# Committee for Risk Assessment

# RAC

## Opinion

proposing harmonised classification and labelling
at EU level of

## Cumene

## EC Number: 202-704-5
## CAS Number: 98-82-8

CLH-O-0000006849-56-01/F

## Adopted

## 17 September 2020

P.O. Box 400, FI-00121 Helsinki, Finland | Tel. +358 9 686180 | Fax +358 9 68618210 | echa.europa.eu

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CLH-O-0000006849-56-01/F

# OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonised classification and labelling (CLH) of:

**Chemical name:** **Cumene**

**EC Number:** **202-704-5**

**CAS Number:** **98-82-8**

The proposal was submitted by **Denmark** and received by RAC on **15 August 2019.**

In this opinion, all classification and labelling elements are given in accordance with the CLP Regulation.

# PROCESS FOR ADOPTION OF THE OPINION

**Denmark** has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **23 September 2019**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **22 November 2019**.

### ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Annemarie Losert**

Co-Rapporteur, appointed by RAC: **Nathalie Printemps**

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The RAC opinion on the proposed harmonised classification and labelling was adopted on **17 September 2020** by **consensus**.

**Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)**

| | Index No | Chemical name | EC No | CAS No | Classification | | Labelling | | | Specific Conc. Limits, M-factors and ATE | 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 | 601-024-00-X | Cumene [1]<br>Propylbenzene [2] | 202-704-5 [1]<br>203-132-9 [2] | 98-82-8 [1]<br>103-65-1 [2] | Flam. Liq. 3<br>STOT SE 3<br>Asp. Tox. 1<br>Aquatic Chronic 2 | H226<br>H304<br>H335<br>H411 | GHS02<br>GHS07<br>GHS08<br>GHS09<br>Dgr | H226<br>H304<br>H335<br>H411 | | | C |
| Dossier submitters proposal | TBD | Cumene | 202-704-5 | 98-82-8 | **Add**<br>Carc. 2 | **Add**<br>H351 | | **Add**<br>H351 | | | |
| RAC opinion | TBD | Cumene | 202-704-5 | 98-82-8 | **Add**<br>Carc. 1B | **Add**<br>H350 | | **Add**<br>H350 | | | |
| Resulting Annex VI entry if agreed by COM | TBD | Cumene | 202-704-5 | 98-82-8 | Flam. Liq. 3<br>Carc. 1B<br>Asp. Tox. 1<br>STOT SE 3<br>Aquatic Chronic 2 | H226<br>H350<br>H304<br>H335<br>H411 | GHS02<br>GHS07<br>GHS08<br>GHS09<br>Dgr | H226<br>H350<br>H304<br>H335<br>H411 | | | |

# GROUNDS FOR ADOPTION OF THE OPINION

## RAC general comment

Cumene is an alkylbenzene mainly used as an intermediate for the production of phenol and acetone. In addition, the substance is a minor constituent of gasoline and solvents. The proposal from the dossier submitter (DS) addressed the following endpoints: mutagenicity, carcinogenicity and toxicity to the reproduction.

The substance has an existing Annex VI entry to CLP (assessed under Directive 67/548/EEC; 25$^{th}$ ATP). The current entry (621-024-00-X) is for cumene (iso-propylbenzene) (EC 202-704-5 [1]; CAS 98-82-8 [2]) and propylbenzene (n-propylbenzene) (203-132-9 [2]; 103-65-1 [2])). The CLH dossier is only on cumene and does not include propylbenzene. A new entry (in addition to the existing one) will thus be created keeping the existing hazard classifications not under discussion in the DS proposal. Note C "*Some organic substances may be marketed either in a specific isomeric form or as a mixture of several isomers*" will be removed from the entry of cumene and propylbenzene.

Following inhalation, cumene is readily absorbed and extensively metabolised by cytochrome P450 enzymes. In both human and experimental animals, the oxidation of the side chain of cumene to 2-phenyl-2-propanol is a key step (both of these compounds are found in human and in animal urine). Other quantitatively less important metabolic pathways observed in mice or rats includes reactive metabolites in animals such as $\alpha$–methyl styrene (side chain oxidation of 2-phenyl-2-propanol) which was observed in expired air of mice (at trace level in rats). This metabolite may be further oxidised to $\alpha$–methyl styrene oxide. In addition, 2-(2-hydroxy-2-propyl)phenylsulfate and 4-(2-hydroxy-2-propyl)phenylsulfate (ring oxidation) were found in the urine of mice and rats. Some studies suggested that metabolism of cumene was more efficient in mice than in rats. These oxidized metabolites are primarily excreted as sulfate or glucuronide conjugates.

# HUMAN HEALTH HAZARD EVALUATION

## RAC evaluation of germ cell mutagenicity

### Summary of the Dossier Submitter's proposal

*In vitro,* six negative Ames tests, one positive and one negative spot test in bacteria and one negative mutation assay in yeast on cumene were available. Cumene was also considered negative by the DS in two studies for *in vitro* gene mutation in mammalian cells. The available *in vitro* cytogenicity test (Putman, 1987a) was negative without metabolic activation, but inconclusive in presence of metabolic activation. A positive result observed in a first unscheduled DNA synthesis (UDS) study (Gulf oil corporation, 1984) was not confirmed in a retest (Curren, 1987). An overall negative outcome was concluded by the DS *in vitro*.

Four *in vivo* micronucleus tests were available, two were performed in mice (inhalation or gavage administration) and two in rats (intraperitoneal or gavage administration). These studies were considered reliable with limitations and similar to OECD TG except the gavage study in mice which was rated as unreliable (Gulf corporation, 1985b). The justification for the Klimish score 3 was not provided. All tests were negative except the study performed by intraperitoneal route in rats (NTP, 2009).

Three *in vivo* comet assays were reported with cumene (NTP, 2012 and Kim *et al.*, 2008). In the NTP gavage studies in male rats and male and female mice, the results were negative in blood and kidney. Weakly positive results were reported in male rats in the liver and in female mice in the lung. A fragment length analysis with repair enzyme (FLARE) combined with a comet assay in lymphocytes and hepatocytes was performed by Kim *et al.*, 2008 after subchronic inhalation exposure in rats. There were some indications of oxidative DNA damage from cumene exposure, but no dose-response was observed in the study.

Analysis of mutations in the cumene-induced lung tumours in mice of the NTP, 2009 carcinogenicity study found significant increases of *K-ras* and *p53* mutations and different types of mutations in cumene exposed mice compared to mutations in spontaneous tumours of the control group (Hong *et al.*, 2008). In addition, loss of heterozygosity (LOH) was detected in cumene induced tumours, not observed in spontaneous tumours.

Data on metabolites were also retrieved by the DS. The metabolite α-methylstyrene was negative in an Ames assay and in an *in vitro* chromosomal aberration assay (NTP, 2007). *In vivo*, α-methylstyrene was weakly positive in female mice but not in male mice in an *in vivo* micronucleus assay following 3-month inhalation exposure (NTP, 2007). Negative results were observed in an *in vivo* single gavage micronucleus study in male mice (Rim *et al.*, 2012). α-methylstyrene is postulated to be further oxidised to α-methylstyrene oxide. This metabolite was reported to be positive in an Ames assay (Rosman *et al.*, 1986).

Overall, the DS concluded that most of the data available on cumene are negative and that there are only few indications of a genotoxic potential:

- DNA damage in male liver or lung of female mice. It is speculated by the DS that the DNA damage may be due to oxidative damage in target organs.
- The postulated α-methylstyrene oxide metabolite may be mutagenic, but the quantitative relevance of this metabolite is unknown and not confirmed by direct observation with cumene.
- Changed profiles and increases of *K-ras* and *p53* mutations in cumene induced lung tumours may either point to mutagenicity of cumene or secondary genotoxicity (e.g. from reactive oxygen species, resulting in genetic instability and/or impairment of repair mechanisms) or epigenetic changes.

Overall, the DS concluded that the CLP criteria for germ cell mutagenicity were not fulfilled as no evidence is available that cumene is a germ cell mutagen and as the evidence for a primarily genotoxic mode of action (MoA) for cumene carcinogenicity is unlikely.

## Comments received during consultation

One MS requested details on the reliability of some studies and highlighted that in some cases, positive intraperitoneal studies may already lead to classification as Muta. 2; H341.

Two industry representatives and two individuals agreed with no classification for germ cell mutagenicity for cumene. They commented that cumene does not pose a mutagenic hazard. They provided some remarks on the DS's proposal:

- K-*ras* and *p53* reported mutation may be more a resulting effect from rapidly dividing tissues than a cause. Moreover, it may be a consequence of irritation combined with inflammation leading to reactive oxygen species (ROS) generation rather than any direct activity from cumene itself.
- Although a positive *in vivo* micronucleus assay was observed in rats *via* intraperitoneal injection, negative results for clastogenicity/aneugenicity were provided by other studies with more relevant route of exposure.

- The borderline increase in the percentage tail DNA in the *in vivo* comet assay may have been related to random background variations and did not correlate with cumene tumorigenic profile.

## Assessment and comparison with the classification criteria

### *In vitro results*

Six negative studies for gene mutation in the Ames test were provided on cumene in various vehicles (ethanol, DMSO, pluronic F127 in 50% ethanol). Only one study was rated reliable with limitation (Monsanto Co, 1985). Klimish score in the other studies was either not reliable or not assignable (due to missing strains, inadequate exposure due to volatility or limited data information on the study). Considering the overall database, all strains recommended in OECD TG 471 were tested up to cytotoxic concentration, including strain *E. Coli* WP2. Both the preincubation methods or direct plate incorporation were used. Sealed tubes were used in the NTP, 2012 study. One negative *in vitro* gene mutation study was also available in yeast. A positive and a negative spot test were also reported but the results of these studies are considered of negligible weight as compared to the six negative Ames assays. Overall, RAC agrees with the DS that cumene did not induce gene mutation in bacteria in presence or absence of metabolic activation.

Two studies for gene mutation in mammalian cells were also available (Gulf oil corporation, 1985a; Yang, 1987). The studies were reported to be similar to OECD TG 476. According to NTP, 2013 evaluation, Gulf oil corporation study had to be retested due to variable background and colony forming efficiency. The same limitations were noted in the retest study from Yang, 1987 (see supplemental information – in depth analysis by RAC). Therefore, reliability of these studies is questionable. In addition, due to the limitations of these studies, RAC considers the increase in mutation frequency in presence of metabolic activation observed in both studies inconclusive rather than positive.

One *in vitro* mammalian chromosome aberration test was available with cumene (Putman, 1987a). The study was performed according to OECD TG 473 but some limitations were noted by RAC: limited information of methods and results provided in the CLH dossier and in the ECHA disseminated database, lower number of metaphases analysed compared to current test guideline, only short-term exposure duration, no repeated experiments and cell growth for main experiment was not reported. The study was negative in absence of rat metabolic activation. A statistically significant increase in cells with structural aberrations were reported at the highest dose tested (156 µg/ml) in presence of metabolic activation compared to the vehicle control. The increase was not statistically significant compared to untreated control and was within historical control range of the laboratory. The increase in chromosomal aberration may thus not be toxicologically relevant.

Cumene did not induce unscheduled DNA synthesis (UDS test) *in vitro*. Although positive results were observed in one study, negative results were obtained following retest. Nevertheless, due to its low sensitivity, the UDS test is considered of low weight.

Overall, cumene was not mutagenic in bacteria in presence or absence of metabolic activation. Inconclusive results were obtained for gene mutation in mammalian cells in presence of metabolic activation due to study limitations (see supplemental information – in depth analysis by RAC). Negative results were observed for cytogenicity in mammalian cells in presence or absence of metabolic activation. Nevertheless, some limitations were noted in the study.

***In vivo results***

In mice, negative results were obtained in a 3-month inhalation exposure bone marrow micronucleus study up to 500 ppm in females and 1000 ppm in males. In male rats, positive results were obtained following intraperitoneal route (NTP, 2009). To clarify the positive result, micronucleus studies in peripheral blood were performed by gavage in male rats up to 800 mg/kg and male and female mice up to 1000 and 1250 mg/kg, respectively during 4 consecutive days (NTP, 2012). The studies were negative. Proof of exposure was observed in rats. The positive results obtained following intraperitoneal route of exposure is of low weight compared to the three negative studies using relevant routes of human exposure (oral, inhalation). Nevertheless, as this is the only micronucleus assay via the intraperitoneal route, the finding cannot be completely neglected. The studies were equivalent to OECD TG 474. Overall, cumene did not induce damage at chromosomal levels in rats and mice *in vivo*.

An *in vivo* rodent comet assay was also performed on the same male rats and male and female mice that were evaluated for the micronucleus endpoint (NTP, 2012). The study was equivalent to OECD TG 489. The substance was tested via gavage application in corn oil. There were two main limitations in this study:

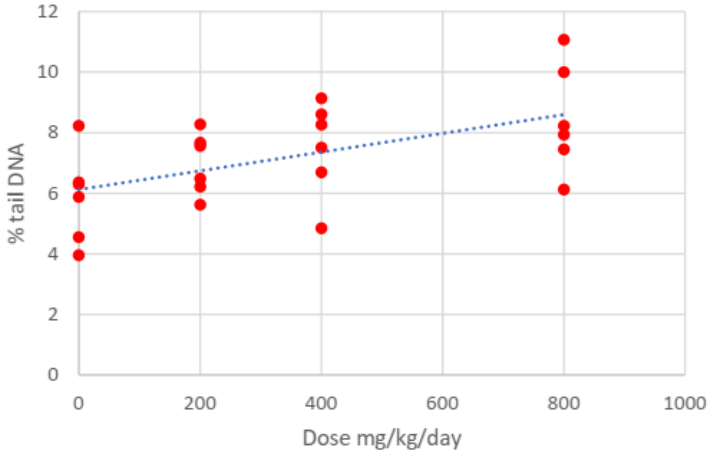
- Historical negative and positive controls were not provided. High variability could be an issue in comet assays.
- No information on cytotoxicity in tested tissues was provided. Histopathology at the top dose in the organs were not available. Nevertheless, Hedgehog cells were excluded from analysis. Moreover, in the 2-week and 13-week inhalation studies in rats and mice (NTP, 2009), only liver weight changes were noted without concomitant necropsy findings up to maximum tolerated dose. Therefore, liver toxicity is not expected to be an issue in the interpretation of the comet assay. No effects on lungs were noted in the 13-week study.

The assay was performed in blood leukocytes, liver, lung and kidney. Negative results were observed in male mice (all tissues), male rat leukocytes, lung and kidney and in female mice leucocytes, liver and kidney. An increase in the % Tail DNA was observed in male rat liver (statistically significant only at the top dose) and female mice lung. The table below reports the results of the comet assay in lung and liver (From NTP, 2012 report).

| Dose (mg/kg) | % Tail DNA (mean ±SD) | | |
|---|---|---|---|
| | Female mice, lung | Male mice, lung | Male rat, liver |
| 0 | 6.8±0.3 | 11.9±1.2 | 5.9±0.6 |
| 250 | 7.3±0.6 | 12.2±0.8 | 7.0±0.4 |
| 500 | 7.8±0.7 | 13.7±1.3 | 7.5±0.6 |
| 1000 | 8.7±0.7* | 13.0±1.3 | 8.5±0.7** |
| EMS | 25±1.2*** | 16.7±1.0** | 37±0.6*** |

*p≤0.05; **p≤0.01; ***p≤0.001; EMS: Ethylmethane sulfonate

The figure below described individual data for % tail DNA in male rat liver with trend line (as provided by one individual during the consultation).




There are three main criteria to conclude on a positive result in a comet assay: dose-relation, statistical significance and the biological relevance (increases above concurrent historical negative control range). In male rat liver and female mice lung, the increase in % tail DNA at the top dose was statistically significant compared to concurrent negative control. Moreover, the increases were also dose-related (trend test). The distribution of the historical negative control data was not provided in the NTP report. Therefore, there are some uncertainties on the toxicological significance of the increase in the % tail DNA in mouse female lung and male liver. According to the DS in the response to comment, an additional comet assay for styrene-acrylonitrile trimer was performed in 2012 by NTP in juvenile F344 rats (3-4 week of age). A higher DNA damage background in liver was found (10.605 ± 2.951 %). Nevertheless, RAC notes that DNA background may differ between F344 young adult rats used in the cumene study (8-weeks of age). Moreover, the high variability observed in the styrene-acrylonitrile trimer study (individual values between 5.3 and 21.9%) was not observed in the case of cumene. Indeed, the variability in the controls and treated groups was similar based on standard variation. Therefore, there are no data suggesting that the current control results for liver would not be reliable. Regarding the lung tissue, higher background values were obtained in negative control and lower background values in positive controls male mice. Variability in background values were thus observed in this tissue.

***Genotoxicity of cumene metabolites***

α–methyl-styrene (AMS) did not induce gene mutation in Ames test (TA 102 and *E. coli* not tested) and chromosomal aberration *in vitro* in mammalian cells. No *in vitro* test for gene mutation in mammalian cells was available. Positive effects in sister chromatid exchange (SCE) *in vitro* were observed in two studies (NTP, 2007; Norppa and Vainio, 1983). Nevertheless, SCE assay is considered to have a lower weight than the negative *in vitro* cytogenicity test. The putative metabolite α-methylstyrene oxide was reported to be mutagenic in an Ames test (TA 100) in the CLH dossier.

*In vivo*, α-methylstyrene was positive in female mice for micronuclei induction (trend, highest dose tested) following 3-month inhalation exposure, but was negative in male mice (NTP, 2007). Another recent test on micronuclei formation in male mice bone marrow cells was negative following single administration by gavage (Rim *et al.*, 2012). There are no *in vivo* data available for the postulated metabolite α-methylstyrene oxide. No data were available on other metabolites of cumene.

RAC notes that the pathways leading to reactive metabolism were only minor pathways of the substance (not fully quantified).

***Mechanism of genotoxicity***

Some studies investigated the genotoxicity MoA of cumene.

In Kim *et al.*, 2008 DNA damage from reactive oxygen species was measured in rats that were treated with cumene by inhalation at doses up to 800 ppm for up to 13 weeks using fragment length analysed with repair enzyme (FLARE) formamidopyrimidine (Fpg)/endonuclease III (Endo III) in conjunction with comet assay. Based on the limitations reported by the DS, RAC considered the study not adequate for the evaluation of cumene (inadequate reporting, unacceptable controls, and inappropriate statistical analysis).

In the published study from Hong *et al.*, 2008, point mutations were evaluated in the *K-ras* (exon 1 and 2) and *p53* genes (exons 5 to 8) in a subset of lung neoplasms observed in the carcinogenicity studies (NTP, 2009). LOH was also analysed at the p16 locus in chromosome 16 and near the *K-ras* gene on chromosome 6. A significant dose-dependent increase of *K-ras* and *p53*- mutations from cumene exposed mice compared to mutations in spontaneous tumours in the control group were observed. *K-ras* codon 12 G to T transversion and *K-ras* codon 61 A to G transitions clearly differs from untreated mice (0.008% vs 2%). The table below presents the *K-ras* and *p53* mutations in lung neoplasms of mice in the two-year study of cumene (Hong *et al.*, 2008).

| Treatment (ppm) | Activate *K-ras* (%) | Activate *p53* (%) |
|---|---|---|
| 0 (Concurrent control) | 1/7 (14) | 0/7 (0) |
| 0 (Historical control) | 33/117 (28) | Not provided |
| Cumene (total of neoplasm) | 45/52 (87) | 27/52 (52) |
| 125 | 1/4 (25) | 0/4 (0) |
| 250 | 10/13 (77) | 5/13 (38) |
| 500 | 17/18 (94) | 11/18 (38) |
| 1000 | 17/17 (100) | 11/17 (65) |

According to the authors, the mutation observed in the *p53* genes (Exon 5 and 7 only, no mutation detected at exon 6 and 8) was clearly induced by cumene as this mutation was not detected in spontaneous tumours. No differences in mutation spectrum was observed between adenomas and carcinomas. Mutations were higher in males than in females. In addition, cumene-induced lung carcinomas showed LOH on chromosome 4 near the *p53* gene (13%) and on chromosome 6 near the *K-ras* gene (12%). No LOH was observed in spontaneous carcinomas or in normal lung tissues examined. The authors concluded that direct and indirect DNA damage may have contributed to the mutations. Direct DNA adducts and subsequent point mutation may have been caused by reactive metabolites of cumene. Indirect damage from oxidative stress may also have contributed to the mutations. G to T transversion are consistent with 8-OH-G adducts produced during oxidative damage. The authors concluded that the patterns of *K-ras* and *p53* mutations identified in the cumene-induced lung tumours suggest that DNA damage and genomic instability may be the contributing factors to the mutation profile and development of lung cancer in mice.

***Comparison with criteria***

**In conclusion**, there are no human data in the literature, and based on the animal data available, there is no concrete evidence that cumene is mutagenic to germ cells or that it distributes to the reproductive tissues. Therefore, the criteria to classify a substance as a germ cell mutagen in Category 1B according to the CLP criteria are not met.

The classification in category 2 is based on positive evidence 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.

Cumene did not induce damage at chromosomal levels *in vitro* in mammalian cells. The positive results observed in the intraperitoneal micronucleus test *in vivo* may indicate an intrinsic genotoxic potential of the substance. It is further noted that a weakly positive result in females was also obtained in a micronucleus test after 3-month inhalation exposure to α-methyl styrene in mice (but not in males). Nevertheless, the negative results observed in the *in vivo* micronucleus assays with cumene using relevant route of exposure (inhalation, gavage) in mice and rats decrease the concern.

As regards gene mutations, gene mutation assays in bacteria were negative. *In vitro* gene mutation assays in mammalian cells were inconclusive due to study deficiencies. *In vivo*, the positive comet assays in liver of male rats and lung of female mice performed according to a relevant route of exposure (inhalation) indicate potential of cumene to induce gene mutation in somatic cells. As discussed above, the absence of historical control range raises some uncertainties on the biological relevance of the results of the comet assay. Hong *et al.*, 2008 study showed that in mice lung tumours induced by cumene increase in point mutation in *K-ras,* and *p53* mutations in specific investigated exons were observed. Although the results may be explained by direct mutagenicity through reactive metabolites, the substance may also act through an indirect MoA *via* oxidative damages. Epigenetic changes may also be involved (Wakamatsu *et al.*, 2008). They are discussed in more detail in the carcinogenicity section under "Assessment and comparison with the classification criteria". Nevertheless, the analysis was only performed on a subset of lung tumour tissues leading to some uncertainties on the results. RAC also notes that except for mice lungs, the positive results of the comet assay were not fully consistent with the tumorigenic profile of cumene in rats (kidney and respiratory epithelium tumours) and mice (lung and liver tumours). Nevertheless, the difference in the route of exposure between the studies (oral vs inhalation) make the comparison difficult.

Overall, although a weak genotoxic potential of cumene cannot be excluded, RAC agrees with the DS that the criteria for Germ cell mutagen in category 2 (H341) are not fulfilled. **No classification for germ cell mutagenicity is warranted for cumene**.

## RAC evaluation of carcinogenicity

### Summary of the Dossier Submitter's proposal

For the assessment of carcinogenicity, the DS included two high-quality carcinogenicity studies in mice and rats via the inhalation route (NTP, 2009). In addition, two transformation assays in mice (Putman, 1987b; Gulf Oil corporation, 1984) and a mechanistic study in mice lung tumours (Wakamatsu *et al.*, 2008) were available.

Based on the available data the DS concluded that a classification of cumene in Category 2, H351 is warranted. Based on the results of the NTP, 2009 study in mice and rats, there is sufficient evidence in animals to demonstrate carcinogenicity.

The DS concluded that the evidence on MoA for the tumours was insufficient to completely dismiss their relevance to humans. The following tumours were taken into account by the DS:

- Kidney tubular cell tumours in male rats. The DS considered it plausible that the α2u - globulin nephropathy MoA, specific to male rats, could be the underlying cause of the observed kidney tumours. Nevertheless, the fact that progressive chronic nephropathy

was also seen in female rats and that there is no specific cumene data on the MoA, leads to uncertainties on the proposed MoA.

- Nasal respiratory epithelium adenoma in male rats. These tumours are assumed to be relevant to human but progression to malignancy was considered uncertain.

- Lung alveolar/bronchiolar adenoma or carcinoma in male and female mice. Transformation of cumene by CYP2F2 in the lower respiratory tract was considered as a possible species specific MoA. Nevertheless, some specific data on cumene suggest that other MoA may be involved.

- Liver adenoma or carcinoma in male mice. There is some indication of a specific MoA *via* nuclear receptors (CAR, PPAR$\alpha$). Nevertheless, no evidence is available on the involvement of these receptors.

## Comments received during consultation

In contrast to the DS, two MSs were in favour of a more stringent classification as Carc 1B. Their conclusion was based on the observation of tumours in both sexes (lung tumours in mice) and two species (tumours in the lung and liver in mice, tumours in the nose and kidney in rat). Although these MSs acknowledged that the kidney tumours in male rat and the liver tumours in female mice could be associated with a MoA non-relevant to humans, they considered the evidence provided on the different modes of action was not sufficient to exclude human relevance. One of the MS placed special emphasis on the significantly increased lung alveolar/bronchiolar adenomas/carcinomas in male and female mice. This MS also pointed out that the mutations observed in these tumours, in contrast to spontaneous tumours, were also relevant in human lung cancer and considered these mutations as evidence for genotoxicity.

One MS was unsure whether the data supported a classification as Cat 1B or 2. This MS recommended a tabular comparison of the arguments in favour and against each proposed MoA and the characteristics of the different tumours observed (significance, malignancy, multi-site, dose-response,…) and the discussed modes of actions. This MS also pointed out that the CLP regulation assumes human relevance of findings in animals "unless there is strong evidence that the mechanism of tumour formation is not relevant for humans" (CLP, Annex I: 3.6.1.1).

The DS prepared the tabular comparison as suggested by the MS, including the results of the pilot study on CAR/PXR MoA provided by industry during PC. This tabular overview can be found attached to the Background document (see page 90).

Three industry representatives and one individual were not in favour of classification.

The individual argued that overall the tumour responses were weak and that the data presented on the different modes of action would either disprove human relevance (considered the evidence presented for CAR MoA, CYP2F2/Clara cell MoA and α2u-globulin MoA as sufficient to rule out human relevance) or that progress to malignancy was not expected (nasal tumours in male and female rats). As for the remaining tumours observed in mice and rat the commenter considered them to fall within the background incidence.

Furthermore, the other industry commenters considered the evidence insufficient to support a classification as carcinogen. With regard to the NTP (2009) studies in rat and mice they pointed out that the CLH report would not address saturation of certain metabolic pathways for cumene resulting in the formation of critical metabolites, however, without providing evidence for this shift towards critical metabolic pathways at these elevated doses.

They further criticised that despite no classification for germ cell mutagenicity is proposed, a lengthy discussion of the genotoxic potential of cumene is included in the dossier. The DS clarified that even though they proposed no classification for germ cell mutagenicity there are remaining uncertainties regarding the genotoxic potential of cumene in somatic cells.

In a similar manner to the individual commenter they were of the view that the provided evidence for the MoA with non-human relevance for lung, liver and kidney tumours was sufficient.

For the remaining tumours they concluded that those were within the spontaneous background ranges and/or did not meet the statistical threshold relevant to common tumours.

Overall Industry was of the view that only limited criteria for the classification as carcinogen were met.

## Assessment and comparison with the classification criteria

Two carcinogenicity assays were included in the CLH report, one in B6C3F1 mice and one in F344 rats (NTP, 2009). Additionally, mechanistic studies were available in the dossier.

***Mouse***

Mice were exposed to cumene vapour concentrations of 0, 125 (female mice only), 250, 500, or 1000 (male mice only) ppm. An exposure concentration-related decrease in survival occurred in male mice, and the survival of 1000 ppm males was significantly less than that of the chamber controls. Mean body weights for the 1000 ppm males were generally less than those of the chamber controls after week 8 of the study, and those of the 500 ppm females were less from week 28 until week 76 of the study (NTP, 2009), but the decrease in body weight did not exceed 10% at any time point. Dose selection was based on the results of a 3-month study in which 8/10 females died at 1000 ppm. The observed mortality occurred in the first week of dosing and was considered an acute effect. Liver weights of mice exposed to 500 or 1000 ppm were significantly increased and mean body weights of males exposed to 500 or 1000 ppm were significantly less than those of the chamber controls (NTP, 2009). Thinness and some difficulties in breathing was seen in some of the top dose males and females, but no other clinical signs were observed.

<u>Lung tumours</u>

Lung tumours were statistically significantly increased in mice as evidenced by alveolar/bronchiolar adenoma or carcinoma in males (19/50, 38/50***, 42/50***, 43/50*** at 0, 250, 500 and 1000 ppm, respectively (*** $p \leq 0.001$; P for trend: $P < 0.001$)) and in females (4/50, 31/50***, 42/50***, 46/50*** at 0, 125, 250 and 500 ppm, respectively P for trend: $P < 0.001$), for further information on lung lesions see the table below. Historical incidence for NTP 2-year inhalation studies in male mice were: 32.5% (Range: 26-44%) and in female mice: 7.6% (range 2-14%) (NTP, 2009).

As noted by the DS not all of the related lesions (e.g. bronchiolar metaplasia in males) followed a clear dose-response, but a dose-related increase in severity was seen for these effects. The data clearly indicate an exposure related neoplastic effect in both sexes, with a slightly higher susceptibility in females.

| | Incidence of lung lesions | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Dose (ppm)** | **0** | **250** | **500** | **1000** | **0** | **125** | **250** | **500** |
| Alveolar epithelium, bronchiale, metaplasia | 5/50 | 43/50 ** | 42/50 ** | 39/50 ** | 0/50 | 42/50 ** | 49/50 ** | 47/50 ** |
| Bronchiale, hyperplasia | 0/50 | 11/50 ** | 17/50 ** | 18/50 ** | 0/50 | 17/50 ** | 10/50 ** | 14/50 ** |
| Alveolar/bronchiolar adenoma, multiple | 1/50 | 12/50 ** | 15/50 ** | 20/50 ** | 0/50 | 13/50 ** | 20/50 ** | 30/50 ** |
| Alveolar/bronchiolar adenoma (includes multiple) | 13/50 | 31/50 *** | 31/50 *** | 29/50 *** | 1/50 | 26/50 *** | 36/50 *** | 38/50 *** |
| Alveolar/bronchiolar carcinoma, multiple | 0/50 | 8/50 ** | 20/50 ** | 17/50 ** | 0/50 | 6/50 * | 7/50 ** | 19/50 ** |
| Alveolar/bronchiolar carcinoma (includes multiple) | 9/50 | 19/50 * | 32/50 *** | 33/50 *** | 3/50 | 16/50 *** | 20/50 *** | 34/50 *** |
| Alveolar/bronchiolar adenoma or carcinoma | 19/50 [a] | 38/50 [a] *** | 42/50 [a] *** | 43/50 [a] *** | 4/50 [b] | 31/50 [b] *** | 42/50 [b] *** | 46/50 [b] *** |

[a] Historical control data: 146/449 (32,5% ± 5,9%), range 26% - 44%

[b] Historical control data: 34/449 (7,6% ± 4,0%), range 2% - 14%

* $\leqslant$ 0.05, ** $\leqslant$ 0.01, *** $\leqslant$ 0.001 (Poly-3 test)

The DS discussed primary genotoxicity, secondary genotoxicity (formation of ROSs), epigenetic factors and cytotoxicity via Cyp2f2 expression in Clara cells (Club cells) as a possible underlying cause of the observed lung tumours.

a) Primary genotoxicity:

The DS considered direct interaction of cumene or metabolites of cumene with DNA (Primary genotoxicity) an unlikely cause of the observed tumours. However, RAC considers the significant increase in DNA damage in lungs of female mice observed in an *in vivo* Comet assay as supportive for a genotoxic MoA. It has to be noted that the Comet assay only gave positive results in female lung tissues, but tumours were seen in both sexes, though female mice were more susceptible than males.

The remaining genotoxicity/mutagenicity tests with cumene were largely negative, but also results for the metabolite AMS were considered by the DS. For this metabolite most of the assays were also negative, but it gave weakly positive results in two SCE assays (Norppa & Vainio, 1983, NTP, 2007). Inconclusive results were observed *in vivo* in the micronucleus assay (positive in female mice and negative in male mice following repeated exposure, negative in male mice following single exposure).

Although not confirmed *in vivo* it is likely that also AMS oxide is formed as a metabolite. For AMS oxide positive results were obtained in a gene mutation test in bacteria.

There was a clear increase in *K-ras* and *p53* mutations in cumene induced tumours compared to spontaneously occurring tumours from concurrent and historical controls. The type of mutations seen in the cumene induced tumours was also different. In addition, an increased loss in heterozygosity was seen in cumene-induced mouse lung tumours, which was not seen in spontaneous tumours in control mice (Hong *et al.*, 2008).

Wakamatsu *et al.* (2008) further investigated gene expression patterns of cumene-induced lung tumours with *K-ras* mutations with those of spontaneously occurring tumours without *K-ras* mutations. The cumene induced tumours with *K-ras* mutations had increased expression for genes involved in the MAPK signalling pathway, inhibition of apoptosis, increased angiogenesis, invasion and metastasis. The authors concluded that these features are indicative of a higher degree of malignancy.

In line with the DS, RAC is of the view that there is no indication for species specificity of the observed *K-ras* mutations seen in mice. This is supported by Hoenerhoff *et al.* (2009) where it is reported that *K-ras*, as well as *p53* mutations, were found in human lung cancer and NTP (2013) where it is pointed out that activation of the proto-oncogen *K-ras* and inactivation of the tumour suppressor gene *p53* were frequently observed in human pulmonary adenocarcinoma.

Overall, it can be concluded that the increased frequency of specific types of mutations observed appear to be characteristic for cumene induced lung tumours. The NTP report suggested that the high frequency of *K-ras* mutation in adenoma was a relatively early event. However, as the sample size was small, it cannot be clearly concluded whether these specific mutations were caused by cumene or its metabolites or if they are a feature of the lung tumours as they developed as a consequence of cumene exposure.

In conclusion, genotoxicity can currently not be excluded as contributing MoA for lung cancer formation.

b) Secondary genotoxicity via formation of ROS:

Hong *et al.* (2008) mentioned that indirect genotoxicity via ROS formation might be involved in the mutations observed in the lung tumours induced by cumene. Indeed, among others, G to T transversions were noted in the *K-ras* genes. This type of transversion is consistent with 8-OH-G adduct formation (see also section on germ cell mutagenicity). A study to clarify the potential of cumene to induce DNA damage through the formation of ROS was conducted by Kim *et al.* (2008), but RAC considers this study as inadequate to draw a conclusion. In their response to comments from the consultation the DS stated that generation of ROS is in general associated with inflammation processes that can be observed in the histopathological examination of the relevant tissues, but in case of the lung of mice, no increased inflammation was observed in exposed mice vs controls.

In conclusion, RAC is of the view that a contribution of ROS to the observed tumours cannot be ruled out.

c) Epigenetic factors:

Wakamatsu *et al.* (2008) also found indications for the action of possible epigenetic mechanisms in cumene-induced lung cancer. They observed significant alterations in genes associated with the histone deacetylase complex (HDAC) in mouse lung carcinomas. A stronger association was observed between altered genes supposedly associated with HDACs and tumours with *K-ras* mutations compared to tumours without *K-ras* mutations. Therefore, *K-ras* activation may affect histone modification or vice versa.

d) Involvement of Cyp2f2 expression in Clara cells (Club cells), and human relevance of the observed tumours:

Lung tumours induced by several alkylbenzenes and other aromatic compounds (e.g. styrene) have been observed in mice but not in rats. Cruzan *et al.* (2009, 2012) mainly investigated styrene and postulated that a mouse specific expression of Cyp2f2 in Clara cells (Club cells), which is supposed to catalyse hydroxylation of the aromatic ring (not the side-chain epoxide), leads to the formation of reactive metabolites. It is postulated that these reactive metabolites lead to cytotoxicity, resulting in hyperplasia and finally (at late stage) in tumours. CYP2F2 expression is lower in club cells than the expression of the orthologous enzymes of rat and human,

and the frequency of club cells in the lower respiratory tract is much lower in humans compared to rodents. In a workshop by Toxicology Excellence for Risk Assessment, TERA (2013) the relevance of the respective mouse tumours for humans was discussed.

The workshop concluded that the MoA is theoretically possible in humans, if sufficient concentrations of active metabolite were produced, but highly unlikely to occur given the cross-compound evidence of the central role of mouse-specific Cyp2f2 in mediating cytotoxicity. An analogy of cumene to styrene was assumed in recent assessments e.g. AGS (2014); DFG (2016) or SCOEL (2015), but IARC (2013) considered the lung tumours seen with cumene relevant for humans.

The TERA (2013) workshop also developed criteria which need to be fulfilled in order to demonstrate the postulated MoA for other compounds, based on styrene:

- Evaluate the ring oxidation potential of the chemical's structure, looking for demonstration of ring-oxidized metabolites, including *in vitro* Cyp2f2 metabolism studies
- Look at the genetic activity profiles (GAP), to determine if mutation is an early and influential key event in the MoA.
- Look for evidence of acute cytotoxicity in mice and rats (*in vivo*).
- If cytotoxicity response is specific to mice (and not rats), then use Cyp2f2 knockout mouse to demonstrate that the response is dependent upon Cyp2f2 metabolism.
- Lastly, test in the humanised lung tumours in a "susceptible" system (TERA, 2013).

The DS concluded that none of the above tests have been performed for cumene, nor do the available studies support the described MoA.

Metabolites for cumene from ring-hydroxylation were found only in small quantities in $^{14}C$ analysis by Chen *et al.* (2011) and there is no obvious quantitative difference indicating that mice were more prone to this metabolic pathway compared to rat.

Cruzan *et al.* (2009) postulated the Cyp2f2 pathway leads to cytotoxicity as an essential step for subsequent hyperplasia and tumours. However, in the long-term NTP studies on cumene there was no observed cytotoxicity in the lower respiratory tract in mice preceding hyperplasia (NTP, 2009, 2013).

There has been Clara cell loss in bronchioles with styrene exposure. However, this loss has not been observed for cumene (US EPA, 2014).

The observed *K-ras* mutations in tumours from cumene exposure may be part of an alternative MoA, which has not been observed or discussed for styrene.

The postulated analogy of cumene to styrene and other alkylbenzenes is therefore not supported and is in line with a recent US EPA workshop that concluded: "Although structurally related chemicals may cause lung tumours in the B6C3F1 mouse, the mechanism may not be similar" (Pandiri, 2015, US EPA, 2014).

In conclusion, there is insufficient evidence that the MoA observed in other alkylbenzenes is applicable to cumene and some findings (e.g. lack of cytotoxicity or lack of difference with regard to metabolites between rat and mouse) also point against this specific MoA.

Overall, it can be concluded that the relevance of the observed lung tumours in mice for human cannot be excluded. It is most likely that a combination of the described modes of action is the underlying cause of the cumene induced lung tumours.

<u>Liver tumours</u>

A weak but statistically significant increase in liver tumours was seen in female mice, as demonstrated by the combined incidence of hepatocellular adenoma and carcinoma in exposed females (25/50, 25/50, 29/50, 36/50* (* $p<0.05$; P for trend = 0.024), for further details see

the table below). There was a slight trend with dose, and historical controls were exceeded at all doses.

| | Incidence of liver lesions | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | |
| **Dose (ppm)** | **0** | **250** | **500** | **1000** | **0** | **125** | **250** | **500** |
| Eosinophilic foci | 6/50 | 5/50 | 16/50 ** | 14/50 * | 8/50 | 11/50 | 7/50 | 14/50 |
| Hepatocellular adenoma, multiple | 17/50 | 20/50 | 22/50 | 26/50 | 9/50 | 13/50 | 9/50 | 10/50 |
| Hepatocellular adenoma (includes multiple) | 34/50 | 33/50 | 37/50 | 35/50 | 18/50 | 23/50 | 27/50 | 29/50 * |
| Hepatocellular carcinoma, multiple | 3/50 | 1/50 | 4/50 | 7/50 | 2/50 | 1/50 | 2/50 | 0/50 |
| Hepatocellular carcinoma (includes multiple) | 13/50 | 18/50 | 21/50 | 17/50 | 10/50 | 7/50 | 6/50 | 12/50 |
| Hepatocellular adenoma or carcinoma | 40/50 [a] | 42/50 [a] | 43/50 [a] | 41/50 [a] | 25/50 [b] | 26/50 [b] | 29/50 [b] | 36/50 [b]* |

[a] Historical control data: 264/449 (58,8% ± 9,6%), range 50% - 80%

[b] Historical control data: 145/447 (32,4% ± 8,8%), range 22% - 50%

* $\leqslant$ 0.05, ** $\leqslant$ 0.01, *** $\leqslant$ 0.001 (Poly-3 test)

a) Genotoxicity:

There are only minor indications for direct interaction of cumene or its metabolites with liver DNA. Though significant DNA damage was seen in the Comet assay in the liver of male rats, this was not observed in the liver of mouse. For a detailed discussion of genotoxicity see section on genotoxicity and lung tumours above. Overall indications for genotoxicity in the liver are limited, but involvement of primary or secondary genotoxicity in the formation of liver tumours cannot be excluded.

b) Cytotoxicity:

In the sub-chronic study in mice some minimal focal liver inflammation was observed, though without dose-response. Liver weight was also increased reaching up to 38% in males and females at the top dose. Eosinic foci and necropsy was increased in the top dose males, but tumours were seen in females. Based on these findings it can be concluded that cytotoxicity is not a relevant contributor to the observed tumours.

c) Possible involvement of nuclear receptor activation in the formation of liver tumours:

In the CLH report the DS concluded that there were no mechanistic data to link cumene induced liver tumours to neither CAR- nor PPAR $\alpha$-activation and, in consequence, potentially non-relevance for humans. An increase in liver weights in the absence of considerable liver toxicity might be supportive for this MoA.

In response to comments received during the consultation, the DS concluded that a CAR mediated MoA was likely and supported by new data from a pilot *in vivo* study on mouse liver provided during consultation. Observations supporting this MoA were increased liver weight and absence of relevant liver toxicity, strong induction of Cyp2b and increased cell proliferation. Deficiencies in the argumentation for this MoA are that key events (KE) 1 to 3 are only supported for oral exposure and hyperplasia and liver foci, which would support that KE 4 was not consistently shown in the NTP study (only in male animals, while tumours were seen in female animals). For details on the postulated MoA see e.g. Peffer *et al.* (2018). The preliminary study submitted by industry during consultation and the results are summarised under "Supplemental information - In depth analyses by RAC".

RAC concludes that the carcinogenic signal in female mice is not very strong and the proposed CAR/PXR mediated MoA is plausible. However, not all mechanistic studies required to demonstrate this MoA are available and some findings in the newly submitted study do not support the proposed MoA. Importantly, human relevance has not been investigated. In conclusion, the relevance of the observed tumours for humans cannot be completely dismissed.

Spleen tumours

A slight increase in haemangiosarcoma of the spleen was seen in male mice at the top dose. The DS did not put much weight on these tumours as the increase was only marginally above the historical control values. The DS further mentioned that haemangiosarcomas occur in multiple tissue types and are not specific to or rare in the spleen (NTP, 2013). When taking the haemangiosaromas of all tissues together, no increase above historical control was evident.

Haemangiosarcoma incidence in spleen: 0/50, P = 0.002, 0/50, 0/49, 4/50* at 0, 250, 500 and 1000 ppm.; HCD: 6/444, (1.4% ± 1.5%), range 0% - 4%.

Haemangiosarcoma incidence, all organs: 0/50, P = 0.015, 1/50, 2/50, 4/50* at 0, 250, 500 and 1000 ppm.; HCD: 21/450, (4.7% ± 3.7%), range 0% - 12%.

(For the chamber control values the P-value is presented which accounts for differential mortality in animals not reaching terminal sacrifice)

RAC concurs with the DS giving low weight to this tumour type.

Follicular-cell adenoma of the thyroid gland

An increase in top dose males was not statistically significant, only the trend was significant (P = 0.01). The increase of 6% was within the historical control range (0 – 6%) for inhalation studies and for all routes. No significant increase in follicular hyperplasia was observed.

Follicular-cell, hyperplasia: 7/50, 7/50, 7/49, 11/50 at 0, 250, 500 and 1000 ppm.

Follicular-cell, adenomas: 0/50, P = 0.010, 0/50, 0/49, 3/50 at 0, 250, 500 and 1000 ppm; HCD: 5/441 (1.1% ± 2.0%), range 0 – 6%.

(For the chamber control values the P-value is presented which accounts for differential mortality in animals not reaching terminal sacrifice)

RAC concludes that the finding is of low biological significance.

***Rats***

In the carcinogenicity study, survival and body weight changes were similar between the exposed groups and the chamber controls. Body weight changes were not observed. There were no clinical signs related to exposure to cumene. Therefore, RAC noted that no excessive general toxicity was observed in the treated groups. The top dose was chosen based on the kidney histopathological findings (suggestive of kidney toxicity) observed at 1000 ppm in the 3-month inhalation rat study.

Kidney tumours

In male rats, renal tubule adenoma was increased in all exposed groups above the historical control range of the laboratory (consisting of nine NTP studies performed by inhalation between 1995 and 2005). The incidences of renal tubule carcinoma were increased at ≥ 500 ppm and exceeded the historical control ranges. The increases were not statistically significant and no dose-response was observed.

A statistically significant dose-related increase in renal tubule hyperplasia was observed in males at 500 ppm. According to NTP, this lesion was distinguished from regenerative epithelial changes and considered as a preneoplastic lesion. Mineralization of the renal papilla was significantly

increased in all dose groups of males, consistent with mineralisation associated with α2u-globulin nephropathy. Additionally, an increase in the severity of chronic progressive nephropathy was observed in both males and females. A significant increase in kidney weight was noted in all exposed groups in males and in the mid and top dose group in females in the 3-month inhalation rat study of cumene.

RAC considers the increase in kidney renal tumours in males treatment related.

The incidences of kidney tumours in male rats are shown in the table below:

| | **Kidney tumour incidence (overall rate, %)** | | | | |
|---|---|---|---|---|---|
| **Dose (ppm)** | **0** | **250** | **500** | **1000** | **HC** |
| **Males: renal tubule** | | | | | |
| Adenoma | 2 | 8 | 10 | 8 | 0-2 |
| Carcinoma | 2 | 2 | 6 | 6 | 0-2 |
| Carcinoma or adenoma | 4 | 10 | 16* | 14 | 0-4 |

HC: historical control; ** p<0.01;

In the table below, selected non-neoplastic kidney findings at termination are provided:

| | **n=50** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Males** | | | | **Females** | | | |
| **Dose (ppm)** | **0** | **250** | **500** | **1000** | **0** | **250** | **500** | **1000** |
| Nephropathy (severity) | 47 (2.3) | 47 (2.6) | 47 (2.9) | 50 (2.7) | 38 (1.4) | 37 (1.5) | 41 (1.9) | 44 (1.9) |
| Renal tubule hyperplasia | - | 3 | 8** | 6* | - | - | - | - |
| Pelvis transitional epithelium hyperplasia | 3 | 5 | 14** | 15** | - | - | - | - |
| Papilla mineralization | 5 | 35** | 44** | 41** | - | - | - | - |

**p≤0.01, *p≤0.05 (poly-3 test)

The DS postulated that the accumulation of a chemical- α2u-globulin complex resistant to lysosomal degradation in male rats results in renal tubular cell death and compensatory cell proliferation and neoplasms. This MoA is specific to male rat, as this protein does not exist in humans and to a much lesser extent in female rats (ECHA guidance on CLP criteria, 2017).

The following key events were considered by the DS:
- reversible binding of cumene or cumene metabolite(s) to α2u-globulin;
- increased number and size of hyaline droplets in renal proximal tubule cells;
- the hyaline droplets contained α2u-globulin;
-histopathological changes in shorter-term studies, renal tubular cell proliferation and induction of tumours.

A treatment-related increase in the amount of α2u-globulin, hyaline droplets accumulation in the cortex and medullary granular cast were observed in the 3-month inhalation rat study (NTP, 2009). According to the NTP report, kidney weight and the incidence and severity of renal cortical tubule regeneration were increased. In cell proliferation analysis, kidney cell labelling index was not statistically significantly different from control, but the number of cells in the S-phase was increased. It is noticeable that incidence of kidney nephropathy was very high in controls (97%).

Overall, RAC considered the proposed MoA plausible. RAC notes the absence of specific data on binding affinity to α2u-globulin to strengthen the case. Moreover, a clear correlation between chronic nephropathy and renal tubule adenoma or carcinoma is difficult to establish due to the very high background in controls.

Nasal tumours

An increased incidence of respiratory epithelium adenoma was noted in all treated groups in both male and female rats above the historical control ranges (0-2% in males, not seen in females). The increase was statistically significant in males only. No dose-relation was seen.

The incidences of neoplastic and non-neoplastic lesions in male and female rats are shown in the table below:

| | **Incidence of nose lesions** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Males** | | | | **Females** | | | |
| **Dose (ppm)** | **0** | **250** | **500** | **1000** | **0** | **250** | **500** | **1000** |
| Number of animals | 50 | 50 | 49 | 50 | 50 | 48 | 50 | 50 |
| Respiratory epithelium Adenoma (multiple and all sites) | 0 | 7** | 18** | 10** | 0 | 5* | 4 | 3 |
| Olfactory epithelium hyperplasia | 0 | 19** | 27** | 26** | 0 | 14** | 25** | 31** |
| Respiratory epithelium hyperplasia | 0 | 15** | 16** | 23** | 0 | 0 | 4 | 6* |
| Goblet cell hyperplasia | 3 | 11* | 7 | 5 | - | - | - | - |

**p ≤ 0.01, *p ≤ 0.05 (poly-3 test)

Overall, although no dose-response was observed, the increase in respiratory epithelium adenoma were clearly above historical control ranges and are considered treatment-related in both males and females. The benign tumour type and the low incidences in females may decrease the concern.

Tumours in testis

A statistically significant increase in top dose males and a positive trend for unilateral and bilateral interstitial cell adenoma of the testis was observed. HCD: 345/449.

| | **Incidence of testis lesions** | | | |
|---|---|---|---|---|
| | **Males** | | | |
| **Dose (ppm)** | **0** | **250** | **500** | **1000** |
| Animal number | 50 | 50 | 50 | 50 |
| Interstitial cell, hyperplasia | 12 | 18 | 19 | 9 |
| Bilateral interstitial cell, hyperplasia | 0 | 0 | 0 | 1 |
| Interstitial cell, adenoma | 18 | 14 | 13 | 9 |
| Bilateral interstitial cell, adenoma | 18 | 24 | 27 | 37 |
| Interstitial cell, adenoma (includes bilateral) | 36* | 38 | 40 | 46** |

*p = 0.006; For chamber control incidence, the p value given is associated with the trend test determined by Poly-3 test (accounts for differential mortality in animals that do not reach terminal sacrifice).
**p ≤ 0.01 (compared to chamber control group determined by Poly-3 test)

As indicated by the historical control data there is a very high background incidence of this tumour type and it is noted that no progression to malignancy is reported (NTP, 2013).

Overall, RAC concludes that the finding is of minor biological relevance.

Other studies relevant for carcinogenicity:

The CLH report also reports two *in vitro* transformation assays (Putman, 1987b, Klimisch 1 and Gulf Oil Corporation, 1984, Klimisch 3). The retest by Putman (1987b) did not confirm the positive result from the study by Gulf Oil Corporation. RAC concludes that the two studies have no strong impact on the conclusion on classification.

***Overall conclusion on classification and comparison with CLP criteria*:**

As there is no evidence of carcinogenicity in humans reported in the dossier, classification in Category 1A is not appropriate.

Based on the results of the NTP (2009) study in mice and rats there is clear evidence for carcinogenicity in animals.

According to the CLP regulation (Annex I: 3.6.2.2.4), additional considerations like human relevance and background incidences as part of a weight of evidence approach have to be taken into account for a classification for carcinogenicity. These are assessed in the following table:

| Factor | Evidence with cumene | Conclusion |
|---|---|---|
| Tumour type<br>Considering background incidence and HCD | Lung adenoma or carcinoma in B6C3F1 male and female mice.<br>High spontaneous tumour.<br>Marked dose-related increase above historical control. | Supportive of classification |
| | Respiratory epithelium adenomas in F344 male and female rats.<br>Above HCD range in both sexes.<br>Low incidences in females. | Supportive of classification |
| | Tubular kidney adenoma or carcinoma in F344 male rats.<br>Above HCD range. | Supportive of classification |
| Multi-site responses | Yes | Increased concern |
| Progression of lesions to malignancy | Yes for kidney and lung tumours.<br>No progression to malignancy for nasal tumours. | Increased concern |
| Reduced tumour latency | Not investigated. | - |
| Whether responses are in single sex or both | Both sexes in rats and mice reported tumours. | Increased concern |
| Whether responses are in a single species or several | Tumour formation occurred in rats and mice. | Increased concern |
| Structural similarity to a substance(s) for which there is good evidence of carcinogenicity | Several aromatic hydrocarbons have been investigated for their tumorigenic potential, with special focus on the induction of lung tumours. For some substances, with styrene being the reference substance, lung tumours were identified as being mouse specific (Cruzan *et al.*, 2009, 2012, TERA, 2013, US EPA; 2014: CYP2F2, Clara cells).<br><br>The NTP has conducted carcinogenicity studies of ethylbenzene administered by inhalation and reported induction of renal tubule neoplasms in male and female F344/N rats, testicular adenoma in male F344/N rats, alveolar/bronchiolar neoplasms in male B6C3F1 mice, and hepatocellular neoplasms in female B6C3F1 mice in 1999 (reported in NTP, 2009).<br><br>Ethylbenzene has an old entry in Annex VI (translated from DSD) and in a RAC opinion from 2012 carcinogenicity was not evaluated. | NI |
| Routes of exposure | Inhalation is a relevant route of exposure | NI |
| Comparison of ADME between test animals and humans | No species specific differences identified in the available toxicokinetics studies. | NI |

| Factor | Evidence with cumene | Conclusion |
|---|---|---|
| MoA and its relevance for humans | Lung tumours in mice:<br><br>a) Genotoxic MoA<br>(Supported by positive results in the COMET assay in the lung and high rate of metabolism in the lung)<br><br>b) Metabolism-specific lung tumours in mice (lower relevance to human).<br><br>c) Epigenetic MoA | <br><br>MoA relevant to human plausible.<br><br>Not substantiated by data<br><br>MoA plausible, supported by related gene expression pattern, possibly more than one MoA active. |
| | α2u-globulin nephropathy MoA of kidney tumours in male rats. Lower relevance to human | MoA not relevant to human. Decreased concern |
| | Epithelial nasal tumours: no data to support that the tumours would not be relevant to human | Human relevance plausible |

NI - no influence on the concern (neither increase nor decrease)

Cumene was demonstrated to be a multi-site carcinogen in two rodent species via inhalation. No data on other exposure routes are available. A slight genotoxic potential was however demonstrated also for the oral route (COMET assays via gavage application).

As the specific tumour types were either seen in mice or in rats, species specific modes of action as underlying cause and human relevance of the neoplastic findings need to be discussed.

For the cumene induced lung tumours seen in male and female mice, although the background incidence of this type of tumour is high in this strain of mice, the increase was considered treatment related and biologically relevant as the increase was of extensive magnitude and statistically significant and already started at the lowest dose tested. A mouse specific MoA via increased transformation of cumene involving ring oxidation by CYP2F2 in Clara cells of the lower respiratory tract was postulated. However, several findings which are normally seen with this MoA were not demonstrated: lack of cytotoxicity, lack of appreciable amounts of ring oxidation in metabolism studies, absence of differences with regard to these metabolites between rats and mice and lack of Clara cell loss (which was demonstrated for styrene). In addition, the observed *K-ras* mutations might be part of an alternative MoA. In conclusion, the lung tumours have to be regarded as relevant for humans.

For liver tumours in mice there are indications for a CAR/PXR dependent MoA. Support for this MoA comes from a pilot study submitted by industry, but not all relevant mechanistic studies were provided and alternative MoA cannot be completely ruled out on the basis of the available data. Cytotoxicity does not appear to be a relevant contributor to tumour formation based on the chronic toxicity studies. Overall, the weak increase of benign tumours in female mice is not considered a strong indication for carcinogenicity.

For the renal tumours seen in male rats the formation of α2u-globulin and hyaline droplet formation were discussed as possible underlying MoA. Several aspects of this MoA have been demonstrated but not all of the requirements according to IARC are fulfilled (e.g. insignificant increase of non-malignant nephropathic effects in female rats). On that basis human relevance cannot be completely excluded.

Neoplasms were also seen at several different sites (i.e. testis, spleen, thyroid), but they occurred at low incidences and did not progress to malignancy.

For nasal tumours in male and female rats, human relevance can be assumed, but progress to malignancy is questionable.

In conclusion several tumours were seen in animal studies for which non-human relevance could not be clearly demonstrated.

The highest weight for classification comes from the lung tumours which provide sufficient evidence of carcinogenicity. The induced tumours were clearly malignant and were seen in both male and female mice. In addition, the mechanistic data clearly indicate that the postulated mouse specific MoA involving increased metabolism in Clara cells of the lower respiratory tract is unlikely for cumene. The NTP, 2009 study was considered reliable.

In addition, nasal tumours observed in male and female rats provide evidence of carcinogenicity but the evidence can be considered limited as only benign tumours were seen.

Regarding kidney tumours the proposed modes of action without human relevance are likely but, as described above, important elements needed to support these MoAs are missing. Therefore, kidney tumours also provide limited supportive evidence of carcinogenicity.

Liver tumours in female mice are considered of very low weight in the overall WoE for classification as background incidence was high in this strain of rats by inhalation. In addition, the proposed MoA (CAR-mediated tumours) is plausible but RAC notes that the MoA was not sufficiently investigated.

Neoplasms with lower weight due to lack of malignancy or low incidences were also seen at several additional sites (testis, spleen, thyroid). Nevertheless, these tumour types were not considered sufficient for classification.

A contribution of genotoxicity to the observed tumour formation cannot be excluded.

On this basis, RAC is of the opinion that the overall weight of evidence warrants a **classification as Carcinogen Category 1B** based on lung tumours in mice supported by nasal and kidney tumours in the rat.

***Specific concentration limit***

SCLs were not covered in the CLH proposal, but one MSCA commented in the PC that cumene might fall in the low potency group and might require adequate SCLs, based on T25 calculations.

In line with the EC (1999) guidance RAC calculated the following T25 values based on the alveolar/bronchiolar adenoma or carcinoma observed in male and female mice after inhalation exposure, after correction for background exposure (NTP, 2009).

**NTP, 2009:** Species and exposure route: mouse, inhalation

6h/day, 5 days/week, 105 weeks

| **Males, endpoint: alveolar/bronchiolar adenoma or carcinoma** | | | | |
|---|---|---|---|---|
| Dose in ppm | 0 | 250 | 500 | 1000 |
| Incidence | 19/50 (38%) | 38/50 (76%) | 42/50 (84%) | 43/50 (86%) |
| Background correction | 0% | 38% | 46% | 48% |

Dose closest to 25%: 250 ppm (38%, after background correction)

25/38 * 250 = 164,5 ppm = 810 mg/m3 = 0,81 mg/l

Inhalation volume mouse (EC, 1999): 1,8 l/h (males & females) ➔ 10,8 l/6h

0,81 mg/l * 10,8 l/6h = 8,75 mg/6h

Weight male mouse: 30g ➔ 8,75 mg * 1000g / 30g = 292 mg/kg bw/6h

Correction for 5 days per week, 105 weeks vs 104 weeks: 292 * 5/7 * 105/104 = 210 mg/kg bw/day.

T25 = 210 mg/kg bw/day ➔ > 100 mg/kg bw/day ➔ low potency group

| **Females, endpoint: alveolar/bronchiolar adenoma or carcinoma** | | | | |
|---|---|---|---|---|
| Dose in ppm | 0 | 125 | 250 | 500 |
| Incidence | 4/50 (8%) | 31/50 (62%) | 43/50 (84%) | 46/50 (92%) |
| Background correction | 0% | 54% | 76% | 84% |

Dose closest to 25%: 125 ppm (54%, after background correction)

25/54 * 125 = 57,9 ppm = 285 mg/m3 = 0,285 mg/l

Inhalation volume mouse (EC, 1999): 1,8 l/h (males & females) ➔ 10,8 l/6h

0,285 mg/l * 10,8 l/6h = 3,078 mg/6h

Weight female mouse: 25g ➔ 3,078 mg * 1000g / 25g = 123 mg/kg bw/6h

Correction for 5 days per week, 105 weeks vs 104 weeks: 123 * 5/7 * 105/104 = 88,6 mg/kg bw/day.

T25 = 88,6 mg/kg bw/day ➔ < 100 mg/kg bw/day ➔ medium potency group

Industry recommended the use of a different inhalation volume than the one proposed by EC (1999), i.e. 3,25 l/h. They considered this value superior to the default value recommended in EC (1999) as it is based on plethysmograph measurements in unanaesthetised mice of the relevant strain, i.e. B6C3F1 (Chang et al., 1981, supported by US EPA, 1988).

In the RAC discussion it was further considered to use the inhalation volume recommended in the REACH guidance document, Chapter R.8 (v2.1, 2012) instead, i.e. males, 2.5 l/h, females 2.2 l/h, as these values are also applied for the derivation of e.g. DNEL values under REACH.

It was, however, noted that the inhalation volume recommended in EC (1999) was the basis for the derived potency classes recommended for classification in the same document. A change of these values would require an in-depth evaluation of all available data relevant for this purpose. A review of the procedure for the derivation of SCLs is currently conducted by the COM expert group on carcinogenicity. In case this group decides that there is a need for revising the currently recommended inhalation volumes, the concentration values could be revisited.

The derived T25 values result in the low potency for males, but in the medium potency group for females. In order to protect the more sensitive sex **RAC recommends the application of the generic concentration limit of 0.1% for the classification of mixtures containing cumene**.

## RAC evaluation of reproductive toxicity

### Summary of the Dossier Submitter's proposal

#### *Sexual function and fertility*

The evaluation of sexual function was based on two 90-day inhalation repeated-dose toxicity studies in rats and mice (NTP, 2009). These studies were similar to OECD TG 413 and GLP-compliant. A third rat 90-day study, investigating some reproductive parameters, published by Cushman *et al.*, 1995 was also considered but rated unreliable by the DS. In addition, some reproductive parameters examined in a developmental rat toxicity study (Darmer *et al.*, 1997) were taken into account by the DS.

No studies on fertility following OECD TG were available. In these studies, the observed effects on oestrous cycle, testicular atrophy, epididymis weight and sperm findings were considered of low or minimal toxicological significance and of questionable human relevance. Therefore, no classification was proposed by the DS for toxicity on sexual function and fertility.

#### *Developmental toxicity*

The assessment of developmental toxicity was based on two studies in rats and rabbits published by Darmer *et al.*, 1997. The study was performed according to OECD TG 414 (reliable with limitations) and was GLP compliant. No classification was proposed by the DS as no developmental toxicity was observed.

### Comments received during consultation

One MS commented that in absence of adequate fertility study and based on the available 90-day studies, data are inconclusive and insufficient to conclude on classification for fertility. The DS responded that although reproductive organs were examined in 90-day studies, the absence of screening of generational studies might indeed lead to the conclusion of "data lacking".

Two industry representatives and one individual agreed with the DS that findings are insufficient to classify cumene as a reproductive toxicant (sexual function, fertility and development).

### Assessment and comparison with the classification criteria

#### *Sexual function and fertility*

The observed findings that may indicate potential effects on sexual function and fertility, highlighted by the DS, are discussed below.

<u>Estrous cycle findings</u>

In the 90-day repeated-dose toxicity studies (inhalation exposure) in rats (NTP, 2009), exposed females spent significantly ($p \leq 0.05$) more time in the estrous stage than chamber control females and less time on proestrus. At the top dose, mean body weight and body weight gains in all tested groups were similar to controls. Liver toxicity consisted of biochemical changes and increased liver relative weight at ≥ 250 ppm. In addition, kidney relative weight was slightly increased in female rats at ≥ 250 ppm. No necropsy findings were observed in these organs.

The table below presents the estrous cycle characterisation in the 3-month NTP study.

| Dose (ppm), n=10 | **0** | **250** | **500** | **1000** |
|---|---|---|---|---|
| Estrous cycle length | 5.06 | 4.85 | 4.80 | 4.90 |
| Estrous stage (% of cycle) | | | | |
| - Diestrus | 49.2 | 41.7 | 41.7 | 44.2 |
| - Proestrus | 19.2 | 14.2 | 9.2 | 11.7 |
| - Estrus | 15.8 | 25.8 | 28.3 | 25 |
| - Metestrus | 15.8 | 18.3 | 20.8 | 19.2 |

Estrous cycle disruption, indicated by an extended vaginal estrous is of concern and may indicate a potential effect on ovulation. Nevertheless, among the exposed groups, no clear dose-response was observed. The increased duration of estrous had no impact on the lengthening of the cycle and acyclicity was not reported. In addition, no histopathological findings in ovary (e.g. atrophy, absence of *corporea lutea*) were noted in this study. In another study using pregnant rats (Darmer *et al.*, 1999), and in the 90-day study published by Cushman *et al.*, 1995, no findings in ovary (weight, necropsy) or other investigated reproductive parameters were noted. Overall, RAC agrees with the DS that the shift in the relative length of time spent in the oestrus stages is insufficient for classification. Nevertheless, RAC notes that a study investigating fertility is missing and would have been necessary to clarify this concern.

Testicular findings in male rats

Testicular atrophy was reported by Cushman *et al.*, 1995 in one out of 15 male rats at the top dose (1200 ppm). RAC agrees with the DS that this finding was only observed in one rat and may thus not be treatment related. Moreover, this finding was not observed in the NTP, 1999 studies. Cushman *et al.*, 1995 also observed at the mid-dose an increase in the frequency of sperm head abnormalities (0.5%, 0.5%, 1.1%, 0.7% at 0, 100, 500, 1200 ppm, respectively). RAC agrees with the DS that the finding provide only very limited indication of some adversity as the effect was not dose-related, not statistically significant and infrequent. No sperm head abnormalities were seen in the NTP, 2009 study.

Epididymides findings in male mice

In the 90-day NTP study (exposure by inhalation), a significant reduction in cauda epididymis weight was observed at the top dose. A dose-related reduction in spermatid counts was also noted, reaching statistical significance at the top dose only. A summary of these effects is presented in the table below (mean values presented).

| **Dose (ppm), n=10** | **0** | **250** | **500** | **1000** |
|---|---|---|---|---|
| Absolute cauda epididymis weight (g) | 38.3 | 36.1 | 36.3 | 34.7** (↓9,4% *vs* control) |
| Spermatid count ($10^6$/cauda epididymis) | 18.05 | 17.62 | 17.53 | 14.70* (↓19% *vs* control) |

**p≤0.01; *p≤0.05

Testis and epididymis weights and epididymal spermatozoal measurements were not affected by treatment. At the top dose, mean body weight in males exposed to 500 and 1000 ppm were significantly less than those of the controls. Liver toxicity (weight, necrosis) was also observed at the top dose. As the effect occurs in presence of toxicity in males, RAC agrees with the DS that the observed effects are of minimal toxicological significance.

Conclusion on fertility and sexual function

Overall, RAC agrees with the DS that the findings in the 90-day studies investigating reproductive parameters are not sufficient to classify cumene for reproductive toxicity. Nevertheless, **due to the lack of data on fertility and sexual function (e.g. a generational study), RAC was unable to evaluate this hazard class.**

***Developmental toxicity***

As no relevant toxicological findings were observed in the two developmental toxicity studies performed with cumene, RAC agrees with the DS that **no classification is warranted for cumene for developmental toxicity**.

***Adverse effects on or via lactation***

**RAC was unable to evaluate on this hazard class due to lack of data**.

## Additional references

Peffer RC, LeBaron MJ, Battalora M, Bomann WH, Werner C, Aggarwal M, Rowe RR, Tinwell H (2018): Minimum datasets to establish a car-mediated MoA for rodent liver tumors. Regula Toxicol Pharmacol, ;96:106-120.

**ANNEXES:**

Annex 1 The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter; the evaluation performed by RAC is contained in 'RAC boxes'.

Annex 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and RAC (excluding confidential information).