 rats had in some cases not been reproduced in a second study in F344 rats of a comparable design (e.g. acetaminophen, butyl benzyl phthalate) and had never been confirmed in another rat strain or in mice. Human relevance of an increase in mononuclear cell leukaemia observed only in F344 rats is generally considered low, although unresolved questions remain (Thomas *et al.*, 2007) and case-by-case evaluation is needed.

2-year inhalation study in rats: mononuclear cell leukaemia					
Concentration (ppm)	**0**	**100**	**300**	**1000**	**HCD[a]**
Males					
No. of males examined	50	50	50	50	
Mononuclear cell leukaemia	26 (52%)	32 (64%)	29 (58%)	38* (76%)	Mean±SD 48%±9% Range 34-66%
First incidence (days)	495	562	558	401	
Females					
No. of females examined	50	50	50	50	
Mononuclear cell leukaemia	18 (36%)	21 (42%)	21 (42%)	22 (44%)	Mean±SD 33%±11% Range 20-52%

* Statistically significant difference from control (Poly-3 test), p ≤ 0.05

[a] 12 two-year studies via inhalation starting between 1997 and 2005 (the current study started in 2001); the same laboratory, source of animals and diet

Testicular tumours in rats

F344 rats used in NTP studies had a high and variable incidence of interstitial cell adenomas. The incidences at 100 and 1000 ppm in the current study were significantly different from concurrent control but there was no dose-response relationship (the incidence at 1000 ppm is equal to that at 100 ppm). The historical control range was marginally exceeded. RAC agrees with the authors of the NTP report and with the DS that the increase in testicular adenomas may be incidental.

2-year inhalation study in rats: testicular tumours					
Concentration (ppm)	**0**	**100**	**300**	**1000**	**HCD[a]**
No. of males examined	50	50	50	50	
Testes, adenoma	33 (66%)	44* (88%)	41 (82%)	44* (88%)	Mean±SD 74%±8% Range 58-84%

* Statistically significant difference from control (Poly-3 test), $p \leq 0.05$

[a] 12 two-year studies via inhalation starting between 1997 and 2005 (the current study started in 2001); the same laboratory, source of animals and diet

Liver tumours in male and female mice

B6C3F1 mice, particularly males, have a high spontaneous incidence of hepatocellular tumours. They are also particularly sensitive to induction of liver tumours by chemicals compared to some other strains of mice and to other species (see e.g. Scheepmaker *et al.*, 2005; Grisham, 1996).

Male mice in this study showed a significantly increased incidence of hepatocellular adenomas and carcinomas combined. It is noted that the top dose incidence remained close to the historical control mean.

Females showed statistically significant increases in hepatocellular adenomas, hepatocellular carcinomas, and adenomas and carcinomas combined. The incidence of carcinoma at the top concentration markedly exceeded the historical control range. There was a biologically plausible sequence of increased eosinophilic foci, adenoma and carcinoma. Part of the liver carcinomas metastasized to the lung (a common site of metastasis of liver tumours). No robust MoA information is available.

Short-term and subchronic inhalation studies with α-methylstyrene reported increased liver weight, hepatocellular hypertrophy and depletion of hepatic glutathione (see 'supplemental information'). Glutathione depletion indicates formation of a reactive metabolite in the liver. Whether this metabolite is responsible for the increase in tumours (e.g. via genotoxicity or cytotoxicity) is currently unknown. Hepatocellular hypertrophy may indicate a MoA via activation of nuclear receptors (e.g. CAR). However, no specific studies on activation of nuclear receptors or other MoAs are available. Thus, the mode of action (or the combination of MoAs) leading to liver tumours in α-methylstyrene-exposed mice remains unknown.

As to the structurally related substances, cumene caused a weak increase in hepatocellular ademonas in female mice but not in male mice or in rats (NTP, 2009). No liver tumours were found in mice exposed to styrene (Cruzan *et al.*, 2001) but the top concentration had to be relatively low (160 ppm) due to mortality and severe toxicity (including liver necrosis) at higher exposure levels.

In summary, the increase in liver tumours in female B6C3F1 mice is clearly related to treatment and the increase in males may also have been caused by the substance. On the other hand, the concern is reduced by the high background incidence and the high susceptibility of this strain.

Liver tumours in mice					
Concentration (ppm)	**0**	**100**	**300**	**600**	**HCD[a]**
Males					
No. of animals examined	50	50	50	50	
Hepatocellular adenoma	24 (48%)	27 (54%)	27 (54%)	25 (50%)	Mean±SD 51%±14% Range 30-74%
Hepatocellular carcinoma	10 (20%)	12 (24%)	11 (22%)	17 (34%)	Mean±SD 30%±11% Range 18-52%
Hepatocellular adenoma or carcinoma	28 (56%)	36* (72%)	33 (66%)	37* (74%)	Mean±SD 67%±13% Range 50-88%
Lung, metastatic hepatocellular carcinoma[b]	4 (8%)	6 (12%)	4 (8%)	7 (14%)	Mean±SD 11%±6% Range 0-20%
Hepatocellular carcinoma first incidence (days)	549	537	565	429	
Females					
No. of animals examined	50	50	50	50	
Eosinophilic focus	2	5	7	12**	
Hepatocellular adenoma	10 (20%)	20* (40%)	21** (42%)	23** (46%)	Mean±SD 29%±10% Range 12-50%
Hepatocellular carcinoma	3 (6%)	9 (18%)	6 (12%)	18** (36%)	Mean±SD 12%±4% Range 8-20%
Hepatocellular adenoma or carcinoma	13 (26%)	26** (52%)	24* (48%)	33** (66%)	Mean±SD 38%±11% Range 22-56%
Lung, metastatic hepatocellular carcinoma[b]	1 (2%)	5 (10%)	3 (6%)	13 (26%)	Mean±SD 6%±3% Range 0-10%
Hepatocellular carcinoma first incidence (days)	634	537	416	612	

Statistically significant difference from control (Poly-3 test): *, $p \leq 0.05$; **, $p \leq 0.01$

[a] 13 two-year studies via inhalation starting between 1997 and 2006 (the current study started in 2001), the same laboratory, source of animals and diet

[b] Statistical analysis not conducted

Comparison with criteria

According to the CLP criteria, sufficient evidence of carcinogenicity in experimental animals, corresponding to Category 1B, is available when a causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or (b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols. An increased incidence of tumours in both sexes of a single species in a well-conducted study can also provide sufficient evidence. A single study in one species and sex might be considered to provide sufficient evidence of carcinogenicity when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites.

In the case of α-methylstyrene there are three neoplastic findings:

- Hepatocellular adenomas and carcinomas in female and male mice
- Renal tubular adenomas and carcinomas in male rats
- Mononuclear cell leukaemia in male rats

Increases in malignant tumours have been observed in two species, so Category 1B has to be considered.

A single study in one species and sex might be considered to provide sufficient evidence of carcinogenicity when malignant neoplasms occur to an unusual degree. However, this is not the case for any of the three tumour types listed above. Further, the findings at multiple sites in one species and sex (here kidney tumours and leukaemia in male rat) cannot be regarded as 'strong'.

To aid in the weight of evidence assessment, the CLP Regulation further provides a list of factors increasing or decreasing the level of concern for human carcinogenicity (CLP, Annex I, 3.6.2.2.6). An overview of these factors together with relevant information on α-methylstyrene is provided in the following table.

Factors increasing or decreasing the level of concern for human carcinogenicity	
Factor	**Evidence for α-methylstyrene**
Tumour type and background incidence	All three tumour types occur in humans High background incidence of hepatocellular tumours in B6C3F1 mice and of mononuclear cell leukaemia in F344 rats
Multi-site responses	Yes, in male rats
Progression of lesions to malignancy	Yes
Reduced tumour latency	Slightly reduced latency of mononuclear cell leukaemia

Whether responses are in single or both sexes	Renal tumours and mononuclear cell leukaemia in a single sex (male rats) Hepatocellular tumours in mice: a marked increase in females, a weak increase in males
Whether responses are in a single species or several species	Response in two species Each tumour type in a single species
Structural similarity to a substance(s) for which there is a good evidence of carcinogenicity	Cumene classified as Carc. 1B, but besides similarities the carcinogenicity profiles of the substances show also significant differences (α-methylstyrene did not cause an increase in respiratory tract tumours)
Routes of exposure	Inhalation is a route relevant for humans
Comparison of ADME between test animals and humans	Information is limited but no indication of marked interspecies differences
The possibility of a confounding effect of excessive toxicity at test doses	No excessive toxicity
Mode of action and its relevance for humans	Renal tumours in male rats partly related to α2μ-globulin accumulation and increased cell proliferation MoA of liver tumours and mononuclear cell leukaemia unknown

To sum up, the main factors increasing the concern are:

- Increases in malignant tumours in two species
- Multi-site response in male rats

The main factors decreasing the concern are:

- High spontaneous incidence of hepatocellular tumours in B6C3F1 mice and high susceptibility of this strain to induction of liver tumours by chemicals
- High spontaneous incidence of mononuclear cell leukaemia in F344 rats, not seen in other strains and species, and poor reproducibility observed for some substances
- Involvement of α2μ-globulin accumulation in the development of renal tumours in male rats (although human relevance cannot be completely excluded)
- The fact that kidney tumours and mononuclear cell leukaemia were limited to a single sex a species

Taking into account all the factors increasing and decreasing the concern, RAC concludes that α-methylstyrene should be classified as **Carc. 2; H351** as proposed by the DS.

Supplemental information - In depth analyses by RAC

Historical control data

The NTP report on α -methylstyrene (2007) contains historical control data from 8 rat inhalation studies and 7 mouse inhalation studies conducted between 1995 and 2000. The

2-year studies with α-methylstyrene started in July/August 2001. RAC compiled a wider historical control database comprising all NTP 2-year inhalation studies in F344/N rats and B6C3F1 starting ±5 years from the current study (i.e. between August 1996 and July 2006) from the online NTP database (NTP, 2022b). The studies were conducted in the same facility (Battelle Northwest, Richland, WA), used animals from the same source (Taconic, Germantown, NY) and the same diet (NTP-2000). The refined HCD compiled by RAC for selected tumour types or their combinations is presented in the tables below.

Historical control data: mononuclear cell leukemia and interstitial cell tumours in male F344 rats

Substance	Start of study	Mononuclear cell leukemia (%)	Testicular adenoma (%)
Vanadium pentoxide	Jan 1997	44	72
Decalin	Aug 1997	38	80
Propylene glycol mono-t-butyl ether	Oct 1997	66	82
Stoddard solvent (type IIC)	Jan 1999	50	82
Divinylbenzene	Sep 1999	44	76
Methyl isobutyl ketone	May 2000	50	84
Cumene	Jun 2001	52	72
Propargyl alcohol	Oct 2001	43	78
Tetralin	Jun 2003	50	58
1-Bromopropane	Jul 2003	58	68
Diethylamine	Aug 2003	50	72
Vinylidene chloride	Jun 2005	34	64
Mean		*48*	*74*
SD		*9*	*8*
Minimum		*34*	*58*
Maximum		*66*	*84*

Historical control data: hepatocellular tumours in B6C3F1 mice

Substance	Start of study	Males				Females			
		A (%)	C (%)	A+C (%)	CML (%)	A (%)	C (%)	A+C (%)	CML (%)
Vanadium pentoxide	Jan 1997	30	28	52	0	12	12	24	4
Decalin	Aug 1997	44	20	56	6	14	8	22	4
Propylene glycol mono-t-butyl ether	Sep 1997	36	18	50	6	29	8	37	2

Stoddard solvent (type IIC)	Dec 1998	46	32	68	12	18	12	26	4
Divinylbenzene	Sep 1999	44	26	60	12	35	10	39	6
Methyl isobutyl ketone	Jun 2000	34	24	54	14	26	12	34	8
Cumene	Jun 2001	68	26	80	14	36	20	50	8
Propargyl alcohol	Sep 2001	43	20	53	6	30	8	34	6
Tetralin	Jun 2003	67	22	76	8	28	14	40	6
1-Bromopropane	Jul 2003	60	30	76	14	38	10	48	0
Diethylamine	Aug 2003	56	40	80	16	28	8	32	4
Vinylidene chloride	Jun 2005	74	52	88	20	50	16	56	10
Cobalt	May 2006	56	50	76	18	38	20	50	10
Mean		*51*	*30*	*67*	*11*	*29*	*12*	*38*	*6*
SD		*14*	*11*	*13*	*6*	*10*	*4*	*11*	*3*
Minimum		*30*	*18*	*50*	*0*	*12*	*8*	*22*	*0*
Maximum		*74*	*52*	*88*	*20*	*50*	*20*	*56*	*10*

A = hepatocellular adenoma; C = hepatocellular carcinoma; A+C = hepatocellular adenoma or carcinoma; CML = lung, metastatic hepatocellular carcinoma (incidence of CML obtained from the individual NTP reports)

Kidney-related findings in short-term and subchronic studies

Short-term inhalation studies in rats and mice (Morgan *et al.*, 1999)

Morgan *et al.* (1999) investigated short-term inhalation toxicity (3, 5 or 12 exposures, 6 h/day) of α-methylstyrene in F344 rats and B6C3F1 mice. The top concentration was set at 1000 ppm because generation of higher concentrations resulted in the production of unwanted aerosols. No changes were observed in the kidneys of mice. The rat findings from Study 2 (12-day study in F344 rats) are summarised in the table below.

12-day inhalation study in rats (Morgan *et al.*, 1999, Study 2): kidney-related parameters

	Males			**Females**		
Concentration (ppm)	**0**	**600**	**1000**	**0**	**600**	**1000**
No. of animals	5	5	5	5	5	5
Kidney weight, relative (% bw)	0.44	0.48*	0.46	0.46	0.52	0.50
Hyaline droplet; incidence (mean severity)	5 (1.0)	5 (3.0)	5 (3.0)	0	0	0
Chronic nephropathy (mean severity)	0	1 (1.0)	1 (1.0)	0	0	1 (1.0)

Mineralization (mean severity)	0	0	0	5 (1.0)	5 (1.2)	5 (1.2)

* Statistically significant difference from control, p ≤ 0.05

The only effect in rats in Study 2 was increased severity of hyaline droplets in males. This effect was further investigated in a follow-up study, Study 3. Study 3 employed, besides male and female F344 rats, also male NCI Black-Reiter (NBR) rats. NBR is a strain deficient in α2μ-globulin. The animals were exposed to 0, 125, 250 or 500 ppm for 9 days (6 h/day). Induction of hyaline-droplet accumulation was confirmed in male F344 rats at ≥250 ppm. Hyaline droplet nephropathy or other effects were not present in female F344 or male NBR rats (data not shown in the publication).

3-month inhalation studies in rats and mice (NTP, 2007)

The following renal pathology investigations were conducted in rats: Formalin-fixed and processed left kidney from male and female rats was cut into three sections. The first section was stained with hematoxylin and eosin for histopathology (males and females), the second section was stained with Mallory-Heidenhain for evaluation of hyaline droplets (males and females). The third section was stained with proliferating cell nuclear antigen (PCNA) complexed with avidin and biotin for determination of cell proliferation (males). For male rats, the slides stained with PCNA were evaluated to determine the labelling index of cells in the S-phase in the proximal tubules; at least 2000 cells per dose group were counted.

Frozen right kidneys from male rats were evaluated for α2μ-globulin and soluble protein. The kidneys were homogenized and centrifuged. The protein content of each supernatant was measured in a 1:50 dilution. Analysis of α2μ-globulin in supernatants was conducted using ELISA.

Urine collection was conducted in week 12. Besides standard urinary parameters, activities of several enzymes whose elevation may indicate nephrotoxicity were determined.

The results of the kidney-related investigations in rats are summarised in the tables below.

3-month inhalation study in rats (NTP, 2007): kidney-related parameters in males						
Concentration (ppm)	**0**	**75**	**150**	**300**	**600**	**1000**
No. of males	10	10	10	10	10	10
Necropsy body weight (g)	330	338	334	329	327	313
Right kidney weight, absolute (g)	1.00	1.07	1.06	1.07	1.09	1.10*
Right kidney weight, relative (‰)	3.04	3.17*	3.19**	3.25**	3.32**	3.53**
Hyaline droplet accumulation in renal tubules; incidence (mean severity)	9 (1.1)	10 (1.2)	10 (1.3)	10 (1.1)	10 (1.8)	10 (1.7)
Renal tubule regeneration (mean severity)	8 (1.0)	4 (1.3)	9 (1.0)	5 (1.0)	9 (1.2)	8 (1.1)
Labelling index[a] (%)	2.3	3.0	3.1*	3.4**	3.1**	3.9**

Soluble protein (mg/ml)	23	28	39**	41**	38**	42**
α2μ-globulin (nmol/g kidney)	195	349*	497**	689**	724**	749**
α2μ-globulin (ng/μg soluble protein)	81	115	119*	161**	176**	167**
Clinical chemistry: urea nitrogen (mg/dl), day 23	9.1	9.3	10.0	9.7	11.1	14.6*
Clinical chemistry: creatinine (mg/dl), day 23	0.84	0.85	0.84	0.84	0.84	0.86
Clinical chemistry: urea nitrogen (mg/dl), week 14	13.7	14.4	15.3*	14.6	13.8	14.2
Clinical chemistry: creatinine (mg/dl), week 14	0.97	0.98	0.97	0.98	0.96	0.96
Urine volume (ml/16 h)	12	23	18	19	25**	25**
Urine specific gravity	1.018	1.011	1.015	1.014	1.013	1.015
Urinanalysis: creatinine (mg/dl)	74	41	59	48	35**	34**
Urinanalysis: protein/creatinine ratio	0.65	0.68	0.72	0.77*	0.94**	1.17**
Urinanalysis: ALP/creatinine ratio	3.1	3.0	3.2	3.7*	3.8*	3.9*
Urinanalysis: AST/creatinine ratio	0.09	0.08	0.12	0.26**	0.39**	0.48**
Urinanalysis: LDH/creatinine ratio	0.54	0.60*	0.70**	0.95**	1.38**	1.59**
Urinanalysis: GGT/creatinine ratio	19.1	21.6	20.1	21.7	20.6	22.1
Urinanalysis: NAG/creatinine ratio	0.16	0.16	0.17	0.21**	0.22**	0.26**

Statistically significant difference from control: *, $p \leq 0.05$; **, $p \leq 0.01$

Severity scores: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked

[a] no. of labelled cells / total no. of counted cells x 100; indicator of cell proliferation

ALP = alkaline phosphatase; AST = aspartate aminotransferase; LDH = lactate dehydrogenase; GGT = γ-glutamyl transferase; NAG = N-acetyl-β-D-glucosaminidase

3-month inhalation study in rats (NTP, 2007): kidney-related parameters in females						
Concentration (ppm)	**0**	**75**	**150**	**300**	**600**	**1000**
No. of females	10	10	10	10	10	10
Necropsy body weight (g)	201	203	203	198	202	192
Right kidney weight, absolute (g)	0.67	0.67	0.68	0.67	0.71*	0.72*
Right kidney weight, relative (‰)	3.31	3.32	3.36	3.41	3.53**	3.73**

Renal tubule regeneration (mean severity)	0	0	3 (1.0)	3 (1.0)	2 (1.0)	1 (1.0)
Clinical chemistry: urea nitrogen (mg/dl), day 23	12.0	11.6	11.7	11.6	10.3	11.7
Urine volume (ml/16 h)	13	11	12	16	17	11
Urine specific gravity	1.011	1.014	1.013	1.011	1.012	1.020**
Urinanalysis: creatinine (mg/dl)	38	48	42	31	30	42
Urinanalysis: protein/creatinine ratio	0.08	0.09	0.09	0.10	0.12**	0.10*
Urinanalysis: ALP/creatinine ratio	2.1	2.0	2.0	2.3	2.9**	3.2**
Urinanalysis: AST/creatinine ratio	0.01	0.03	0.01	0.02	0.02	0.02
Urinanalysis: LDH/creatinine ratio	0.31	0.34	0.38*	0.46**	0.60**	0.69**
Urinanalysis: GGT/creatinine ratio	8.2	7.7	10.3	9.7	13.6**	13.9**
Urinanalysis: NAG/creatinine ratio	0.12	0.14	0.14	0.13	0.15*	0.16**

No kidney findings (organ weight, standard histopathology) were observed in mice up to the top concentration of 1000 ppm.

Reproductive toxicity screening in rats according to OECD TG 422 via oral route (1997)

The following information related to renal toxicity is available in the summary on the ECHA dissemination website:

Effects in males:

- 1000 mg/kg bw/d: increased urea nitrogen, enlarged kidney, increased kidney weight (absolute and relative), increased hyaline droplets and basophilic change of the renal tubular epithelium; micro-granular calculi in the urinary bladder, hyperplasia of the mucosal epithelium of the urinary bladder; 1 mortality due to ischuria with urinary calculi
- 200 mg/kg bw/d: increased hyaline droplets and basophilic change of the renal tubular epithelium

Effects in females:

- 1000 mg/kg bw/d: enlarged kidney, increased kidney weight (absolute and relative)
- 200 mg/kg bw/d: increased kidney weight (relative), vacuolation of the renal tubular epithelium (vacuoles identified as lipid droplets)

Liver-related findings in short-term and subchronic studies

Short-term inhalation studies in rats and mice (Morgan *et al.*, 1999)

In the main study (Study 2), B6C3F1 mice (18/sex/concentration) were exposed to 0, 600, 800 or 1000 ppm α-methylstyrene for 6 h/day, 5 days/week for up to 12 exposures. An

extra 6 mice/sex were added to the 1000 ppm chamber to ensure there would be enough survivors for tissue collection. Six mice/sex/concentration were killed after 1 exposure, and the liver was weighed and analysed for glutathione concentration. After 5 and 12 exposures, six mice/sex/concentration were used for clinical chemistry (including ALT and SDH) and histopathology, and the day 5 group also for glutathione analysis.

High mortality occurred in female mice. In addition, all treated male and female mice appeared sedated after the first exposure; the effect disappeared by the second week. Relative liver weights were significantly increased in most treated groups without a histopathological correlate and without a change in clinical chemistry. Liver glutathione was significantly decreased at all concentrations. No treatment-related effects were observed at 500 ppm in a previous study of a similar design (Study 1).

Morgan *et al.* 1999, Study 2: liver-related parameters in mice

	Males				**Females**			
Concentration (ppm)	**0**	**600**	**800**	**1000**	**0**	**600**	**800**	**1000**
No. of animals	18	18	18	24	18	18	18	24
Mortality	0	0	0	0	0	1	10	5
Liver weight, relative (% bw), after 1 exposure; (no. of animals examined)	5.50 (6)	6.04 (6)	6.21* (6)	6.24* (6)	5.47 (6)	5.88 (6)	6.36* (6)	6.23* (6)
Body weight (g), after 5 exposures	24.6 (6)	22.4* (6)	22.8* (6)	22.3* (6)	20.4 (6)	19.4 (6)	n.d.	19.4 (6)
Liver weight, relative (% bw), after 5 exposures	5.00 (6)	5.84* (6)	6.09* (6)	6.43* (6)	5.19 (6)	5.95* (5)	n.d.	6.18* (6)
Body weight (g), after 12 exposures	28.1 (6)	24.6* (6)	25.2* (6)	25.1* (12)	23.3 (6)	21.7 (6)	22.3 (2)	21.5 (7)
Liver weight, relative (% bw), after 12 exposures	5.74 (6)	6.19 (6)	6.68* (6)	6.55* (12)	5.77 (6)	6.69* (6)	7.47* (2)	6.94* (7)
Hepatic glutathione (µg/g), after 1 exposure	1427 (6)	825* (6)	1066* (6)	868* (6)	1348 (6)	627* (6)	900* (6)	758* (6)
Hepatic glutathione (µg/g), after 5 exposures	1681 (6)	1499 (6)	1263* (6)	1009* (6)	1821 (6)	1507* (5)	n.d.	918* (6)

* Statistically significant difference from control, $p \leq 0.05$; n.d. = not determined

F344 rats (5/sex/concentration) were exposed to α-methylstyrene at 0, 600 or 1000 ppm for 6 h/day, 5 days/week for up to 12 exposures. The investigations included clinical chemistry, organ weight and histopathology (including the liver). Liver glutathione levels were not investigated in rats. Similar to mice, there was an increase in liver weight without a histopathological correlate or a change in clinical chemistry.

3-month inhalation studies in rats and mice (NTP, 2007)

In the 3-month rat study a significant increase in liver weight was observed in both sexes (absolute by 32%/22% m/f, relative by 39%/27 at 1000 ppm) without a histopathological correlate or a change in clinical chemistry.

In the 3-month mouse study a statistically significant increase in absolute liver weight was observed in females and increased incidence of hypertrophy (minimal to mild) was seen in both sexes from 600 ppm, see the table below. Clinical chemistry parameters were unaltered.

3-month inhalation study in mice (NTP, 2007): liver-related parameters						
Concentration (ppm)	**0**	**75**	**150**	**300**	**600**	**1000**
Males						
No. of males	10	10	10	10	10	10
Necropsy body weight (g)	38.7	37.8	38.5	36.8	33.7**	32.3**
Liver weight, absolute (g)	1.48	1.50	1.58	1.57	1.55	1.63
Liver weight, relative (‰)	38.4	39.6	41.0*	42.8**	46.0**	50.6**
Hypertrophy, centrilobular; incidence (mean severity)	0	0	0	0	4* (1.0)	10** (1.9)
Females						
No. of females	10	10	10	10	10	8
Necropsy body weight (g)	31.0	28.3*	30.7	28.3*	29.7	27.7**
Liver weight, absolute (g)	1.36	1.28	1.37	1.33	1.57**	1.60**
Liver weight, relative (‰)	43.8	45.1	44.8	47.1**	52.8**	57.6**
Hypertrophy, centrilobular	0	0	0	0	5* (1.0)	8** (1.6)

* Statistically significant difference from control: *, $p \leq 0.05$; **, $p \leq 0.01$

Severity scores: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked

Reproductive toxicity screening in rats according to OECD TG 422 via oral route (1997)

The following information related to liver toxicity is available in the summary on the ECHA dissemination website:

Effects in males:

- 1000 mg/kg bw/d: increased liver weight, acidophilic change of hepatocytes (micro-granular acidophilic cells diffusely spread in the entire small lobules, coincided with loss of fatty droplets that normally are present), increased ALT
- 200 mg/kg bw/d: acidophilic change of hepatocytes, increased ALT

Effects in females:

- 1000 mg/kg bw/d: increased liver weight, acidophilic change of hepatocytes (micro-granular acidophilic cells around the centre of the lobules), enlarged hepatocytes
- 200 mg/kg bw/d: increased liver weight, acidophilic change of hepatocytes

10.10 Reproductive toxicity

Hazard class not assessed in this dossier.

10.11 Specific target organ toxicity-single exposure

Hazard class not assessed in this dossier.

10.12 Specific target organ toxicity-repeated exposure

Table 26: Summary table of animal studies on STOT RE

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td colspan="4">inhalation exposure</td></tr>
<tr>
<td>2-years Carcino-genicity study

Similar to OECD TG 451 (NTP standards)

<u>GLP:</u> yes (21 CFR, Part 58)

Supporting study

<u>Species:</u> rats

<u>Strain:</u> F344/N

<u>No/group:</u>
n = 50 / sex / treatment group</td>
<td>2-phenyl-propene
(> 99 % purity)

Whole-body inhalation

<u>Conc.:</u>
♂/♀: 0, 100, 300, 1000 ppm (approx. 0.48, 1.45, 4.83 mg/l)

<u>Duration of exposure:</u>
2 years (105 weeks)

6 h/d plus T_{90} (12 minutes)

5 d/w (except holidays)</td>
<td><u>General effects:</u>

- no treatment-related effect on survival rates
- reduced body weight in ♂ and ♀ rats at 1000 ppm (5 – 10 %) during the 2nd year of the study

<u>Neoplastic effects:</u>

Male rats positive (kidney, mononuclear cell leukaemia, testis)

Female rats negative

In ♂ rats:
<table>
<tr><th>Neoplastic effects:</th><th>No. of animals with lesions / no. of animals in the group</th></tr>
<tr><td>Kidney (renal tubule adenoma or carcinoma [combined])#

HCI†: 4/399 (1.0 % ± 1.1 %; range 0 % - 2 %)</td><td>Ctrl: 1/50 (2 %)
100 ppm: 2/50 (4 %)
300 ppm: 3/50 (6 %)
1000 ppm: 7/50* (14 %)
Trend test: p = 0.006</td></tr>
<tr><td>Kidney (renal tubule adenoma [includes multiple])#

HCI†: 3/399 (0.8 % ± 1.0 %; range 0 % - 2 %)</td><td>Ctrl: 1/50 (2 %)
100 ppm: 2/50 (4 %)
300 ppm: 2/50 (4 %)
1000 ppm: 5/50 (10 %)</td></tr>
<tr><td>Kidney (renal tubule carcinoma)

HCI†: 1/399 (0.3 % ± 0.7 %; range 0 % - 2 %)</td><td>Ctrl: 0/50 (0 %)
100 ppm: 0/50 (0 %)
300 ppm: 1/50 (2 %)
1000 ppm: 2/50 (4 %)</td></tr>
<tr><td>Mononuclear cell leukaemia

HCI†: 188/399 (47.1 % ± 10.3 %; range 32 % - 66 %)</td><td>Ctrl: 26/50 (52 %)
100 ppm: 32/50 (64 %)
300 ppm: 29/50 (58 %)
1000 ppm: 38/50* (76 %)
Trend test: p = 0.018</td></tr>
<tr><td>Testis (interstitial cell adenoma)

HCI†: 316/399 (79.2 % ± 3.9 %; range 72 % - 84 %)</td><td>Ctrl: 33/50 (66 %)
100 ppm: 44/50* (88 %)
300 ppm: 41/50 (82 %)
1000 ppm: 44/50* (88 %)
Trend test: p = 0.007</td></tr>
</table>
* $p \leq 0.05$
# data of the standard and extended evaluations together (single and step section combined)
† HCI: historical control incidence (inhalation studies) as reported within the NTP report (data as of January 28th, 2005) (NTP, 2007)

In ♀ rats: no neoplastic effects observed</td>
<td>(NTP, 2007)

ECHA dissemination page (Carc.): 001 Key | Experimental results</td>
</tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td></td><td></td><td>
<u>Pre-/Non-neoplastic effects:</u>

In ♂ rats:
<table>
<tr><th>Pre-/non-neoplastic effects:</th><th>No. of animals with lesions / no. of animals in the group (0, 100, 300, 1000 ppm):</th></tr>
<tr><td>Kidney (papilla, linear mineralisation†)</td><td>12/50 (24 %), 16/50 (32 %), 10/50 (20 %), 33/50** (66 %)</td></tr>
<tr><td>Kidney (hyperplasia)#</td><td>1/50 (2 %), 0/50, 1/50 (2 %), 4/50 (8 %)</td></tr>
<tr><td>Nose (olfactory epithelium, hyperplasia, basal cell⸸)</td><td>0/50 (0 %), 17/50** (34 %), 18/50** (36 %), 43/49** (88 %)</td></tr>
<tr><td>Nose (olfactory epithelium, degeneration⸸)</td><td>1/50 (2 %), 3/50 (6 %), 3/50 (6 %), 16/49** (33 %)</td></tr>
</table>
* p ≤ 0.05 ** p ≤ 0.01
† severity: minimal (cf. Annex I 2.3.1.1)
# data of the standard and extended evaluations together (single and step section combined)
⸸ severity: minimal

In ♀ rats:
<table>
<tr><th>Pre-/ non-neoplastic effects:</th><th>No. of animals with lesions / no. of animals in the group (0, 100, 300, 1000 ppm):</th></tr>
<tr><td>Kidney (papilla, mineralisation#)</td><td>1/49 (2 %), 6/50 (12 %), 8/50* (16 %), 7/50* (14 %)</td></tr>
<tr><td>Nose (olfactory epithelium, hyperplasia, basal cell†)</td><td>0/49 (0 %), 14/49** (29 %), 30/50** (60 %), 49/50** (98 %)</td></tr>
<tr><td>Nose (olfactory epithelium, degeneration†)</td><td>1/49 (2 %), 1/49 (2 %), 7/50* (14 %), 24/50** (48 %)</td></tr>
</table>
* p ≤ 0.05 ** p ≤ 0.01
# characterised by laminated concretions / severity: minimal (cf. Annex I 2.3.1.1)
† severity: minimal
</td><td></td></tr>
<tr><td>2-years Carcino-genicity study

Similar to OECD TG 451 (NTP standards)

<u>GLP:</u> yes (21 CFR, Part 58)</td><td>2-phenyl-propene
(> 99 % purity)

Whole-body inhalation

<u>Conc.:</u>
♂/♀: 0, 100, 300, 600 ppm (approx. 0.48, 1.45, 2.90 mg/L)</td><td><u>General effects:</u>

- no treatment-related effect on survival rates
- reduced body weight in ♂ and ♀ mice at 600 ppm (final mean body weight: - 8 % in ♂ and - 9 % in ♀; final mean body weight gains: - 12 % in ♂ and - 20 % in ♀)

<u>Neoplastic effects:</u>

Male mice positive (liver)

Female mice positive (liver)</td><td>(NTP, 2007)

ECHA disseminatio n page (Carc.): 001 Supporting | Experimental results</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr>
<td>Supporting study

Species: mice

Strain: B6C3F$_1$

No/group:
n = 50 / sex / treatment group</td>
<td>Duration of exposure:
2 years (105 weeks)

6 h/d plus T_{90} (12 minutes)

5 d/w
(except holidays)</td>
<td>
In ♂ mice:
<table>
<tr><th>Neoplastic effects:</th><th>No. of animals with lesions / no. of animals in the group</th></tr>
<tr><td>Liver (hepatocellular adenoma or carcinoma [combined])

HCI†: 196/350 (56.0 % ± 6.2 %; range 50 % - 68 %)</td><td>Ctrl: 28/50 (56 %)
100 ppm: 36/50* (72 %)
300 ppm: 33/50 (66 %)
600 ppm: 37/50* (74 %)
Trend test: p = 0.093</td></tr>
<tr><td>Liver (hepatocellular adenoma [includes multiple])

HCI†: 134/350 (38.3 % ± 6.3 %; range 30 % – 46 %)</td><td>Ctrl: 24/50 (48 %)
100 ppm: 27/50 (54 %)
300 ppm: 27/50 (54 %)
600 ppm: 25/50 (50 %)
Trend test: p = 0.453</td></tr>
<tr><td>Liver (hepatocellular carcinoma [includes multiple])

HCI†: 85/350 (24.3 % ± 4.8 %; range 18 % – 32 %)</td><td>Ctrl: 10/50 (20 %)
100 ppm: 12/50 (24 %)
300 ppm: 11/50 (22 %)
600 ppm: 17/50 (34 %)
Trend test: p = 0.081</td></tr>
</table>
* p ≤ 0.05
† HCI: historical control incidence (inhalation studies) as reported within the NTP report (data as of January 28th, 2005) (NTP, 2007)

In ♀ mice:
<table>
<tr><th>Neoplastic effects:</th><th>No. of animals with lesions / no. of animals in the group</th></tr>
<tr><td>Liver (hepatocellular adenoma or carcinoma [combined])

HCI†: 108/347 (31.1 % ± 6.8 %; range 22 % - 39 %)</td><td>Ctrl: 13/50 (26 %)
100 ppm: 26/50** (52 %)
300 ppm: 24/50* (48 %)
600 ppm: 33/50** (66 %)
Trend test: p < 0.001</td></tr>
<tr><td>Liver (hepatocellular adenoma)

HCI†: 78/347 (22.5 % ± 8.1 %; range 12 % - 35 %)</td><td>Ctrl: 10/50 (20 %)
100 ppm: 20/50* (40 %)
300 ppm: 21/50** (42 %)
600 ppm: 23/50** (46 %)
Trend test: p = 0.014</td></tr>
<tr><td>Liver (hepatocellular carcinoma)

HCI†: 37/347 (10.7 % ± 1.8 %; range 8 % - 12 %)</td><td>Ctrl: 3/50 (6 %)
100 ppm: 9/50 (18 %)
300 ppm: 6/50 (12 %)
600 ppm: 18/50** (36 %)
Trend test: p < 0.001</td></tr>
</table>
* p ≤ 0.05 ** p ≤ 0.01
† HCI: historical control incidence (inhalation studies) as reported within the NTP report (data as of January 28th, 2005)(NTP, 2007)

<u>Pre-/Non-neoplastic effects:</u>
</td>
<td></td>
</tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td></td><td></td><td>In ♂ mice:
<table><tr><td>Pre-/non-neoplastic effects:</td><td>No. of animals with lesions / group (0, 100, 300, 600 ppm):</td></tr><tr><td>Nose (olfactory epithelium, metaplasia†)</td><td>6/50 (12 %), 47/50** (94 %), 49/50** (98 %), 49/50** (98 %)</td></tr><tr><td>Nose (olfactory epithelium, glands, hyperplasia†)</td><td>4/50 (8 %), 50/50** (100 %), 50/50** (100 %), 50/50** (100 %)</td></tr><tr><td>Nose (olfactory epithelium, atrophy#)</td><td>0/50 (0 %), 2/50 (4 %), 8/50** (16 %), 12/50** (24 %)</td></tr><tr><td>Forestomach (hyperplasia of the epithelium)</td><td>1/50 (2 %), 4/49 (8 %), 7/49* (14 %), 11/48** (23 %)</td></tr><tr><td>Forestomach (inflammation)</td><td>0/50 (0 %), 2/49 (4 %), 1/49 (2 %), 5/48* (10 %)</td></tr></table>* p ≤ 0.05 ** p ≤ 0.01
† severity: mild at 100 ppm, moderate at ≥ 300 ppm
# severity: minimal at ≥ 300 ppm

In ♀ mice:
<table><tr><td>Pre-/non-neoplastic effects:</td><td>No. of animals with lesions / group (0, 100, 300, 600 ppm):</td></tr><tr><td>Nose (olfactory epithelium, metaplasia†)</td><td>2/49 (4 %), 49/49** (100 %), 47/50** (94 %), 50/50** (100 %)</td></tr><tr><td>Nose (olfactory epithelium, glands, hyperplasia#)</td><td>3/49 (6 %), 49/49** (100 %), 50/50** (100 %), 50/50** (100 %)</td></tr><tr><td>Liver (eosinophilic foci)</td><td>2/50 (4 %), 5/50 (10 %), 7/50 (14 %), 12/50** (24 %)</td></tr><tr><td>Kidney (nephropathy#)</td><td>16/50 (32 %), 21/49 (43 %), 12/50 (24 %), 26/50* (52 %)</td></tr></table>* p ≤ 0.05 ** p ≤ 0.01
increased incidence and severity (cf. Annex I 2.3.1.2)
† severity: mild at 100 ppm, moderate at ≥ 300 ppm
# severity: mild at 100 and 300 ppm, moderate at 600 ppm</td><td></td></tr>
<tr><td>Non-guideline repeated dose toxicity study

<u>GLP:</u> no

Supporting study

<u>Species:</u></td><td>2-phenyl-propene (98.6 – 99.1 % purity)

Whole-body inhalation

<u>Conc.:</u>
- rat: 0, 200, 600, 800, 3000</td><td><u>General effects:</u>
Body weight and body weight changes:
- rat: slight growth depression at 800 ppm
- guinea pig: slight growth depression at 800 ppm
- rabbit: slight growth depression at 600 ppm
- rhesus monkey: no effects
Mortality and time to death:</td><td>(Wolf et al., 1956)

Specific target organ toxicity – repeated exposure by inhalation</td></tr>
</table>

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
- Wistar rats - guinea pigs - rabbits - rhesus monkeys <u>No/group:</u> - rat: 10 – 25 (♂/♀) - guinea pig: 5 – 10 (♂/♀) - rabbit: 1 – 2 (♂/♀) - rhesus monkey: 1 – 2 (♂/♀ low dose, ♀ high dose)	ppm (approx. 0.97, 2.9, 3.86, 14.5 mg/L); - guinea pig: 0, 200, 600, 800, 3000 ppm (approx. 0.97, 2.9, 3.86, 14.5 mg/L); - rabbit: 0, 200, 600 ppm (approx. 0.97, 2.9 mg/L); - rhesus monkeys: 0, 200, 600 ppm (approx. 0.97, 2.9 mg/L) <u>Duration of exposure:</u> **Six months** (depending on the concentration) 7-8 h/d 5 d/w	- rat: severe mortality at 3000 ppm following 3 – 4 days of exposure - guinea pig: severe mortality at 3000 ppm following 3 – 4 days of exposure - rabbit: slight mortality at 600 ppm - rhesus monkey: no findings reported ***<u>Gross pathology findings :</u>*** (see table below) - rat: slightly increased average liver and kidney weight at 600 and 800 ppm - guinea pig: slightly increased average liver weight at 600 and 800 ppm; slightly increased average kidney weight at 800 ppm - rabbit: no findings reported - rhesus monkey: no findings reported ***<u>Histopathology findings:</u>*** - no findings reported	ECHA disseminatio n page: 004 – 007 Supporting | Experimental results

Table: Results as reported within the publication

Species	Conc. (ppm)	Sex	No. of 7 h exposures	Duration of experiment (days)	Effect: growth depression	Effect: organ weight	Mortality
Rat	3000	♂/♀	3-4	3-4	-	-	severe
	800	♂/♀	28	38	slight	liver and kidney, slight	-
	600	♂/♀	149	212	-	liver and kidney, slight	-
	200	♂/♀	139	197	-	no effect	-
Guinea pig	3000	♂/♀	3-4	3-4	-	-	severe
	800	♂/♀	27	38	slight	liver and kidney, slight	-
	600	♂/♀	144	212	-	liver, slight	-
	200	♂/♀	139	197	-	no effect	-
Rabbit	600	♂/♀	152	212	slight	-	slight
	200	♂/♀	139	197	-	no effect	-
Rhesus monkey	600	♀	149	212	-	no effect	-
	200	♂/♀	139	197	-	no effect	-

90-day subchronic inhalation	**2-phenyl-propene** (> 99 % purity)	***<u>General effects</u>*** - no effect on body weight gains and final mean body weight	(NTP, 2007) Specific

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr>
<td>toxicity study

Similar to OECD TG 413 (NTP standards)

GLP: yes (21 CFR, Part 58)

Key study

Species: rats

Strain: F344/N

Sex: ♂/♀

No/group: n = 10 / sex / treatment group (core + clinical pathology group)</td>
<td>Whole-body inhalation

Conc.: ♂/♀: 0, 75, 150, 300, 600, and 1,000 ppm (approx. 0.36, 0.73, 1.45, 2.9, 4.83 mg/l)

Duration of exposure: 14 weeks (core study), 23 days (clinical pathology study)

6 h/d plus T_{90} (12 minutes)

5 d/w (except holidays)</td>
<td>- no mortality, all animals survived
Clinical signs:
- no effects observed
Haematological findings:
- slightly decreased erythron (lower values (~ -5 %) of haematocrit, haemoglobin, and erythrocytes counts) in ♂ at ≥ 150 ppm (no effects in ♀) with statistical significance
Clinical biochemistry findings:
- increased bile acid concentration in ♂ and ♀ at 1000 ppm
- increased urinary parameter indicative of adverse effects[34] on renal tubular epithelium in ♂ at ≥ 300 ppm and ♀ at ≥ 600 ppm
Gross pathology findings:
- no gross lesions
- increased absolute/relative liver weight in ♂ at ≥ 150 ppm and ♀ at ≥ 600 ppm with statistical significance
- increased absolute/relative[35] kidney weight in ♂ at 1000 ppm and ♀ at ≥ 600 ppm with statistical significance
Histopathology findings:
- hyaline droplet accumulation slightly more severe (minimal to mild) in ♂ at ≥ 600 ppm (altered morphology of hyaline droplets (larger more variable in shape) without changes in hyaline droplet accumulation incidences)

♂ rats (0, 75, 150, 300, 600, 1000 ppm): 9/10[36] (1.1)[37], 10/10 (1.2), 10/10 (1.3), 10/10 (1.1), 10/10 (1.8), 10/10 (1.7)
- increased renal cell proliferation (labelling index) and α2u-globulin concentrations[38] in the kidney of ♂ at ≥ 150 ppm with statistical significance
<table>
<tr><th>Conc. (ppm)</th><th>renal cell proliferation (labelling index [%])</th><th>α2u-globulin (ng/µg soluble protein)</th></tr>
<tr><td>0</td><td>2.339 ± 0.110</td><td>81.32 ± 8.87</td></tr>
<tr><td>75</td><td>3.032 ± 0.316 [+30 %]</td><td>115.46 ± 16.81 [+42 %]</td></tr>
<tr><td>150</td><td>3.083 ± 0.226* [+32 %]</td><td>119.29 ± 11.28* [+46 %]</td></tr>
</table>
</td>
<td>target organ toxicity – repeated exposure by inhalation

ECHA dissemination page: 001 Key | Experimental results</td>
</tr>
</table>

[34] increased ratios of protein/creatinine, alkaline phosphatase/creatinine, aspartate aminotransferase/creatinine, lactate dehydrogenase/creatinine, γ-glutamyltransferase/creatinine, and N-acetyl-β-glucosaminidase/creatinine

[35] relative kidney weight in ♂ rats already increased at ≥ 75 ppm

[36] no. of animals affected / no. of animals examined

[37] severity score on a scale from 1 to 4

[38] α2u-globulin concentration was determined in the supernatant of whole kidney homogenate using a competitive indirect enzyme-linked immunosorbent assay (ELISA)

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td></td><td></td><td>
<table>
<tr><td>300</td><td>3.353 ± 0.230** [+43 %]</td><td>160.82 ± 16.51** [+98 %]</td></tr>
<tr><td>600</td><td>3.050 ± 0.159** [+30 %]</td><td>176.02 ± 26.18** [+117 %]</td></tr>
<tr><td>1000</td><td>3.935 ± 0.307** [+68 %]</td><td>167.42 ± 20.50** [+106 %]</td></tr>
</table>
- no evidence of granular casts within the renal tubules

(tissues examined to the no-effect level: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, eyes, gallbladder (mice), harderian gland, heart and aorta, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, larynx, liver, lung (with mainstem bronchus), lymph nodes (mandibular, mesenteric, bronchial, mediastinal), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, skin, spleen, stomach (forestomach and glandular), testis (with epididymis and seminal vesicle), thymus, thyroid gland, trachea, urinary bladder, and uterus)</td><td></td></tr>
<tr><td>90-day subchronic inhalation toxicity study

Similar to OECD TG 413 (NTP standards)

GLP: yes (21 CFR, Part 58)

Key study

Species: mice

Strain: B6C3F1

Sex: ♂/♀

No/group: n = 10 / sex / treatment group (core + clinical pathology group)</td><td>2-phenyl-propene (> 99 % purity)

Whole-body inhalation

Conc.: ♂/♀: 0, 75, 150, 300, 600, and 1,000 ppm (approx. 0.36, 0.73, 1.45, 2.9, 4.83 mg/L)

Duration of exposure: 14 weeks (core study), 23 days (clinical pathology study)

6 h/d plus T_{90} (12 minutes)

5 d/w (except holidays)</td><td>General effects:
- reduced final mean body weight in ♂ and ♀ with statistical significance
▪ ♂: 600 ppm (- 13 %), 1000 ppm (- 17 %)
▪ ♀: 75 ppm (- 9 %), 300 ppm (- 9 %), 1000 ppm (- 11 %)
- reduced final mean body weight gains in ♂ and ♀ at ≥ 300 ppm with statistical significance
▪ ♂: 300 ppm (- 14 %), 600 ppm (- 34 %), 1000 ppm (- 43 %)
▪ ♀: 300 ppm (- 23 %), 600 ppm (- 12 %), 1000 ppm (- 32 %)
- mortality: all ♂ survived, two ♀ died on day 3 in the highest concentration group (1000 ppm)
Clinical signs:
- sedation (moderate to severe) in ♂ at 1000 ppm
- ataxia in ♂ and ♀ at 1000 ppm
Haematological findings:
- slightly decreased erythron (lower values (~ -4 %) of haemoglobin and erythrocytes counts) in ♀ at 1000 ppm with statistical significance
Gross pathology findings:
- no gross lesions
- increased absolute liver weight in ♀ at ≥ 600 ppm with statistical significance and in ♂ at 1000 ppm without statistical significance</td><td>(NTP, 2007)

Specific target organ toxicity – repeated exposure by inhalation

ECHA disseminatio n page: 002 Key \| Experimental results</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td></td><td></td><td>
- decreased epididymal weights in ♂ at 1000 ppm with statistical significance
- prolonged oestrous cycle in ♀ at ≥ 600 ppm with statistical significance

<u>Histopathology findings:</u>

- minimal to mild centrilobular hypertrophy in the liver of ♂ and ♀ at ≥ 600 ppm which contributed to the increased liver weight
- high incidences numerous nasal lesions in ♂ and ♀ at ≥ 75 ppm (atrophy and metaplasia of the olfactory epithelium[39]; atrophy and hyperplasia of Bowman's glands[40]) with statistical significance (incidence table below)
- hyaline degeneration of the respiratory epithelium[41] in ♀ at ≥ 150 ppm with statistical significance (incidence table below)

(tissues examined to the no-effect level: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, eyes, gallbladder (mice), harderian gland, heart and aorta, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, larynx, liver, lung (with mainstem bronchus), lymph nodes (mandibular, mesenteric, bronchial, mediastinal), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, skin, spleen, stomach (forestomach and glandular), testis (with epididymis and seminal vesicle), thymus, thyroid gland, trachea, urinary bladder, and uterus)
</td><td></td></tr>
</table>

Incidence table of nasal lesions observed in the 90-day NTP study in mice:

	Ctrl	75 ppm	150 ppm	300 ppm	600 ppm	1000 ppm
Male						
Atrophy of the Bowman's glands	0/10	7/10**(1.0)$^{\#}$	10/10** (1.3)	10/10** (1.9)	10/10** (2.0)	10/10** (2.0)
Hyperplasia of the Bowman's glands	0/10	9/10** (1.1)	10/10** (1.6)	10/10** (2.3)	10/10** (2.9)	10/10** (2.7)
Atrophy of the olfactory epithelium	0/10	10/10** (1.1)	10/10** (1.4)	10/10** (2.0)	10/10** (2.0)	10/10** (2.1)
Metaplasia of the olfactory epithelium	0/10	5/10* (1.2)	10/10** (1.4)	10/10** (2.0)	10/10** (2.0)	10/10** (2.0)
Hyaline degeneration of the respiratory epithelium	0/10	1/10 (1.0)	2/10 (1.0)	1/10 (1.0)	2/10 (1.0)	0/10
Females						
Atrophy of the Bowman's glands	0/10	8/10** (1.0)	9/10** (1.3)	10/10** (2.0)	10/10** (2.0)	8/8** (2.5)

[39] characterised by a decreased layers of neuronal cells accompanied by a loss of associated axons and replacement by simple columnar ciliated respiratory epithelium

[40] characterised by a loss of Bowman's glands and replacement of the glandular epithelium with hyperplastic epithelium (characterised by an elevated number of cuboidal cells, distended with mucin, cell debris, and inflammatory cells)

[41] characterised by an accumulation of eosinophilic globules in the cytoplasm of the respiratory epithelium

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference

Hyperplasia of the Bowman's glands	0/10	5/10* (1.0)	10/10** (1.7)	10/10** (2.3)	10/10** (2.6)	8/8** (2.6)
Atrophy of the olfactory epithelium	0/10	10/10** (1.0)	10/10** (1.6)	10/10** (2.0)	10/10** (2.0)	8/8** (2.0)
Metaplasia of the olfactory epithelium	0/10	4/10* (1.0)	9/10** (1.7)	10/10** (2.0)	10/10** (2.0)	8/8** (2.0)
Necrosis of the olfactory epithelium	0/10	0/10	0/10	0/10	0/10	2/10 (3.0)
Hyaline degeneration of the respiratory epithelium	0/10	2/10 (2.0)	6/10** (1.3)	9/10** (1.6)	8/10** (1.4)	4/8* (1.0)

* $p \leq 0.05$, ** $p \leq 0.01$ (number of animals with lesions / number of animal examined), # average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked

Non-guideline repeated dose toxicity study (three sub-studies) GLP: no **Supporting study** Species, strain, sex, No/group: *Sub-study 1:* **mice**, B6C3F1, ♂/♀, n = 6[42] *Sub-study 2:* **mice**, B6C3F1, ♂/♀, n = 6[43] and **rats,** F344, ♂/♀, n = 5[44] *Sub-study 3:* **rats**, F344, ♂/♀, n = 4[b] and rats, NCI Black-Reiter, ♂, n = 4[b]	**2-phenyl-propene** (99 % purity) Whole-body **inhalation** Conc. and duration of exp.: *Sub-study 1:* 0, 125, 250, or 500 ppm (approx. 0.6, 1.21, 2.42 mg/m^3) 3, 5, 12 days *Sub-study 2:* Mice: 0, 600, 800, 1000 ppm (approx. 2.9, 3.87, 4.83 mg/L) Rats: 0, 600, 1000 ppm (approx. 2.9, 4.83 mg/L) 6 h, 5 and 12 days (mice); 12 days (rats) *Sub-study 3:* 0,	*Sub-study 1 (♂/♀ B6C3F1 mice):* - no effects observed *Sub-study 2 (♂/♀ B6C3F1 mice and ♂/♀ F344 rats):* ***General effects:*** Mice - reduced body weight in ♂ at 600, 800, 1000 ppm after 5 and 12 days with statistical significance - mortality: o no mortality in ♂ o 1/18 ♀ at 600 ppm o 10/18 ♀ at 800 ppm o 5/24 ♀ at 1000 ppm Rats - no effects observed ***Clinical signs:*** Mice - sedated appearance after 6 h exposure at 600, 800, 1000 ppm (effect disappeared after prolonged exposure) - hyperactivity and unresponsive to noise Rats - no effects observed ***Clinical biochemistry findings:*** Mice - **decreased liver glutathione** in ♀ at ≥ 600 ppm after 1 or 5 days and in ♂ at ≥ 600 ppm after 1 day of exposure and ≥	(Morgan et al., 1999) Specific target organ toxicity – repeated exposure by **inhalation** ECHA disseminatio n page: 003 Supporting | Experimental results

[42] number of animals / sex / concentration / time point
[43] number of animals / sex / concentration / time point (except for the highest dose group which included 12 mice)
[44] number of animals / concentration

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
	125, 250, or 500 ppm (approx. 0.6, 1.21, 2.42 mg/l) 9 days	800 ppm after 5 days of exposure with statistical significance (the depletion was concentration-depended after day 5 in both sexes) Rats - no effects observed ***Gross pathology findings:*** Mice - **reduced relative spleen weight** in ♂ and ♀ at all exposure levels with statistical significance - **increased relative liver weight** in ♂ at 600 ppm after 5 days of exposure and at ≥ 800 ppm after 1, 5, and 12 days with statistical significance; in ♀ at ≥ 600 ppm after 1, 5, and 12 days with statistical significance (except for ♀ at 600 ppm after short-term, 1 day, treatment) Rats - **increased relative liver weight** in ♂ and ♀ at all exposure levels with statistical significance (concentration-dependent) - **increased relative lung weight** in ♂ at 1000 ppm with statistical significance - **increased relative kidney weight** only in ♂ at 600 ppm with statistical significance ***Histopathology findings:*** Mice *(tissues examined on day 5: lungs, kidney, spleen, and liver; on day 12: lungs, liver, kidney, spleen, nasal cavity, brain, stomach, heart, thymus, adrenal glands)* - **no effect observed** in spleen, liver, lung, kidney, nasal cavity, brain, stomach, heart, thymus, adrenal glands Rats *(tissues examined: lungs, liver, kidney, spleen, nasal cavity, brain, stomach, heart, thymus, adrenal glands)* - no effect observed in liver, spleen, lung, nasal cavity, brain, stomach, heart, thymus, adrenal glands - abnormal accumulation of cytoplasmic eosinophilic granules (hyaline droplets) in the renal tubules in ♂ at all exposure levels (indicative of hyaline droplet nephropathy; mild to moderately severe) but not in ♀	

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		♂ rats (0, 600, 1000 ppm): 5/5[45] (1.0)[46], 5/5 (3.0), 5/5 (3.0); ♀ rats (0, 600, 1000 ppm): 0/5, 0/5, 0/5 - no granular casts in the renal tubules *Sub-study 3 (♂/♀ F344 rats and ♂ NBR rats):* ***General effects:*** - no effect observed ***Gross pathology findings:*** - no effect on kidney weight ***Histopathology findings:*** *(tissues examined: kidney)* - hyaline droplet accumulation (Mallory-Heidenhain staining method) confirmed in ♂ at ≥ 250 ppm - no other related effects in the kidney - no signs of hyaline droplet nephropathy in ♀ or ♂ NBR rats	
oral exposure			
Combined Repeated Dose Toxicity Study with the Reproduction / Developmental Toxicity Screening Test Equivalent or similar to **OECD TG 422** GLP: yes **Key study** Species: **rats** Strain: Crj: CD(SD)	**2-phenyl-propene** (99.6 % purity) **Oral** (gavage) Dose: ♂/♀: 0, 40, 200 or 1000 mg/kg bw/d Duration of exposure: ♂: 43 days (14 days prior mating until after mating) ♀: ~53 days[47] (14 days prior mating until post-partum day 3)	***General effects*** - reduced body weight gain in ♂ at 1000 mg/kg bw/d - decreased food consumption on day 7 in ♂ at 1000 mg/kg bw/d - one ♂ died on day 23 at 1000 mg/kg bw/d ***Clinical signs:*** - slight or moderate level of salivation in ♂ at ≥ 200 mg/kg bw/d and ♀ at 1000 mg/kg bw/d ***Haematological findings:*** - no effects observed ***Clinical biochemistry findings:*** - increased GPT in ♂ at ≥ 200 mg/kg bw/d - increased urea nitrogen and potassium in ♂ at 1000 mg/kg bw/d - decreased triglyceride in ♂ at 1000 mg/kg bw/d ***Gross pathology findings:*** - enlarged liver in ♂/♀ at 1000 mg/kg bw/d (dark reddish	(Study report RDT, 1997) Specific target organ toxicity – repeated **oral** exposure ECHA dissemination page: key study Unpublished study report in Japanese Information taken as reported on ECHAs dissemination page

[45] no. of animals affected / no. of animals examined

[46] severity score on a scale from 1 to 4

[47] 14 days prior mating + up to 14 days mating* + 22 days gestation* + 3 days lactation (* information taken from OECD TG 422)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
Sex: ♂/♀ No/group: n = 10 / sex / treatment group Deviation: - shorter treatment time in ♀ (lactation) Full study report not available (limited reporting)		change in ♂ at ≥ 200 mg/kg bw/d) - enlarged kidney in ♂/♀ at 1000 mg/kg bw/d (discolouration of the cortico-medullary junction in ♀ at ≥ 200 mg/kg bw/d) - yellow micro-granular calculi in the urinary bladder of ♂ at 1000 mg/kg bw/d - enlarged adrenals (greyish colour) and thymus atrophy individually found in ♀ at 1000 mg/kg bw/d - increased absolute and relative liver weight in ♂ at 1000 mg/kg bw/d and ♀ at ≥ 200 mg/kg bw/d - increased absolute and relative kidney weight in ♂/♀ at 1000 mg/kg bw/d (relative kidney weight increased in ♀ at 200 mg/kg bw/d already) - decreased absolute and relative thymus weight in ♀ at 1000 mg/kg bw/d ***Histopathology findings:*** (no statistic provided) Liver - acidophilic change of the hepatocytes in ♂/♀ at ≥ 200 mg/kg bw/d o micro-granular acidophilic cells were diffusely spread in the entire small lobules, loss of fatty droplets in ♂ o micro-granular acidophilic cells distinctively around the centre of the lobules and enlarged hepatocytes in ♀ Kidney - increased hyaline droplets in the renal tubular epithelium of ♂ at 200 mg/kg bw/d accompanied in most cases by basophilic changes of the renal tubular epithelium - vacuolation in the renal tubular epithelium and lymphocyte infiltration in adjacent areas in some animals in ♀ at ≥ 200 mg/kg bw/d (vacuoles identified as lipid droplets by oil red O staining) Adrenals - increased number of lipid droplets in the fascicular zone of ♂/♀ at 1000 mg/kg bw/d Urinary bladder - hyperplasia of the mucosal epithelium in ♂ at 1000 mg/kg bw/d including thickened mucosal epithelium layer, tissue erosion and infiltration of inflammatory cells Thymus - atrophy in ♀ at ≥ 200 mg/kg bw/d (unclear boundary between the cortex and medulla)	

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		LOAEL: 200 mg/kg bw/day (as stated on ECHAs dissemination page based on histological changes in liver (♂/♀), kidneys (♂/♀), and thymus (♀) and increased GPT (♂) *(HE stains prepared for the brain, heart, liver, spleen, kidneys, adrenals, testes, epididymides, urinary bladders, thymus [♀])*	
Non-guideline repeated dose toxicity study on ototoxicity GLP: no **Supporting study** Species: **rats** Strain: Sprague–Dawley Sex: ♂ No/group: n = 7 – 8	**2-phenyl-propene** (99 % purity) **Oral** (gavage) Dose: ♂: 8.47 mmol/kg bw/d (~1000 mg/kg bw/d) Duration of exposure: 14 days + 10 days post-treatment (5 days / week	***General effects*** - no effects observed (body weight change or lethality) ***Clinical signs:*** - no effects observed ***Histopathology findings:*** mild ototoxicity - lesions of the organ of Corti: loss of outer hair cells (OHC) in the area of the cochlea that is responsive to medium frequencies (third row)	(Gagnaire and Langlais, 2005)

10.12.1 Short summary and overall relevance of the provided information on specific target organ toxicity – repeated exposure

Human evidence for specific target organ toxicity caused by repeated exposure is not available. Pertinent information for the purpose of hazard characterisation can be obtained from studies in experimental animals. The effects of subacute to chronic exposure with 2-phenylpropene have been studied in rodents. Animal data on two different routes of exposure, oral and inhalation, are available. The latter has been described as a relevant route of human exposure, for instance, at the workplace (Morgan et al., 1999; NTP, 2007). The most appropriate information can be derived from three repeated dose toxicity (RDT) studies conducted according to or similar to standard test guidelines with GLP compliance. These RDT studies include an unpublished OECD TG 422 (GLP) study in rats with oral administration and two subchronic RDT NTP studies (similar to OECD TG 413) conducted in rats and mice exposed to 2-phenylpropene via inhalation (NTP, 2007; Study report RDT, 1997). In addition, two NTP carcinogenicity inhalation studies (similar to OECD TG 451, cf. section 10.9) in rats and mice are available (NTP, 2007). Beyond the standard information provided by these guideline studies, three non-guideline studies are available. In an early study by Wolf et al. (1956), rats, guinea pigs, rabbits, and rhesus monkeys were chronically exposure (up to six months) to 2-phenylpropene via inhalation. Morgan et al. (1999) conducted three sub-studies in rats and mice exposed via inhalation for up to 12 days. The aim of the study was to compare the effects of 2-phenylpropene with those of styrene. Another study by Gagnaire and Langlais (2005), examined ototoxic effect conferred by repeated (14 days) oral administration of 2-phenylpropene in male rats.

inhalation exposure

In the **2-year carcinogenicity NTP study** in **rats**, non-neoplastic histopathological findings were noted in the kidney including hyperplasia of the renal tubules in males at the highest concentration (1000 ppm). The incidence of mineralisation of the renal papilla was elevated in males (1000 ppm) and females (≥ 300 ppm). In the nose, higher incidences of basal cell hyperplasia (all treatment groups) and degeneration of the olfactory epithelium (1000 ppm in ♂; ≥ 300 ppm in ♀) were found (NTP, 2007). In **mice**, lesions of the olfactory epithelium such as metaplasia (all treatment groups; severity grade mild to moderate), atrophy (≥ 300 ppm in ♂; severity grade minimal) and hyperplasia of the submucosal glands (all treatment groups; severity grade mild to moderate) were seen. There was an increased incidence and severity of renal nephropathy as well as an elevated incidence of eosinophilic foci in the liver of female mice at the highest concentration (600 ppm). In the forestomach, hyperplasia of the epithelium was obvious in males (≥ 300 ppm) (NTP, 2007).

In the **90-day NTP study** in **rats**, the *kidney* was considered the primary target organ. Increased renal cell proliferation (≥ 150 ppm), elevated α2u-globulin concentration (≥ 150 ppm), and altered hyaline droplet morphology (≥ 600 ppm) were exclusively seen in the kidneys of male mice. Increased urinary marker for renal toxicity (≥ 300 ppm in ♂; ≥ 600 ppm in ♀) and increased absolute and relative kidney weight (1000 ppm in ♂; ≥ 600 ppm in ♀) were found in both sexes (NTP, 2007). Compared to rats, **mice** showed a higher sensitivity to 2-phenylpropene exposure in the 90-day NTP study. The final mean body weights (≥ 600 ppm in ♂; 75, 300, and 1000 ppm in ♀) and body weight gains (≥ 300 ppm in ♂/♀) were reduced and two female mice died on day 3 at the highest concentration (1000 ppm).

The *nose* was identified as the primary target organ. Incidences of multiple nasal lesions (atrophy and metaplasia of the olfactory epithelium, atrophy and hyperplasia of Bowman's glands) were increased in both sexes starting at the lowest concentration tested (≥ 75 ppm in ♂/♀) with statistical significance. The severity of these nasal lesions was rated minimal at low concentrations (75 – 150 ppm) and mild at 300 ppm and above. Atrophic and metaplastic lesions of the olfactory epithelium included decreased layers of neuronal cells accompanied by a loss of associated axons and replacement by simple columnar ciliated respiratory epithelium. Atrophic and hyperplastic effects on the Bowman's glands comprised a loss of glands and replacement of the glandular epithelium with hyperplastic epithelium. The latter was characterised by an elevated number of cuboidal cells, distended with mucin, cell debris, and inflammatory cells. A statistically significantly increased incidence of hyaline degeneration (accumulation of eosinophilic globules in the cytoplasm of the respiratory epithelium) of the respiratory epithelium was seen in female mice (≥ 150 ppm). The severity of this effect was rated minimal at all concentrations. Eosinophilic globules are infrequently observed in untreated control animals with increasing incidences during ageing. Chemical exposure via inhalation has been shown to increase the incidence and severity of these droplets (Monticello et al., 1990). The toxicological relevance is, however, unknown. More severe lesions such as necrosis of the olfactory epithelium were only seen in some females at the highest concentration.

Apart from the nasal lesions, other effects such as centrilobular hypertrophy in the liver, increased absolute liver weight, prolonged oestrous cycle, and decreased epididymal weights were observed only at high concentrations (≥ 600 ppm) in the presence of general toxicity. There were no effects on other male reproductive endpoints (epididymal sperm concentration and motility, spermatid heads/testis) and no histopathology changes in the reproductive tract were noted (NTP, 2007).

In the subacute repeated dose toxicity study by **Morgan et al. (1999)**, mortality was seen in female and reduced body weight in male mice at ≥ 600 ppm. No histopathological findings were observed up to the highest concentration tested (1000 ppm) for as long as 12 days. Other effects such as decreased liver glutathione, reduced relative spleen weight, and increased relative liver weight were observed at ≥ 600 ppm. In the same study in rats, hyaline droplet morphology was distinctly changed as compared to untreated animals at ≥ 600 ppm in F344 males but not F344 female or NBR male rats. Relative weights of the liver (≥ 600 ppm in ♂/♀), kidney (600 ppm in ♂), and lung (1000 ppm in ♂) were increased.

Wolf et al. (1956) conducted several long-term (up to 6 months) inhalation experiments in rats, guinea pigs, rabbits, and rhesus monkeys using concentrations up to 600 ppm (rabbits, rhesus monkeys) and 3000 ppm (rats, guinea pigs). The latter concentration induced severe mortality in the respective species. A slight growth depression was noted in rats (800 ppm), guinea pigs (800 ppm), and rabbits (600 ppm) but not in rhesus monkeys. Rats and guinea pigs exhibited slightly increased liver and kidney weights at ≥ 600 ppm. No histopathological findings were reported in either species or concentration.

oral exposure

According to the information available on ECHAs dissemination page, 2-phenylpropene was orally tested in rats in an unpublished combined RDT study with the reproduction / developmental toxicity screening test (**OECD TG 422**). No effects were found at the lowest dose tested (40 mg/kg bw/d). At 200 mg/kg bw/d, serum levels of GTP were increased in males and acidophilic change of the hepatocytes were noted in both sexes. The liver weight (absolute and relative) was increased in females. The renal tubular epithelium was characterised by an increase in hyaline droplets of males and vacuolation (lipid droplets) and lymphocyte infiltration in females. The relative kidney weight was elevated in both sexes (1000 ppm in ♂; 200 and 1000 ppm in ♀). Atrophy was seen in the thymus of females at ≥ 200 mg/kg bw/d (unclear boundary between the cortex and medulla). At the highest dose (1000 ppm), effects beyond the liver, kidney, and thymus were observed including hyperplasia of the mucosal epithelium of the urinary bladder in males and increased number of lipid droplets in the fascicular zone of the adrenals in both sexes.

Mild ototoxicity (loss of outer hair cells) was observed following oral administration of ~1000 mg/kg bw/d 2-phenylpropene for 14 days (+ 10 days post-exposure) (**Gagnaire and Langlais, 2005**).

Table 27: Summary table of evidence for specific target organ toxicity – repeated exposure including extrapolated effective doses for toxicity studies of greater or lesser duration than 90 days

Study reference and species	Toxicological effects and derived effective dose (ED)	Length of exposure in days	ED extra-polated to 90-day	Classification supported by the study
inhalation exposure				
NTP (2007) Rats	**Kidney** (increased incidence of mineralisation of the renal papilla) ED: 1000 ppm in ♂ (~ **4.83** mg/L) ED: 300 ppm in ♀ (~ **1.45** mg/L)	~ 720 (2 years)	♂: ~ **39** / ♀: ~ **12** mg/L	no support for classification (above GV[48] of 1 mg/L)
	Nose (increased incidence of basal cell hyperplasia of the olfactory epithelium) ED: 100 ppm in ♂/♀ (~ **0.48** mg/L)		♂/♀: ~ 4 mg/L	no support for classification (above GV of 1 mg/L)
	Nose (increased incidence of degeneration of the olfactory epithelium) ED: 1000 ppm in ♂ (~ **4.83** mg/L) ED: 300 ppm in ♀ (~ **1.45** mg/L)		♂: ~ **39** / ♀: ~ **12** mg/L	no support for classification (above GV of 1 mg/L)
NTP (2007) Mice	**Nose** (increased incidence of both, metaplasia of the olfactory epithelium and hyperplasia of the submucosal glands (olfactory epithelium))	~ 720 (2 years)	♂/♀: ~ **4** mg/L	no support for classification (above GV of 1 mg/L)

[48] GV – Guidance Values as given in table 3.9.2 and 3.9.3 of the CLP Regulation (1272/2008)

Study reference and species	Toxicological effects and derived effective dose (ED)	Length of exposure in days	ED extra-polated to 90-day	Classification supported by the study
	ED: 100 ppm in ♂/♀ (~ **0.48** mg/L)			
	Nose (increased incidence of atrophy of the olfactory epithelium) ED: 300 ppm in ♂ (~ **1.45** mg/L)		♂: ~ **12** mg/L	no support for classification (above GV of 1 mg/L)
	Forestomach (increased incidence of hyperplasia of the epithelium) ED: 300 ppm in ♂ (~ **1.45** mg/L)		♂: ~ **12** mg/L	no support for classification (above GV of 1 mg/L)
	Forestomach (inflammation) ED: 600 ppm in ♂ (~ **2.9** mg/L)		♂: ~ **23** mg/L	no support for classification (above GV of 1 mg/L)
	Kidney (increased incidence and severity of renal nephropathy) ED: 600 ppm in ♀ (~ **2.9** mg/L)		♀: ~ **23** mg/L	no support for classification (above GV of 1 mg/L)
	Liver (increased incidence of eosinophilic foci) ED: 600 ppm in ♀ (~ **2.9** mg/L)		♀: ~ **23** mg/L	no support for classification (above GV of 1 mg/L)
NTP (2007) Rats	**Kidney** (increased renal cell proliferation) ED: 150 ppm in ♂ (~ **0.73** mg/L)	90		no support for classification (toxicological relevance unclear although below GV of 1mg/L)
	Kidney (elevated α2u-globulin concentrations) ED: 150 ppm in ♂ (~ **0.73** mg/L)			no support for classification (species-specific although below GV of 1mg/L)
	Kidney (urinary markers for kidney toxicity increased) ED: 300 ppm in ♂ (~ **1.45** mg/L) ED: 600 ppm in ♀ (~ **2.9** mg/L)			no support for classification (above GV of 1 mg/L)
	Kidney (increased weight) ED: 1000 ppm in ♂ (~ **4.83** mg/L) ED: 600 ppm in ♀ (~ **2.9** mg/L)			no support for classification (above GV of 1 mg/L)
NTP (2007) Mice	**Nose** (multiple nasal lesions[49]) ED: 75 and 150 ppm ♂/♀ (~ **0.36** and **0.73** mg/L)	90		no support for classification (minimal severity of lesions - biological significance uncertain although below GV of 1mg/L)
	Liver (increased absolute liver weight) ED: 1000 ppm in ♂ (~ **4.83** mg/L) ED: 600 ppm in ♀ (~ **2.9** mg/L)			no support for classification (above GV of 1 mg/L)
	Liver (centrilobular hypertrophy in the liver)			no support for classification (above GV of 1 mg/L)

[49] high incidences of atrophy and metaplasia of the olfactory epithelium and atrophy and hyperplasia of Bowman's glands in ♂ and ♀ at ≥ 75 ppm with statistical significance relative to the control; degree of tissue damages (mean severity) at 75 ppm and 150 ppm rated minimal

Study reference and species	Toxicological effects and derived effective dose (ED)	Length of exposure in days	ED extra-polated to 90-day	Classification supported by the study
	ED: 600 ppm in ♂/♀ (~ **2.9** mg/L)			
oral exposure				
Study report RDT (1997), rats	**Liver** (increased GPT) ED: 200 mg/kg bw/d in ♂	43	~ **96** mg/kg bw/d	no support for classification (toxicological relevance unclear although below GV of 100 mg/kg bw/d)
	Liver (acidophilic change of the hepatocytes) ED: 200 mg/kg bw/d in ♂/♀	♂: 43 / ♀: 53	♂: ~ **96** / ♀: ~ **118** mg/kg bw/d	no support for classification (toxicological relevance unclear although below GV of 100 mg/kg bw/d)
	Liver (increased absolute and relative liver weight) ED: 1000 mg/kg bw/d in ♂ ED: 200 mg/kg bw/d in ♀	♂: 43 / ♀: 53	♂: ~ **478** / ♀: ~ **118** mg/kg bw/d	no support for classification (above GV of 100 mg/kg bw/d)
	Kidney (increased hyaline droplets in the renal tubular epithelium + basophilic changes of the renal tubular epithelium) ED: 200 mg/kg bw/d in ♂	43	~ **96** mg/kg bw/d	no support for classification (toxicological relevance unclear although below GV of 100 mg/kg bw/d)
	Kidney (vacuolation and infiltrated lymphocytes in the renal tubular epithelium) ED: 200 mg/kg bw/d in ♀	53	~ **118** mg/kg bw/d	no support for classification (above GV of 100 mg/kg bw/d)
	Kidney (increased relative liver weight) ED: 1000 mg/kg bw/d in ♂ ED: 200 mg/kg bw/d in ♀	♂: 43 / ♀: 53	♂: ~ **478** / ♀: ~ **118** mg/kg bw/d	no support for classification (above GV of 100 mg/kg bw/d)
	Thymus (atrophy) ED: 200 mg/kg bw/d in ♀	53	~ **118** mg/kg bw/d	no support for classification (toxicological relevance unclear and slightly above GV of 100 mg/kg bw/d)
	Thymus (decreased absolute and relative thymus weight) ED: 1000 mg/kg bw/d in ♀	53	~ **589** mg/kg bw/d	no support for classification (above GV of 100 mg/kg bw/d)

10.12.2Comparison with the CLP criteria

"Target organ toxicity (repeated exposure) means specific, target organ toxicity arising from a repeated exposure to a substance or mixture. All significant health effects that can impair function, both reversible and irreversible, immediate and/or delayed are included." (CLP Regulation 1272/2008, 3.9.1.1.)

Hazard categories for carcinogens (CLP Regulation 1272/2008, Table 3.9.1)

Category 1:

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON 2-PHENYLPROPENE; α-METHYLSTYRENE

"Substances that have produced significant toxicity in humans or that, on the basis of evidence from studies in experimental animals, can be presumed to have the potential to produce significant toxicity in humans following repeated exposure. Substances are classified in Category 1 for target organ toxicity (repeat exposure) on the basis of:

- *reliable and good quality evidence from human cases or epidemiological studies; or*
- *observations from appropriate studies in experimental animals in which significant and/or severe toxic effects, of relevance to human health, were produced at generally low exposure concentrations. Guidance dose/ concentration values are provided below (see 3.9.2.9), to be used as part of a weight-of- evidence evaluation."*

Category 2:

"Substances that, on the basis of evidence from studies in experimental animals can be presumed to have the potential to be harmful to human health following repeated exposure.

Substances are classified in category 2 for target organ toxicity (repeat exposure) on the basis of observations from appropriate studies in experimental animals in which significant toxic effects, of relevance to human health, were produced at generally moderate exposure concentrations. Guidance dose/concentration values are provided below (see 3.9.2.9) in order to help in classification. In exceptional cases human evidence can also be used to place a substance in Category 2 (see 3.9.2.6)."

inhalation exposure

As described above, a number of experimental animal studies addressing the toxicity of 2-phenylpropene following repeated exposure are available. Relevant effects are summarised in Table 27 including the derived effective dose levels and a short conclusion as to whether the effects may justify classification.

The most reliable information is provided by the two **90-day NTP** inhalation studies in rats and mice. The authors of the study identified the kidney in rats and the nose in mice as the primary target organ. In rats, an increase of renal cell proliferation and elevated α2u-globulin concentrations in the kidney were found at 150 ppm and may justify classification into Category two. However, while the latter effect is clearly male rat-specific (cf. section 10.9.2 [k]), the increase in renal cell proliferation may not be considered toxicological relevant at this concentration as the finding is neither significant nor severe[50]. Urinary markers for renal toxicity that may indicate a functional disturbance of the kidney were elevated only at higher concentrations above the GV in males and females. Other effects in support of kidney toxicity such as higher incidences of mineralisation of the renal papilla were noted in both sexes in the 2-years NTP inhalation study. Thus, renal findings potentially relevant for STOT RE classification have only been found at concentrations above the GV values. However, the renal findings both in rats and mice obtained in subchronic and chronic NTP inhalation studies may be relevant for purpose of classification for carcinogenicity and thus are already covered by the carcinogenicity classification as described in section 10.9.
In mice, multiple nasal lesions were seen at all concentrations in both sexes in the 90-day NTP inhalation studies, potentially allowing classification for STOT RE. As a result of repeated, subchronic insult, degenerative changes of the olfactory mucosa were noted, including atrophy of the olfactory epithelium (decreased layers of neuronal cells with loss of associated axons) and Bowman's glands beneath (loss of Bowman's glands). Following repeated injuries, morphological changes of the nasal epithelium range from associated regeneration of epithelium, atrophy, metaplasia, hyperplasia, and in some case with progression neoplasia (Renne et al., 2009). While the latter was not observed upon 2-phenylpropene exposure, metaplasia of the olfactory epithelium and a hyperplastic replacement of the glandular epithelium was observed. The degree of tissue damage, e.g. severity of the lesions, were rated minimal at the two lowest concentration and mild at 300 ppm and greater. The incidence of hyaline degeneration of the respiratory epithelium was increased in females with statistically significance at 150 ppm and greater. The severity of these lesions was rated minimal at all concentrations. Necrotic alterations were noted at the highest concentration in females. The observed alterations, especially of the olfactory epithelium, are morphological changes of toxicological relevance. The observed incidences are remarkably high, even at the lowest concentration tested. However, the degree of severity of these histopathological nasal changes was rated minimal (lowest possible severity grade) at concentrations relevant for classification (≤ 150 ppm). The biological significance of the findings is, therefore, uncertain and classification for STOT RE may not be justified.
Non-neoplastic effects in the liver observed in the 2-year and 90-day NTP inhalation study in mice may be related to liver carcinogenicity (cf. section 10.9).

[50] as stated in ECHAs guidance on the application of the CLP criteria (version 5, 2017): *"[…] 'significant' means changes which clearly indicate functional disturbance or morphological changes which are toxicologically relevant. 'Severe' effects are generally more profound or serious than 'significant' effects and are of a considerably adverse nature which significantly impact on health."*

No indication for effects that would justify a STOT RE classification can be derived from the two non-guideline inhalation studies by **Wolf et al. (1956)** and **Morgan et al. (1999)**. Apart from morphological changes of the hyaline droplets in the renal tubules of male rats seen in the latter study, no other histopathological findings were observed in rats, mice, guinea pigs, rabbits, and rhesus monkeys. The kidney effects may be related to α2u-globulin nephropathy and are consequently of unknown relevance. Changes in organ weights (liver, kidney, lung, spleen) were observed in the absence of evidence of organ dysfunction and may, therefore, not support classification.

oral exposure

Next to the inhalation studies, two oral studies provide information on the endpoint. As reported on ECHAs dissemination page, toxicological effects of 2-phenylpropene were studies in an **OECD TG 422** by oral administration for 43 and ~53 days in male and females, respectively. Treatment-related effects were noted in the liver, the kidney, and the thymus. Higher serum levels of GTP (glutamic oxaloacetic transaminase; aspartate transaminase AST) were found in males exposed to 200 mg/kg bw/d (equivalent to 95 mg/kg bw/d when extrapolated to 90 days of exposure) or more. Elevated GTP levels are indicative of hepatocellular damages (Ozer et al., 2008). The levels of other enzymatic markers (AST, ALP, GGT) were analysed but no treatment-related changes were reported. Histomorphological alterations described as acidophilic change of the hepatocytes[51] were noted in the liver of male and female mice at 200 mg/kg bw/d or more. The absolute and relative weight of the kidney was higher in females at the same dose and in males at 1000 mg/kg bw/d. The toxicological importance of the liver effects observed at 200 mg/kg bw/d is uncertain. It is doubtful as to whether they reflect significant organ damage at least at this dose level. Apart from organ weight change and an increase in bile acid concentration, no other relevant findings related to hepatotoxicity was seen in inhalation studies with rats. In the same study, histopathological changes were also reported in the kidney. While male rats exhibited alterations of the hyaline droplets and renal epithelium (basophilic changes) at 200 mg/kg bw/d, females displayed vacuolation and lymphocyte infiltration in the renal tubular epithelium at the same dose. The relative kidney weight was already increased at 200 mg/kg bw/d in females. The results in the kidney are considered neither significant nor severe. There is no evidence for a functional disturbance of the kidney at 200 mg/kg bw/d. In addition to the liver and kidney responses, some effects were observed in the thymus of females exposed to 200 mg/kg bw/d. The findings were consistent with atrophic changes that manifested in a decrease of absolute and relative thymus weight at higher doses in females (1000 ppm). The thymus is a very sensitive organ, which is susceptible to the effects of stress and ageing. Involution of the thymus is a normal age-related mechanism. Chemical-induced thymic atrophy must, therefore, be differentiated from changes induced by stress and ageing. Enhanced histopathology of the immune system is, therefore, recommended (Elmore, 2006). Other effects indicative of thymus toxicity or general immunotoxicity have not been reported in studies listed in Table 26. The toxicological relevance of this finding is therefore unknown. The week ototoxic effects described by Gagnaire and Langlais (2005) do not qualify for STOT RE classification as they were seen only at a concentration above the GV.

10.12.3Conclusion on classification and labelling for ~~aspiration hazard~~ STOT RE

Classification of 2-phenylpropene for STOT RE is not recommended.

[51] micro-granular acidophilic cells were diffusely spread in the entire small lobules, loss of fatty droplets in ♂ /micro-granular acidophilic cells distinctively around the centre of the lobules and enlarged hepatocytes in ♀

RAC evaluation of specific target organ toxicity – repeated exposure (STOT RE)

Summary of the Dossier Submitter's proposal

Repeated dose toxicity of α-methylstyrene has been investigated in 3-month and 2-year inhalation studies in rats and mice and in an oral study in rats according to OECD TG 422. Several non-guideline studies are also available.

The kidney has been identified as the main target organ in rats. The renal effects were not considered sufficient for classification by the DS mainly because the changes below the guidance value (GV) were of low severity and some of them are likely to be related to α2μ-globulin nephropathy (male rat-specific phenomenon). Nasal lesions were the most sensitive effect upon inhalation exposure in mice but the effects below the GV were also of a rather low severity. The DS further discussed findings in the liver and the thymus.

The DS concluded that criteria for a STOT RE classification are not met.

Comments received during consultation

Comments were received from 1 MSCA, who pointed out the high incidence (in nearly all animals) of nasal lesions (atrophy, metaplasia) in the mouse study. They were of the view that, despite their low severity, the findings should be considered as significant toxic effects warranting classification as STOT RE 2. The DS acknowledged that the nasal lesions at 75 and 150 ppm might represent a borderline case. Still, they retained their proposal of no classification due to the low severity and the existing classification as STOT SE 3; H335, which at least partly covers the findings in the respiratory tract.

Assessment and comparison with the classification criteria

Effects in the key studies are summarized in Table 27 of the CLH report. The RAC assessment focuses on effects below the (extrapolated) GVs.

3-to 12-day inhalation studies in rats and mice (Morgan et al., 1999)

These short-term studies in F344 rats and B6C3F1 mice reported hyaline droplet accumulation in the kidneys of male rats, increased liver weight in both species without a histopathological correlate and decreased spleen weight in mice. No histopathological changes were observed in the nasal cavity of mice. Further details can be found under 'supplemental information' in the carcinogenicity section. However, the shorter-term studies are considered less informative in relation to STOT RE classification than the available 3-month studies via the same route (studies of longer duration are generally given more weight in the classification, according to the Guidance on the application of the CLP criteria, version 5.0 (2017), section 3.9.2.3.2).

3-month inhalation studies in rats and mice (NTP, 2007)

F344 rats (10/sex/group) and B6C3F1 mice (10/sex/group) were exposed to α-methylstyrene via inhalation (whole body) for 14 weeks (6 hours/day, 5 days/week) at 0, 75, 150, 300, 600 and 1000 ppm.

The effects at 150 and 75 ppm are below the GV for classification in category 2 (the 90-day inhalation study GV is 250 ppm).

The following effects were observed at 150 ppm in the 3-month rat study:

- Increased liver weight in males (relative by 10%)
- Increased α2μ-globulin and increased kidney cell proliferation (measured by a labelling technique) in males

RAC agrees with the DS that these findings are not sufficiently severe to warrant classification.

As to the mouse study, the histopathological changes in the nasal tissues are presented in the table below.

Nasal lesions in the 3-month mouse inhalation study (NTP, 2007)						
Concentration (ppm)	**0**	**75**	**150**	**300**	**600**	**1000**
Males						
No. of males per group	10	10	10	10	10	10
Bowman's glands atrophy: incidence (mean severity score)	0	7** (1.0)	10** (1.3)	10** (1.9)	10** (2.0)	10** (2.0)
Bowman's glands hyperplasia (mean severity score)	0	9** (1.1)	10** (1.6)	10** (2.3)	10** (2.9)	10** (2.7)
Olfactory epithelium atrophy (mean severity score)	0	10** (1.1)	10** (1.4)	10** (2.0)	10** (2.0)	10** (2.1)
Olfactory epithelium metaplasia (mean severity score)	0	5* (1.2)	10** (1.4)	10** (2.0)	10** (2.0)	10** (2.0)
Respiratory epithelium hyaline degradation (mean severity score)	0	1 (1.0)	2 (1.0)	1 (1.0)	2 (1.0)	0
Females						
No. of females per group	10	10	10	10	10	10[a]
Bowman's glands atrophy (mean severity score)	0	8** (1.0)	9** (1.3)	10** (2.0)	10** (2.0)	8** (2.5)
Bowman's glands hyperplasia (mean severity score)	0	5* (1.0)	10** (1.7)	10** (2.3)	10** (2.6)	8** (2.6)
Olfactory epithelium atrophy (mean severity score)	0	10** (1.0)	10** (1.6)	10** (2.0)	10** (2.0)	8** (2.0)
Olfactory epithelium metaplasia (mean severity score)	0	4* (1.0)	9** (1.7)	10** (2.0)	10** (2.0)	8** (2.0)

Olfactory epithelium necrosis (mean severity score)	0	0	0	0	0	2 (3.0)
Respiratory epithelium hyaline degradation (mean severity score)	0	2 (2.0)	6** (1.3)	9** (1.6)	8** (1.4)	4* (1.0)

Statistically significant difference from control: *, $p \leq 0.05$; **, $p \leq 0.01$

Severity scores: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked

[a] Two top concentration females died before exposure on day 3; they had necrosis of the olfactory epithelium

The substance affected mainly the olfactory epithelium and virtually all animals were affected already at the lowest concentration tested (75 ppm). Atrophy and metaplasia of olfactory epithelium are generally significant toxic effects that may warrant classification. However, in this case the severity at relevant concentrations (150 ppm and below) was relatively low (minimal to mild; cf. the criteria of "significant organ damage" or "marked organ dysfunction" according to CLP, Annex I, 3.9.2.7.3) and no substantial increase in severity was observed at higher levels. A STOT RE classification for respiratory tract effects is therefore not considered justified.

Reproductive toxicity screening in rats according to OECD TG 422 via oral route (1997)

CD(SD) rats (10/sex/group) were administered α-methylstyrene in olive oil via gavage at 0, 40, 200 and 1000 mg/kg bw/d. Males were dosed for 43 days, females for up to 53 days (14 days prior to mating and then throughout mating and gestation until termination on lactation day 4). The following effects were observed at the mid-dose of 200 mg/kg bw/d, which is around the extrapolated GV for classification in Category 2:

- Liver: increased liver weight in females, increased ALT in males, acidophilic change in hepatocytes (both sexes)
- Kidney: increased hyaline droplets and basophilic change of the renal tubular epithelium in males, vacuolation of the renal tubular epithelium in females (vacuoles identified as lipid droplets)
- Thymus: thymus atrophy in females

Further details (incidence, severity, magnitude of increases) are not provided in the CLH report.

The 'acidophilic change' in the liver was described as micro-granular acidophilic cells around the centre of the lobules (females) or diffusely spread (males), in males associated with loss of fatty droplets. Based on the available description, the liver findings are not considered sufficient for a STOT RE classification.

Hyaline droplets in male rats are probably related to accumulation of α2μ-globulin, an effect specific to male rats. Basophilic change of the renal tubular epithelium may indicate renal damage, possibly related to an additional mode of action besides α2μ-globulin nephropathy. Still, the kidney effects at 200 mg/kg bw/d are not considered sufficient for a STOT RE classification.

The severity of thymus atrophy in females at 200 mg/kg bw/d is not described in the CLH report. In general, thymus atrophy may represent a specific toxic effect or a non-specific response to stress. As there was no effect on thymus weight or histopathology in males in

this study up to 1000 mg/kg bw/d, and no effect on the thymus was reported to occur in the inhalation studies by NTP (2007), it is uncertain whether the substance specifically affects this organ.

Conclusion

Kidney is the main target organ of α-methylstyrene in rats. However, the effects below the GVs are not sufficient for classification.

Nasal lesions represent the most sensitive effect in the mouse inhalation studies. Although atrophy of olfactory epithelium is generally a significant toxic effect, the severity of the finding at relevant concentrations was low in this case and no substantial increase in severity was observed at higher levels. Therefore, a STOT RE classification for respiratory tract effects is not considered justified.

In conclusion, RAC agrees with the DS's proposal of **no classification for STOT RE**.

10.13 Aspiration hazard

Hazard class not assessed in this dossier.

11 EVALUATION OF ENVIRONMENTAL HAZARDS

Hazard class not assessed in this dossier.

12 EVALUATION OF ADDITIONAL HAZARDS

Not assessed in this dossier.

13 ADDITIONAL LABELLING

Not assessed in this dossier.

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15 ANNEXES

Annex I