Givaudan

21 September, 2023

Givaudan Response to CLH report Proposal for Harmonized Classification and Labelling of 4-ISOPROPYLBENZOIC ACID (4-iPBA) AND SUBSTANCES FORMING 4-iPBA

Chemical names:

a) p-cymene; 1-isopropyl-4-methylbenzene

b) 3-p-cumenyl-2-methylpropionaldehyde; 2-methyl-3-(4-isopropylphenyl)propanal [1]; 3-(p-cumenyl)propionaldehyde; 3-(4-isopropylphenyl)propanal [2]; 4-isopropylbenzaldehyde; cuminic aldehyde [3];

EC Numbers:

a) 202-796-7

b) 203-161-7 [1]; 231-885-3 [2]; 204-516-9 [3]; 208-642-5 [4]

CAS Numbers:

a) 99-87-6

b) 103-95-7 [1]; 7775-00-0 [2]; 122-03-2 [3]; 536-66-3 [4]

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1. Summary

The proposed harmonized classification (CLH) submitted by the Swedish Competent Authority as Dossier Submitter (DS) for para-cymene, 3-*p*-cumenyl-2-methylpropionaldehyde (Cyclamen Aldehyde), 3-(*p*-cumenyl)propionaldehyde (Cyclemax) and 4-isopropylbenzaldehyde (Cuminic Aldehyde) as reproductive toxicant Repr. 1B is inconsistent with the criteria provided by the CLP Regulation[1]. This is largely due to inaccuracies in the DS dossier, such as the statement that "two studies of limited quality demonstrate similar findings in one additional species (rabbit)[2]" and the suggestion that mechanistic data, data on species difference and human relevance were already sufficiently considered in the classification of 2-(4-tert-butylbenzyl)-propionaldehyde (Lysmeral). The DS also does not adequately account for recent data under REACH or in the literature.

According to Section 3.7.2.1.1, a classification as Repr. 1B is conditioned by the existence of data providing "clear evidence of an adverse effect on sexual function and fertility or on development", in the absence of secondary non-specific consequences of other toxic effects. In the present case, a pattern of evidence demonstrates that such conditions are not met and hence that a classification as Repr. 1B is not warranted.

First, based solely on the rabbit study[3], without consideration to any other information, cyclemax is not classifiable as a reproductive toxicant. Indeed, classification criteria of "statistically or biologically significant positive results" are simply not evident in the rabbit. The CLP Regulation further emphasizes that classification may not be needed in cases when "only effects recorded are considered to be of low or minimal toxicological significance, such as small changes in semen parameters". This is the best what can be observed in the rabbit study.

Secondly, the DS evaluation of the rabbit study with cyclamen aldehyde contradicts the original study authors, as well as a peer reviewed publication, without substantive expert explanation. The DS approach is also contrary to the CLP rules for Weight of Evidence (WoE) determination, according to which a "Classification as a reproductive toxicant is made on the basis of an assessment of the total weight of evidence." For WoE, it is indeed essential to

[1] Furthermore the proposed classification entry does not conform with Section 1.6.3.3.3 of the ECHA Guidance on CLP for 'Additivity vs non-additivity of hazards for mixtures, as the need for classification of mixtures based on the sum of the concentrations of individual substances forming the same metabolite is not normally applicable to reproductive hazards under ECHA Guidance on CLP (Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, 2017). Summing up chemicals forming the same metabolite does not taking into account vastly different pharmacokinetics to form the metabolite. Furthermore, it is a generic requirement with implications on compliance and enforcement, as it may not be known for many chemicals whether actually they also could lead to formation of such a common small molecule metabolite.

2 Short-term repeated dose toxicity study in Male New Zealand White rabbits with 3-p-cumenyl-2-methylpropionaldehyde; Short-term repeated dose toxicity study in Male New Zealand White rabbits with 3-(p-cumenyl)propionaldehyde.

[3] Short-term repeated dose toxicity study in Male New Zealand White rabbits with 3-(p-cumenyl)propionaldehyde.

provide a reliable evaluation of each piece of evidence independently. Integration and weighing of evidence should occur as a subsequent and distinctly separate step. In the present case, the DS dossier even characterizes the findings from the rabbit study as 'similar' to those in the rat, which is technically incorrect.

Thirdly, through new research and published studies referenced in the CLH dossier, the mode of action of these substances to produce the common metabolite 4-iPBA with subsequent conjugation to Coenzyme A (CoA) has been firmly established. The DS has already acknowledged that the grouping of substances based on tBBA metabolism should be separated due to differentiation of the metabolites, and has submitted a separate CLH proposal (ECHA, 2023a). The mechanistic data demonstrate that the DS's conclusion, stating that hazard characterization of 4-iPBA can be made based on the classification of another substance, tBBA, is scientifically incorrect, as mechanistic data were not sufficiently considered in classification of tBBA (See section 5.1).

In particular, the DS does not take into account more recent mechanistic information that further substantiates the pattern of evidence that supports species differences in the Mode of Action (MoA) for precursors of these small acids between lower mammals and humans. Following the formation of 4-iPBA, the conjugation with Coenzyme A leading to 4-iPBA-CoA is the key event leading to toxicity. Whereas there is clearance of this metabolite over time in rabbits, and to an even higher degree in human cells, accumulation is observed in rats. Thus, there are clear differences in the stable accumulation, and lack of clearance of 4-iPBA-CoA between species, which has been acknowledged by the DS on pages 11-13 of the CLH dossier. This lack of clearance in rats leads to toxicity through 4-iPBA-CoA accumulation in the testis and liver and effects on lipid metabolism. This species-specific mechanistic knowledge raises significant doubt about relevance to humans.

In that regard, according to Section 3.7.2.1.1 of Annex I to the CLP, "when there is mechanistic information that raises doubt about the relevance of the effect for humans", a Repr. 1B is not warranted. In the present case, the ECHA Guidance on the Application of the CLP Criteria (ECHA, 2017) needs to be considered when evaluating justification for a Repr. 1B classification. The document states that "Where it is known that the mode or mechanism of action is not relevant for humans or is of doubtful relevance to humans, this should be taken into account in the classification and should not be used again as a modifying factor for potency." and "In cases where mechanistic information shows a lower sensitivity in humans than in experimental animals, this may move substances which are close to the potency boundaries to a lower potency group." (3.7.2.6.5. Modifying factors). Moreover, given the species differences and the presence of p-cymene at high levels in a wide variety of plant-derived products, the criterion of needing "data which provide a strong presumption that the substance has the capacity to interfere with reproduction in humans" for classification in Repr. 1B cannot be considered as met. There is published and peer reviewed evidence that Cyclamen Aldehyde, *p*-cymene and other compounds lead to the formation of 4-iPBA in rats and induce 4-iPBA-CoA accumulation in the testes and livers, with a convincing pattern of evidence of a marked species difference and of low or absent relevance to humans. The substantial mechanistic data presented below

raises doubt about the relevant of the effect for humans, that is not addressed by the DS while supporting that a CLH as Repr. 1B is not warranted.

Fourthly, regarding fertility in female rats, reported effects may either be attributed to the male reproductive toxicity or are effects occurring with maternal toxicity. Overall, there is no clear evidence for a selective developmental toxicity as the statistically significant and dose-related effects are consistent with effects likely to occur as a result of maternal toxicity, reduced feed consumption and/or body weight. Again, as further evidence, the DS could also consider that no developmental effects in absence of maternal toxicity were evident in a study with a material containing p-cymene 4, as provided in the plant protection product registration of QRD460 (EFSA, 2014), which the CLH dossier references but excludes as information in the evaluation.

For developmental toxicity, the DS proposes a Repr. 1B without following the CLP Regulation requirement to evaluate whether maternal toxicity is "likely to have influenced these effects", such as delayed development. The DS dossier fails to consider the toxic effects on organs and evidence of stress in the study with p-cymene, leading to the need to euthanize one female rat prior to pregnancy.

Fifthly, there is literature on substances in plants, foods, essential oils, and products thereof, which contain *p*-cymene often present at concentrations >3% for example in extracts from Cardamom, Citrus fruits, Coriander seed, Cumin seed and Nutmeg and at even higher concentrations in various plant oils such as Thyme oil (see section 5.3). Given the varied human consumption of *p*-cymene from multiple natural sources, including in traditional medicines, it is inappropriate to classify the substances for Repr. 1B when there are no corresponding human adverse events reported (Section 5.3).

Many EU regulatory frameworks reference hazard classification under CLP with a differentiation between category 1 and 2 reproductive toxicants. For substances present in many product types including a large array of natural oils and food products, like it is the case for p-cymene, ensuring that relevant and appropriate CLP criteria for classification are met is critical for further evaluations by other expert bodies and regulatory authorities. Implications of a Repr. 1B classification for p-cymene and related substances would result in the allocation of significant resources for risk assessment, which would be disproportionate to the intrinsic hazard.

In conclusion, the DS dossier lacks sufficiently robust scientific assessment of the MoA and species differences. Based on all the data available and clear mechanistic differences between rats, rabbits and humans, a Repr. 1B classification is <u>not</u> warranted neither for effects on sexual function and fertility, nor for effects on development, for the substances of the group assessed (Cyclamen Aldehyde, p-cymene, Cyclemax and Cuminic Aldehyde).

In the following, the scientific evidence for these conclusions is presented.

[4] https://efsa.onlinelibrary.wiley.com/doi/pdf/10.2903/j.efsa.2014.3816

Background data availability:

This document is accompanied by the following background documents as Annex II - IV:

- Report 154'270 contains the detailed data of the review on Cyclamen aldehyde (Natsch et al., 2021) - along with details on the individual experiments and with additional data on the effects on the endogenous C8-CoA pool in the published experiments. (Annex II)

- Report 154'305 contains new data on 4-iPBA precursors of the Dossier with a detailed analysis of dose-dependent effects on the endogenous C8-CoA pool (Annex III)

- Charles River Study No. 20185914, a 28-Day Study of Cyclamen Aldehyde by Oral Gavage in Wistar Han Rats is including as Appendix 9 report RCR 153'889, i.e. the bioanalysis report on *in vivo* metabolism of Cyclamen aldehyde and analysis of testes, liver and plasma samples. (Annex IV)

2. Adverse testicular effects in male animals (rats vs. rabbits)

2.1 Evidence of Species Differences

As summarised in the CLH report and in Natsch et al. (Natsch et al., 2021), effects for Cyclamen Aldehyde are observed in male rats. In repeated dose toxicity studies, this is evident by marked reduction in the % motile spermatozoa and % of spermatozoa with a normal morphology. In parallel, adverse degeneration of elongating spermatids and spermatid retention were observed in histopathological examinations of the testes next to further alterations.

In contrast, no adverse effects were seen in the rabbit study. Treatment with up to 300 mg/kg bw/d of Cyclamen Aldehyde **did not affect** motility and morphology of spermatozoa (see Table 11 in CLH report and Figure 3 in (Natsch et al., 2021)). The number of motile sperms and total sperm counts was very variable with high standard deviations, varied within historical control range and no significant differences were found (Table 2). The study authors stated that "individual values were highly variable, and the lowest reported individual values were within the range of the control group values. In general, **all values across all treated groups were within the range of the concurrent control group and/or the historical control range at the Testing Facility**". The cauda epididymal sperm counts were less variable and both cauda epididymal sperm counts and density were unaffected by treatment up to 300 mg/kg bw/d, too (Table 1). Based on this analysis, the study authors concluded that "no noteworthy changes in sperm parameters were observed", hence *a fortiori* no adverse effects.

Table 1. Sperm counts and data variability in rabbit study on Cyclamen Aldehyde

	Historical controls			**Control animals**	**Top dose (300 mg/kg bw/d)**
	Average	Min	Max	Average ± standard deviation	Average ± standard deviation
Number motile sperms	674	405	786	627 ± 425	431 ± 351
% motile sperm	93.9	90.6	97.2	83 ± 26	88 ± 8
Total sperm counts	700	432	841	679 ± 387	469 ± 339
Cauda epididymal sperm counts	*no data* [1)]	*no data*	*no data*	151 ± 78	150 ± 80
Cauda epididymal sperm density	*no data*	*no data*	*no data*	435 ± 166	390 ± 190

[1)] sperm count given in a different scale in the historical control

Given those results, the short, one-sentence conclusion in the CLH report is inadequate and not supported by these findings ("*A dose-related trend of the mean number of motile sperm and total sperm count from ejaculated samples in treated groups was observed in rabbits in absence of adverse general toxicity.*"). Further down, the conclusion on the rabbit study on Cyclamen aldehyde is generalised as "***two studies*** *of limited quality demonstrate* ***similar findings*** *in one additional species (rabbit).*" This is manifestly incorrect since both studies in rabbits (one with Cyclamen Aldehyde and one with Cyclemax, 14-day) showed no effect of toxicological relevance on the key sperm parameters which are affected in rats (reduced motile spermatozoa and spermatozoa with a normal morphology). Besides, sperm counts were not affected in a statistically significant or dose-related manner and were within historical control ranges. **Claiming this a "similar finding" to the rat situation is therefore erroneous**. Also, DS attributes to the rabbit studies a limited quality, but at the same time gives a high weight to the findings claiming that they prove a species-independent effect. This double standard is contradictory and contrary to the basic rules of a weight of evidence determination.

Cyclemax had clear adverse effects in rats on sperm parameters: the average sperm count and density from the cauda epididymis were significantly reduced, with an increased percentage of abnormal sperm at 75 mg/kg bw/d. At 250 mg/kg bw/d, male reproductive organ weights, decreased sperm motility, count, density and increased sperm with detached or no heads and minimal or mild microscopic changes in the testis and epididymis were found (ECHA, 2023b).

A rabbit study was performed on Cyclemax at the same testing facility as for Cyclamen Aldehyde, and no adverse effects were found at 300 mg/kg bw/d for the number of motile sperm, total sperm count, cauda epididymal sperm counts and density. Values in the control groups were lower (226 ± 122 for motile and 301 ± 88 for total count) as compared to the Cyclamen Aldehyde study reflecting the variability in controls and the broad range of historical control data for these parameters in rabbits as reported above. At 300 mg/kg bw/d of Cyclemax, minimal or mild increases in residual bodies in the testes in five rabbits and mild or minimal depletion of spermatozoa in the epididymis in three rabbits were noted microscopically and of these three, two also had detachment of the seminiferous tubules of the testes. The percentage of abnormal sperm on study day 15 was the highest at 300 mg/kg bw/d. The abnormal sperm counts consisting primarily of sperm with detached heads were mainly due to two animals with 49.5% and 60% abnormal sperms. However, abnormal sperm counts also varied in individual animals in pre- and post-dose control groups between 8 - 52%, indicating a high variability of this parameter in rabbits.

For this rabbit study, the claim that the findings were "**similar to the rat studies"** in the CLH report **is not accurate since there were no effects of toxicological relevance** on the key sperm parameters affected in rats and minimal to mild histopathological effects were noted only at clearly higher doses in rabbits. Thus, the results from the rabbit studies would indicate **at the utmost a clear potency difference in rabbits vs. rats**, with slight histopathological effects for one of the tested molecules (Cyclemax) only and only at the highest dose, not yet translating to significant effects on key sperm parameters.

Conclusion from adverse effects in male rats

Based on this assessment, **male reprotoxicity is reproducibly observed in rats, and there is a clear species difference with no effects of toxicological relevance** on key sperm parameters observed in rabbits, and only weak histopathological effects seen at 300 mg/kg bw/d in one out of two studies.

2.2 Mode of Action: CoA-conjugate accumulation in rat liver and testes and species difference

The detailed metabolism studies of Cyclamen Aldehyde to 4-iPBA and further to 4-iPBA-CoA (CoA-conjugation) have been summarised in the peer reviewed literature (Laue et al., 2020; Natsch et al., 2021). Two key metabolic events were measured *in vitro:* (i) formation and kinetics of accumulation of 4-iPBA-Coenzyme A-conjugate as key metabolite (4-iPBA-CoA) and (ii) impact on the endogenous formation of the most dominant CoA-conjugates (octanoyl/octenoyl-CoA esters; C8-CoAs) in plated rat, rabbit and human hepatocytes over 22 h incubation time (Figure 1).

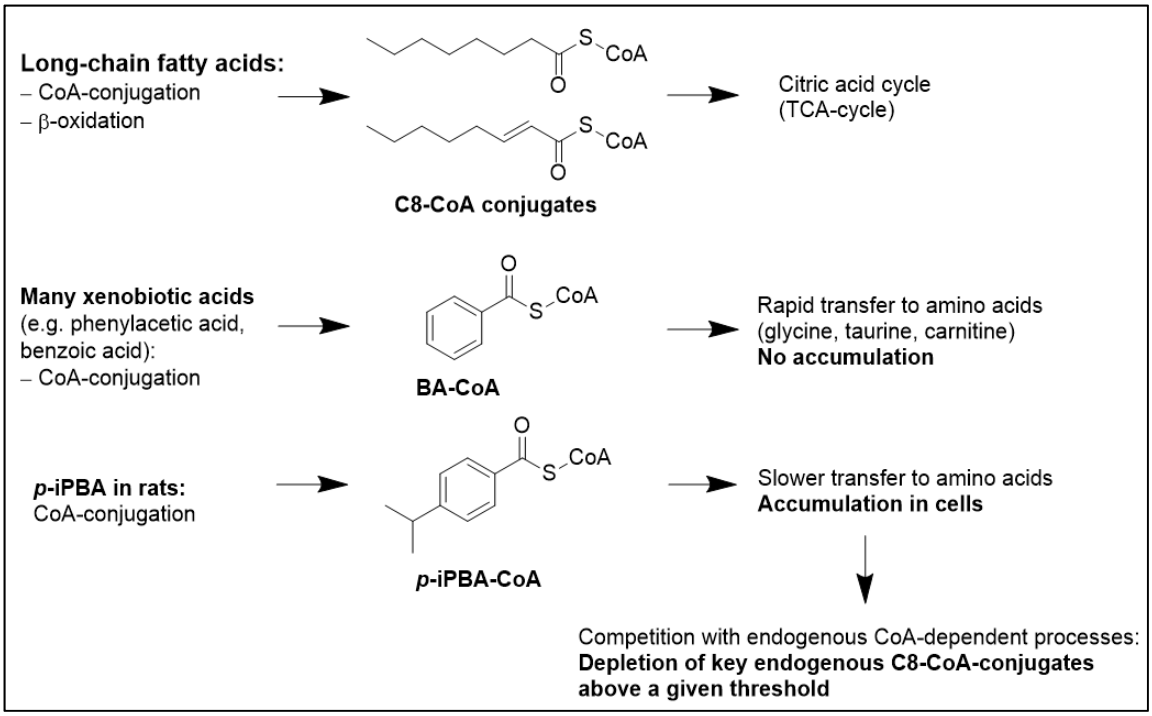

Figure 1. 4-iPBA-CoA and C8-CoA conjugates studied in the mechanistic *in vitro* studies on CoA-homeostasis in liver cells

A summary of the in vitro studies (Laue et al., 2020; Laue et al., 2017; Natsch et al., 2021) is listed below. Further new data including *p*-cymene and detailed analysis of dose-response data are given in additional reports submitted with this document as Annex II and III (RCR 154'270 and RCR 154'305).

<u>Rats:</u>

- Adverse effects in rats on spermatogenesis are reproducible
- 4-iPBA is the key circulating metabolite *in vivo* at a reprotoxic dose of Cyclamen Aldehyde (RCR 153'889)
- 4-iPBA-CoA is formed *in vivo* in liver and testes at a reprotoxic dose of Cyclamen Aldehyde (RCR 153'889)
- 4-iPBA-CoA is accumulating to stable levels over the 22 h incubation time *in vitro* in plated rat hepatocytes exposed to Cyclamen Aldehyde (Figure 2; RCR 154'270), *p*-Cymene or Cyclemax (RCR154'305);
- In parallel to the 4-iPBA-CoA accumulation, there is a sustained drop of the key endogenous C8-CoA metabolites in rat hepatocytes *in vitro* (Figure 3; RCR 154'270) with a clear dose-response relationship and a threshold of Cyclamen Aldehyde or *p*-cymene for the C8-CoA decrease (Figure 6; RCR 154'305);
- A similar metabolic fate in hepatocytes is observed for Cyclamen Aldehyde, *p*-cymene and Cyclemax (RCR 154'305).

Rabbits:

- No statistically significant or dose-response adverse effects on key sperm parameters by Cyclamen Aldehyde and Cyclemax. Cyclemax had only minor effects on testes histopathology at 300 mg/kg bw/d;
- 4-iPBA-CoA is mostly transiently formed and not accumulating to stable levels over 22 h incubations in plated rabbit hepatocytes exposed to Cyclamen Aldehyde *in vitro* (Figure 2; RCR 154'270);
- There is no sustained drop of the key endogenous C8-CoA metabolites in rabbit hepatocytes upon exposure to Cyclamen Aldehyde (Figure 3; RCR 154'270).

Humans:

- Significantly less 4-iPBA is formed in human hepatocytes (RCR154'270; Natsch *et al.*, 2021) and human metabolism is more directed towards Cyclamen alcohol formation
- 4-iPBA-CoA is only transiently formed and not accumulating to stable levels over the 22 h incubation time in plated human hepatocytes (Figure 2);
- There is no lasting drop of the key endogenous C8-CoA metabolites in human hepatocytes exposed to Cyclamen Aldehyde (Figure 3, RCR 154'270).

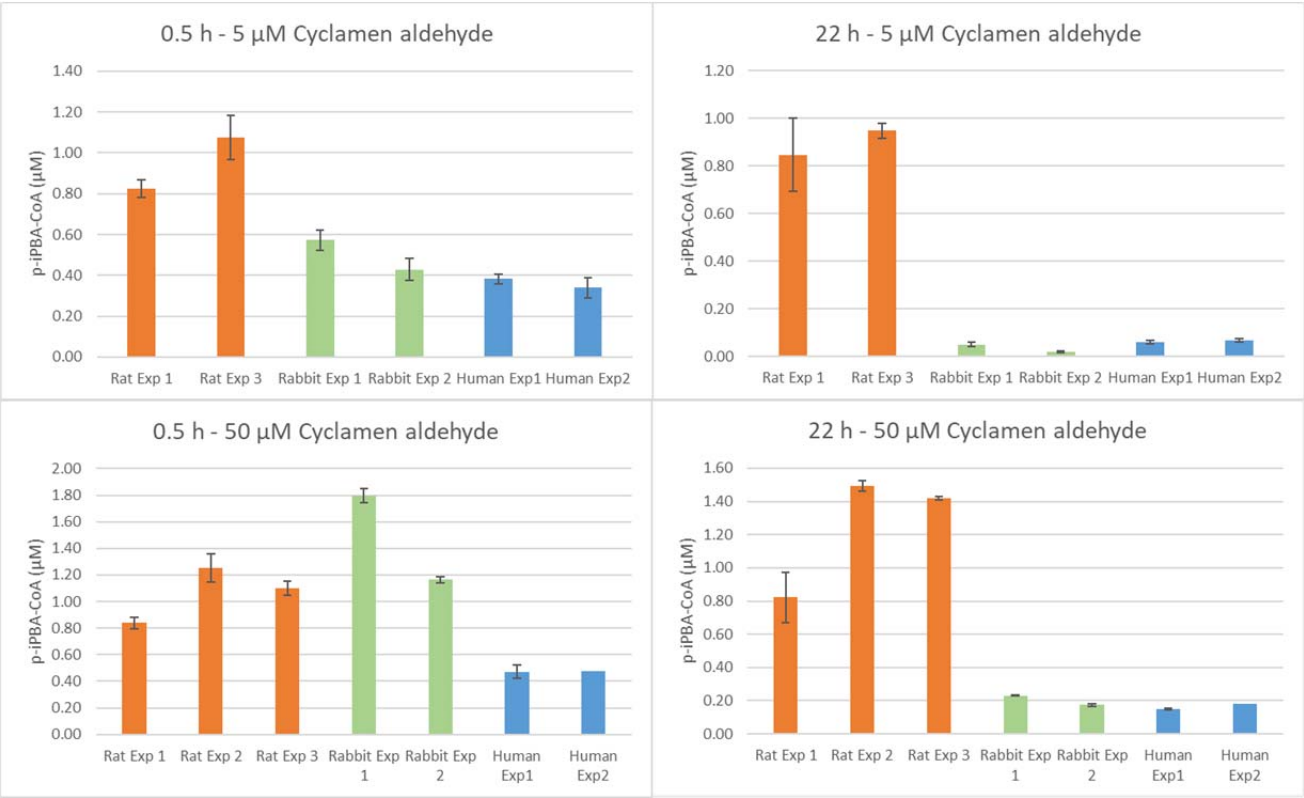

Figure 2. Species difference for p-iPBA-CoA accumulation in hepatocytes from different species. Plated hepatocytes from rat (orange), rabbit (green) and human (blue) were incubated with 5 µM Cyclamen aldehyde (top) or 50 µM Cyclamen aldehyde (bottom) for 0.5 h (left) or 22 h (right) and the level of p-iPBA-CoA quantified with HR LC-MS. For details see RCR 154'270.

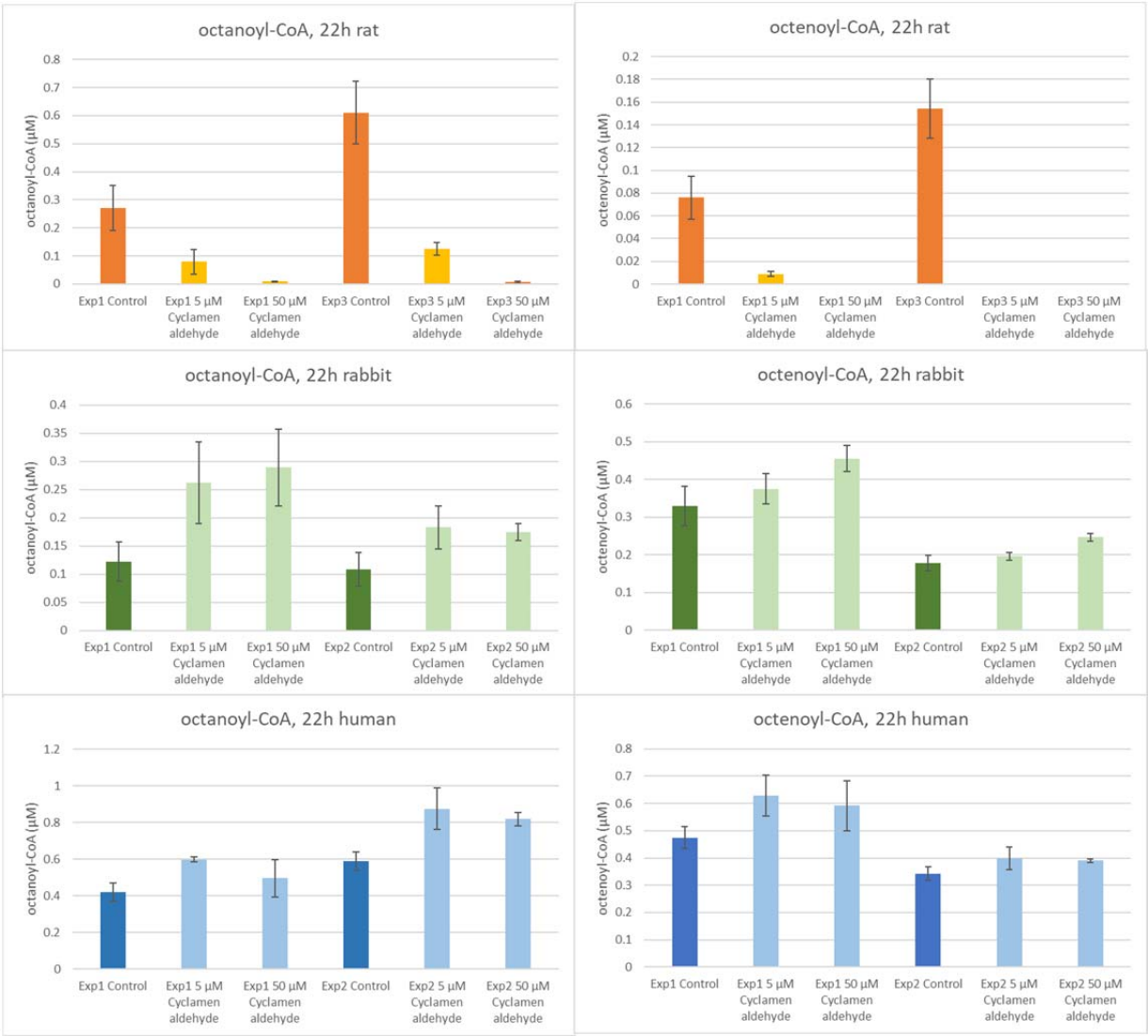

Figure 3. Species difference for effects on C8-CoA levels in hepatocytes from different species 22 h after treatment with Cyclamen aldehyde. For details see RCR 154'270. Plated hepatocytes from rats (orange), rabbits (green) and humans (blue) were incubated with 5 or 50 µM Cyclamen aldehyde for 22 h and the level of octanoyl-CoA quantified with HR LC-MS. Dark colors indicate the values of solvent treated cells in the respective experiment, while light colors indicate the chemical treated cells.

There is evidence that aromatic aldehydes metabolized to benzoic acids with subsequent formation and accumulation of Coenzyme A (CoA) conjugates could impair male reproduction in rats by adversely affecting CoA-dependent processes required for spermatogenesis (Laue et al., 2020). Based on this evidence, **an Adverse Outcome Pathway (AOP) of male reprotoxicity in rats for this group of substances is proposed as the most plausible** in Figure 4.

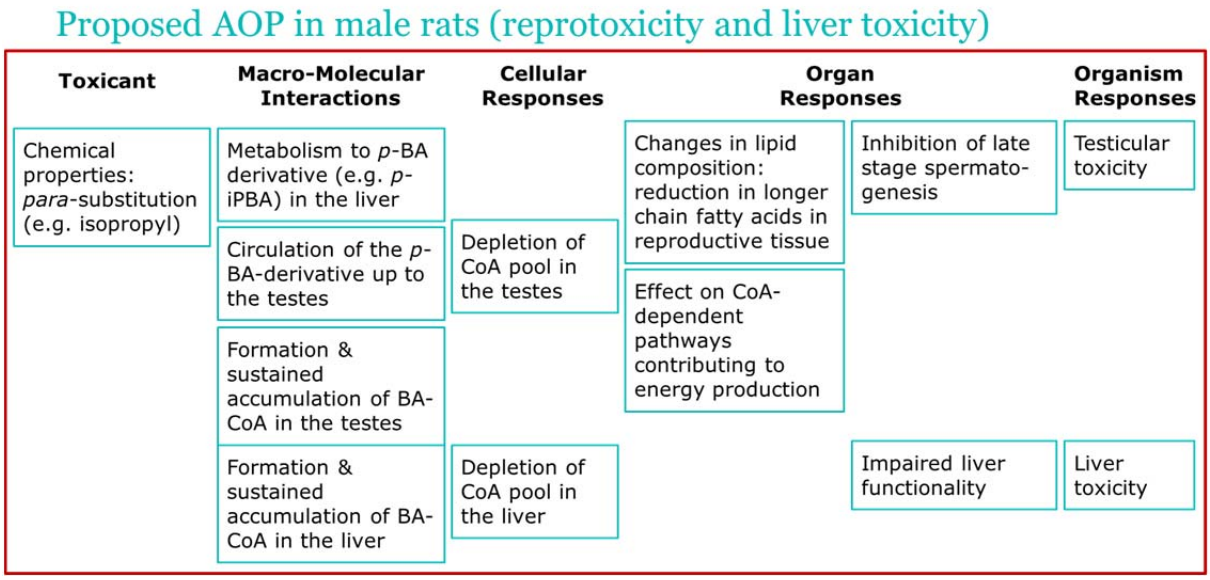

Figure 4. AOP for toxicity mediated by 4-iPBA-CoA

The AOP is supported by the following observations:

- There is a correlation between rat reprotoxicity and benzoyl-CoA accumulation in rat hepatocytes (Laue et al., 2020)
- There is a correlation between C8-CoA depletion and xenobiotic benzoyl-CoA accumulation in rat hepatocytes (Laue et al. 2020; Supporting Information), with those chemicals leading to C8-CoA depletion expressing male rat reprotoxicity;
- There is a quantitative correlation between species specificity for toxicity and (i) *in vitro* xenobiotic benzoyl-CoA-conjugate formation and (ii) C8-CoA depletion in hepatocytes of different species.

Thus, **both (i) differences in toxicity between related chemicals and (ii) differences in potency of reproductive toxicity between species are paralleled by differences in the accumulation of CoA-conjugate and C8-CoA depletion**.

The **pronounced differences in the metabolic fate between rat and rabbit and more importantly between rat hepatocytes and human hepatocytes** have been summarized in Table 6 of the CLP report, yet they **have not been taken into account in the assessment of human relevance in the current CLP report although they are of primary importance to discuss a Repro 1B classification** (see also section 5.4). The report only refers to a prior discussion on species difference on a member of a different group, which is not adequate (see also section 5.1).

An *ex vivo* study to examine the mechanism of toxicity of *p*-alkyl-benzoic acids in rat seminiferous further proved the accumulation of CoA-conjugates by the *para*-substituted derivative as key event affecting lipid homeostasis in the target tissue (Hareng et al., 2023):

- tBBA-CoA accumulation upon exposure to *p*-tBBA was paralleled by effects on late-stage spermatogenesis and a dramatic impact on the lipidome in the rat tissue samples;
- A lack of tBBA-CoA accumulation following *meta*-tBBA exposure was paralleled by a lack of effects on late-stage spermatogenesis and no large impact on the lipidome in the tissue samples.

Thus p-Benzoyl-CoA conjugates do accumulate in *ex vivo* treated rat testes tissue (Hareng et al., 2023) and *in vivo* in rat testes (Laue et al., 2020). This accumulation does occur locally, since CoA-conjugates are not transported across cell membranes but are kept in the cells. Coenzyme A mediates the synthesis and oxidation of fatty acids as well as the oxidation of pyruvate in the citric acid cycle. Reproductive tissues have a high energy demand. Changes in primary metabolism and lipid metabolism as a consequence of CoA-conjugate accumulation above a certain threshold therefore appear as a key organ response due to these biochemical disturbances.

Based on the knowledge summarized in the AOP, the most plausible conclusion is:

a) Benzoyl-CoA-conjugate formation is a hallmark of the MoA for reproductive and liver toxicity in rats;

b) Benzoyl-CoA-conjugate formation above a threshold affects markers of lipid metabolism in both hepatocytes (as evidenced by endogenous C8-CoA conjugates) and *ex vivo* testes tissue (effects on lipidome) of rats;

c) Significant Benzoyl-CoA-conjugate accumulation and sustained depletion of C8-CoA is not observed in rabbit hepatocytes, which is consistent with of a lack of adverse effects on key sperm parameters in rabbits *in vivo*, while all these effects are seen in rats;

d) Benzoyl-CoA-conjugate accumulation and effects on endogenous C8-CoA formation are lower or absent in human cells because human cells can clear these conjugates by further transferring the 4-iPBA to terminal acceptors. This is indicating the lowest response in human cells for these biochemical events among the species studied.

Next to the species difference in the formation and accumulation of the key toxic metabolite 4-iPBA-CoA, also at the level of the formation of Phase I metabolites, clear species difference between rat and human hepatocytes were noted (RCR 154'270, (Natsch et al., 2021)). Thus Cyclamen aldehyde can first be either reduced to the alcohol and then conjugated to glucuronic acid or oxidized to Cyclamen acid. In human cells the reduction is an important step, while in rat the oxidation is the dominant step. In humans, the alcohol is then also transferred to glucuronic acid at higher levels as compared to rats, thus a much higher proportion of the applied dose is directed towards the metabolites based on initial reduction, which then is not further transformed to 4-iPBA (Referred to as metabolites M1, M20, M21, M41, see Table 10 in RCR 154'270).

As a consequence of this deviation of the metabolism towards Cyclamen alcohol, much lower levels of 4-iPBA are formed in human cells (5 µM in humans as compared to 31 µM in rat cells; See Figure 3 in RCR 154'270). Once 4-iPBA is formed, this metabolite can then also be more efficiently detoxified by glucuronidation in human cells as compared to rat cells (See Table 10 in RCR 154'270). This is in line with observations from (Sakaguchi et al., 2004), who studied glucuronidation of various carboxylic acids and noted that "human UGT1A9 showed the highest glucuronidation rates with cuminic acid (=4-iPBA) compared to any of the other carboxylic acids tested in this study", thus confirming very efficient detoxification of 4-iPBA in human cells.

Conclusion on metabolic differences

The pronounced metabolic differences indicate **that the 4-iPBA-CoA-dependent biochemical MoA is likely not to be similarly active in humans exposed to chemicals leading to 4-iPBA**. Thus the Dossier's Submitter conclusion on page 45 of the CLP report, namely that "*There is no mechanistic evidence to indicate that the observed effects are not relevant for humans*" **is inadequate, as clear mechanistic evidence for the mode of action and a clear species difference for the toxic principle of this mode-of-action** has been published (Natsch et al., 2021) and is further substantiated in attached report (RCR154'270). Next to the difference in CoA-formation, already at the level of 4-iPBA formation and detoxification of 4-iPBA by glucuronidation, pronounced species differences exist between rat and human cells which will add to the difference in CoA-conjugate accumulation.

3. Adverse reproductive and developmental effects in female rats, insufficient evidence across multiple species

3.1. Reproduction effects in female are a secondary and non-specific consequence of male infertility

As summarised in the CLH report, adverse effects related to maternal exposure of Cyclamen Aldehyde include reduced number of implantation sites, increased pup mortality, reduced ovaries weights at 150 mg/kg bw/d as well as reduced uterus weights at ≥ 75 mg/kg bw/d.

Marked liver weight increase is reported for Cyclamen Aldehyde and *p*-cymene studies, reaching on average +20% and going up to 45% (relative to body weight) at 150 mg/kg bw/d and 100 mg/kg bw/d, respectively, indicating there are clear effects on overall metabolism. **It is likely that effects on pup body weight and survival discussed below are secondary to the maternal effects** (i.e. maternal reduced body weight/body weight gain for Cyclamen Aldehyde and increased maternal liver weight).

In the OECD 415 study conducted on Cyclamen Aldehyde, the adverse effects were observed in the presence of decreased body weight gain occurring at 150 mg/kg bw/d during the dosage period prior to cohabitation, with statistically significant reductions ($p \leq 0.01$) occurring on SD 1 to 8 and overall on SD 1-15, in comparison to the

vehicle control group values. Body weight gains in the 150 mg/kg bw/d group was 65% of the vehicle control group during the dosage period prior to cohabitation (SD 1 to 15).

The differences on body weight and body weight gain were observed in the presence of statistically significant absolute and relative feed consumption changes during the cohabitation (SD 1 to 8).

The decrease of absolute and relative weights of the non-gravid uterus and the ovaries following Cyclamen Aldehyde exposure was observed in the absence of microscopic changes, without dose-dependent effect, in the OECD 415 study. Therefore, the **biological relevance of these findings is questionable**, since no microscopic correlates were reported in the histopathology of these rat uterus and ovaries. Furthermore, no effects on estrous cyclicity or mating and fertility parameters were seen at doses up to 150 mg/kg bw/d. Also, the reduced average number of implantation sites seen at the highest dose in the OECD 415 study (13.8 implantation sites vs 15.5 in the control group) described in the CLH report were within the historical control data range (12.9 - 16.8) of the lab and **are therefore not considered as adverse.** Important to note the results of the OECD 414 study on Cyclamen Aldehyde that shows no relevant effect on numbers of corpora lutea and implantation sites or mean litter proportions of pre-implantation loss or differences in normal gravid uterine weights at 150 mg/kg bw/d.

For *p*-cymene, a decrease in thymus, uterus and cervix organ weight is highlighted in one single female of the 500 mg/kg bw/d group, observed in the non GLP dose range finder study, with no information on the statistical significance. Important to note, this female was sacrificed as moribund for welfare reasons (thin, rapid breathing, decreased activity and hunched posture) on study day 13, indicating this dose exceeded the maximum tolerated dose, and results observed at this level are considered **inconclusive or irrelevant**.

Also, irregular cycles during pre-cohabitation (<4 or >5 days) and increased pre-implantation loss were reported in the OECD 422 study on *p*-cymene. The number of animals having regular cycles (4-5 days) was reduced in an adverse way at the high dose (200 mg/kg bw/d). However, the number of cycles and the cycle duration of all dose groups were comparable with the control values. Furthermore, no statistically significant difference compared to the control group is reported for the increased pre-implantation loss seen at the low and mid dose. This finding may likely be attributed to the male infertility. The number of pregnant females was significantly reduced (9, 9, 4 and 0 females pregnant) in the control, 50, 100 and 200 mg/kg bw/d dose groups, respectively, indicating treatment-related reductions in fertility at 100 and 200 mg/kg bw/d, clearly related to **the infertility of male rats and not a direct reprotoxic effect in females**. This conclusion is corroborated by the OECD 415 study of Cyclamen Aldehyde, where lack of pregnancy was only seen with treated males, but not in treated females mated with non-treated males. *P*-cymene induced testicular changes observed at 200 mg/kg bw/d (lower organ weights, germ cell depletion/degeneration, depletion, and/or sperm retention), along with correlative changes in the epididymis that were considered adverse. These changes were considered the likely cause of the total absence of

pregnancy among P0 females in the 200 mg/kg bw/d dose group. At 100 mg/kg bw/d, a marginal degree of sperm retention was observed, and this may also have contributed to the absence of pregnancy in dams.

3.2. Developmental effects are explained by and secondary to maternal toxicity

According to the CLH report, developmental toxicity is evidenced by reduced post-implantation survival, reduced number of live pups at birth, reduced pup survival at the beginning of lactation, reduced foetal and offspring weight at birth in rat reproductive and developmental toxicity studies conducted on Cyclamen Aldehyde (OECD 414, OECD 415) and *p*-cymene (OECD 422). The CLH also highlights the significantly increased number of lens opacities observed in pups of treated females mated with untreated males as well as the reduced weight of several organs in both male and female pups in the Cyclamen Aldehyde OECD 415 study.

Treatment-related alterations in pup survival and mean pup/litter weight have been observed in Cyclamen Aldehyde and *p*-cymene studies. Mortality of pups occurred significantly at 150 mg/kg bw/d and 100 mg/kg bw/d for Cyclamen Aldehyde and *p*-cymene, respectively. Decreases in pups body weight are reported at ≥ 75 mg/kg bw/d and 100 mg/kg bw/d following Cyclamen Aldehyde and *p*-cymene exposure. These adverse effects on F1 generation are **likely explained by maternal toxicity**, occurring at these dose levels and expressed as marked liver weight increases, as well as marked body weight/body weight gain reductions for Cyclamen Aldehyde (see below). They are therefore **considered secondary to maternal toxicity**.

The decreased post-implantation survival index reported for *p*-cymene has no statistically significant differences and can therefore not be considered as adverse.

In the OECD 415 study with Cyclamen Aldehyde, body weight losses in dams were observed at all doses during gestation and lactation. The changes reached statistical significance at 150 mg/kg bw/d during gestation (GD 0-21, $p \leq 0.01$) and lactation (LD 1-5, $p \leq 0.01$ and LD 5-8, $p \leq 0.05$) periods when compared to the vehicle control group value. Body weight gains in the 150 mg/kg bw/d group were 85% of the vehicle control group during the gestation period (GD 0 to 21) and 61% of the vehicle control group value during the lactation period (LD 5 to 8). The body weight was also affected in a significant proportion during gestation (on GD 18 and 21) and lactation (on LD 5, 8 and 11) periods at 150 mg/kg bw/d as presented in Figure 5.

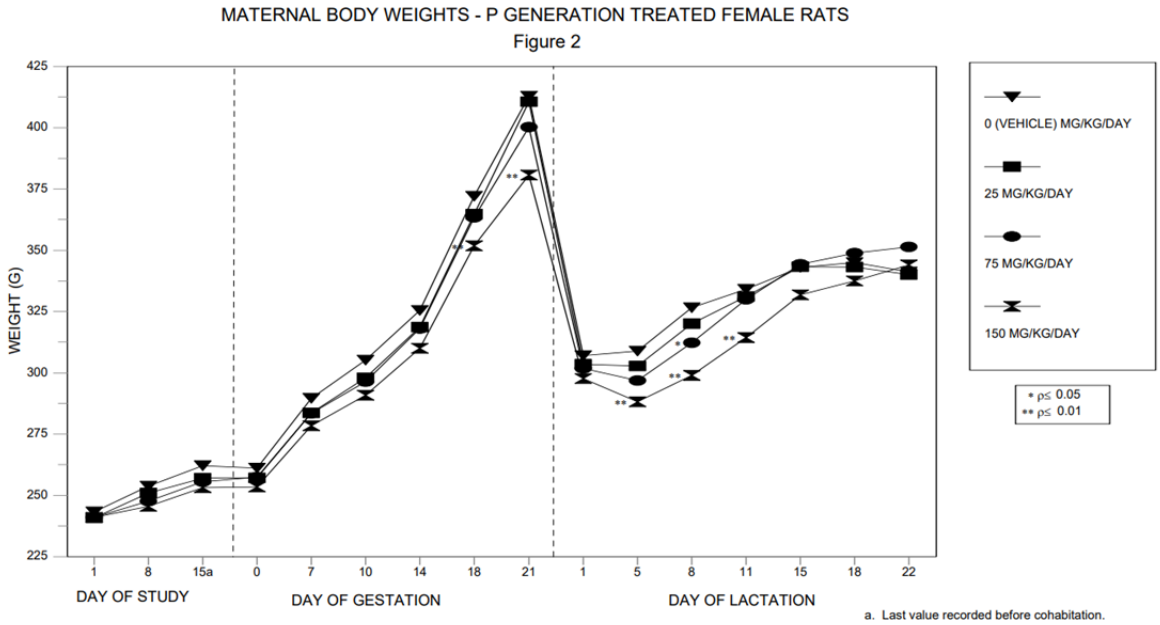

Figure 5: Maternal body weights of P generation female rats treated with Cyclamen Aldehyde (OECD 415 study).

During lactation (LD 1 to 15), absolute and relative feed consumption values were also significantly reduced ($p \leq 0.05$ or $p \leq 0.01$) in the 75 and 150 mg/kg bw/d groups, in comparison to the vehicle control group values. Absolute feed consumption values in the 75 and 150 mg/kg bw/d groups were 89% and 80% of the vehicle control group value, respectively, on LD 1 to 15. **There were significant toxic effects on the female animals during gestation, which were accentuated during lactation. Therefore, the hypothesis that the effects observed in pups are a consequence of maternal toxicity is plausible**. To corroborate this hypothesis, Cyclamen Aldehyde at 150 mg/kg bw/d had no effects on intrauterine survival, mean number of live foetuses, and foetal sex ratios observed in the OECD 414 Developmental toxicity study, during gestation day 6-20.

Differences in organ weights of F1 generation male and female rats treated with Cyclamen Aldehyde were observed mostly when considering the absolute organ weight, which is inappropriate due to the difference in pups body weights. Table 2 summarises the main findings described in the CLH report (left side), with the responder comments (right side).

High incidence of lenticular opacity in pups (from treated females mated with untreated males) was observed at the highest dose (150 mg/kg bw/d) of Cyclamen Aldehyde. The effect was observed at only one dose level, meaning that it occurs only above a certain threshold. No ophthalmological or histopathological evaluation was performed, and the information this study provided for this adverse effect is clearly limited. Most importantly, no other study with Cyclamen Aldehyde or similar substances has reproduced this adverse effect, thus, **the toxicological relevance of this finding is considered questionable**. Lenticular opacities have been found as side-effects for multiple drugs which infer with lipid metabolism by reducing cholesterol levels or changing the profile of sterols in the lens (Iwasaki et al., 2020; Nakano-Ito et al., 2014). Thus, for example inhibitors of the enzyme hydroxyl-

methylglutaryl-Coenzyme A reductase (statins) administered at high dose cause lenticular opacities in dogs by a biochemical mode of action (Gerson et al., 1989).Whether the opacities observed in the OECD 415 study at the top dose is linked to the interference with lipid metabolism by *p*-iPBA-CoA is currently unknown.

Table 2. Developmental effects in pups reported in the OECD 415 study for Cyclamen Aldehyde described in the CLH report, with the responder comments

F1 generation	
Pups of treated males	
No litters produced at 150 mg/kg bw/d (p≤0.01).	Due to adverse effects observed in males, with no relevance for humans.
↓ relative brain weight in males at 25 (-6%) and 75 (-6%) mg/kg bw/d (p≤0.05).	Not a dose response effect, not reproduced in F1 female rats, no other effects observed in pups of treated males. Biological relevance questionable.
Pups of treated females	
↑ litters with pups with lenticular opacities at 150 mg/kg bw/d (20 of 24 litters; p≤0.01).	High incidence of lenticular opacity at top dose, meaning there is a threshold to occur. No ophthalmological or histopathological evaluation was performed. No other study performed with Cyclamen Aldehyde or similar substances has presented lens opacity, thus, the toxicological relevance of this finding is considered questionable.
↓ mean pup body weight/litter at 75 mg/kg bw/d (ranging from -10% on day 1; p≤0.01 to -13% on day 22; p≤0.01) and at 150 mg/kg bw/d (ranging from -11% on day 1; p≤0.01 to -14% on day 22; p≤0.01).	Pup body weight change is not dose-dependent and not progressing from day 1 - 22. It is observed in combination with maternal toxicity at 150 mg/kg bw/d.
↑ pup mortality (days 1-5 postpartum) (p≤0.01) at 150 mg/kg/d ↓ viability index (p≤0.01) at 150 mg/kg/d ↓ number of surviving pups per litter and live litter size on days 1-22 postpartum (p≤0.01)	Pup mortality is observed in combination with maternal toxicity at 150 mg/kg bw/d.

Male pups	
↓ anogenital distance at 75 and 150 mg/kg bw/d (-7% and -6%, respectively, day 22 postpartum; p≤0.05).	When covaried with foetal body weights per litter, the statistically significant reduction in anogenital distance was no longer apparent. This developmental delay correlated with an overall reduction in pup body weights on day 22 postpartum.
↓ absolute left epididymis weight (-10%) at 150 mg/kg bw/d (p≤0.01).	Male pups at this dose had a decrease in body weight, organ differences disappear when values are converted to ratio % of organ weight to terminal body weight.
↑ relative testis weight at 75 mg/kg bw/d (left and right +5%; p≤0.05) and at 150 mg/kg bw/d (left +5% and right +6%; p≤0.05).	Effect contradictory to everything known about this substance and effects observed in adults, which is a decrease in testis organ weight. Relevance of this isolated data of low magnitude is questionable.
↓ absolute pituitary weight at 150 mg/kg bw/d (-17%; p≤0.01).	Pituitary weight at 150 mg/kg bw/d is not different from controls when values are corrected to ratio % of organ weight to terminal body weight.
↓ absolute brain weight at 150 mg/kg bw/d (-4%; p≤0.01).	Brain weight at 150 mg/kg bw/d is not different from controls when values are corrected to ratio % of organ weight to terminal body weight.
↓ absolute and relative adrenals weight at 75 mg/kg bw/d (-15% and -16%, respectively; p≤0.01) and at 150 mg/kg bw/d (absolute only, -13%; p≤0.05).	Statistically significant difference observed at 75 mg/kg/d (absolute and relative to the body weight). Results are not dose dependent considering the relative weight, as the difference is not observed at 150 mg/kg bw/d when results are corrected to ratio % of organ weight to terminal body weight.
Female pups	
↑ anogenital distance at 150 mg/kg bw/d (+4%, day 1 postpartum; p≤0.01 when covaried with foetal bw/litter).	This increase in anogenital distance was no longer apparent by day 22 postpartum. Transient effect only observed in female pups.
↓ absolute brain weight at 75 mg/kg bw/d (-3%; p≤0.05) and at 150 mg/kg bw/d (-6%; p≤0.01).	Brain weight at 75 and 150 mg/kg bw/d is not different from controls when values are corrected to ratio % of organ weight to terminal body weight.
↓ absolute ovary (left) weight at 150 mg/kg bw/d (-17%; p≤0.05).	Ovary left weight at 150 mg/kg bw/d is not different from controls when values are corrected to ratio % of organ weight to terminal body weight.

3.3. Plausible MoA for female effects

The DS relies on a read-across for the effects on females / development within the group of substances forming 4-iPBA and the CLH report does apply the conclusions on female / developmental findings to all members of the group. This generalization made by the DS assumes that the observed effects are due to the common metabolite 4-iPBA defining the group, although this is neither explicitly stated nor demonstrated. While there is less strong mechanistic evidence as compared to the male reprotoxic effects and most female effects are observed in presence of maternal toxicity, this appears as a plausible assumption made by the DS. Thus, a hallmark also in the developmental studies is an increased liver weight in dams and reduced body weight/body weight gain and reduced body weight of pups. Effects on the liver in rats are common and often occurring at the same dose for multiple precursors of *p*-alkyl-benzoic acids (Laue et al., 2020). Given the high accumulation of 4-iPBA-CoA in rat livers observed in an *in vivo* study (Laue et al., 2020; Natsch et al., 2021) and the strong effects of *p-alkyl*-benzoic acids on the metabolome, the lipidome and key metabolites of the primary metabolism in seminiferous tubule of the rat, we may indeed expect that this metabolic imbalance is not specific to reproductive tissues and has effects on multiple organs, since CoA-dependent processes are vital to multiple organs. The different effects observed at doses which also affect parameters in the dams in rat studies may then also be due to the formation of the *p*-alkyl-benzoic acid CoA. Thus, **with the assumption by the DS that 4-iPBA, which is defining the group, is causing these effects on females and some developmental parameters, it indeed appears likely (i.e. the most plausible) that effects are secondary and also due to a strong metabolic imbalance after administration of precursors of 4-iPBA leading to 4-iPBA-CoA mediated effects above a given threshold**. Although it cannot be ruled out that 4-iPBA has a secondary, independent toxicity mechanism, it appears rather unlikely.

4. Key toxicological conclusions

The **testicular effects in rats appear species-specific with strong mechanistic evidence that these effects are not relevant to humans**, due to pronounced differences in hepatic metabolism in humans and clearance of the toxic metabolite. Thus, a C&L of Repro 1B is not justified.

In rabbits, no statistically significant or dose-response effects on key sperm parameters by Cyclamen Aldehyde and Cyclemax are observed. The minor effects on testes histopathology at 300 mg/kg bw/d in the Cyclemax study are not seen in the Cyclamen Aldehyde study at the same dose.

In female rats, the reduced number of implantation sites, uterus and ovaries weights in dams treated with Cyclamen Aldehyde **are not considered as adverse due to absence of biological relevance**. Absence of pregnancy and/or reduced implantations seen in *p*-cymene OECD 422 study are **likely the consequence of disturbed spermatogenesis**. Effects on sexual organ weights observed in *p*-cymene dose range finding study are

isolated and not significant. The female reproduction effects are occurring together with **maternal toxicity**. Based on these findings, a Repr. 1B classification is not warranted for female reproductive toxicity.

The **developmental effects on pups** in rat studies which show adverse effects on pups/fetus survival and pups growth **appear related to maternal toxicity** as evidenced by reduction in body weight, food consumption (for Cyclamen Aldehyde) and marked liver weight increase (for Cyclamen Aldehyde and *p*-cymene). The effects observed on these developmental parameters occur together with maternal toxicity. The lenticular opacity observed in pups from Cyclamen Aldehyde treated females is of questionable relevance due to lack of reproducibility in other studies and uncertainties remain due to limited information and absence of confirmatory ophthalmological/histopathological evaluation.

There is no female reproductive toxicity nor developmental toxicity data available for Cyclemax, Cuminic Aldehyde and 4-iPBA. Besides, the effects described above for Cyclamen Aldehyde and *p*-cymene are only observed in female rats. Contrary to the male fertility studies, no animal study is available in females of other species for the full assessment of female fertility and developmental effects, thus preventing the evaluation of the species specificity aspect for this gender.

5. Additional Comments

5.1 The previous CLH conclusion for Lysmeral did not take into account the relevant Mode of action and is thus not relevant to the evaluation of the MoA of 4-iPBA.

The DS has acknowledged that the grouping of substances based on tBBA metabolism should be separated and has submitted a separate CLH proposal, yet contradicting this separation of the groups the CLH report states that: "*Relevance to humans was discussed in the RAC opinion of the structurally similar substance 2-(4-tert-butylbenzyl)propionaldehyde (lysmeral) (ECHA, 2019), in which a similar mechanism of action was proposed by the DS (BASF SE) based on formation of 4-tert-butylbenzoic acid (tBBA) and stable CoA-tBBA conjugates*".

Such reference is incoherent as the DS has already agreed that the tBBA grouping should be considered separately to the substance group metabolising to 4-iPBA. Furthermore, this statement suggests that the mechanistic relevance question had been settled by RAC for the MoA on which the current grouping is based. However, this is a misrepresentation. Indeed, in the original assessment of Lysmeral by Sweden, a high weight was given to the very weak estrogenic activity of parent Lysmeral *in vitro* based on an *in vitro* study in MCF7 human breast cancer cells (Charles & Darbre, 2009). Given that the effect was several magnitudes lower compared to the positive control 17 -estradiol, the biological relevance of this effect is more than doubtful. At the time of the RAC Opinion, Lysmeral was also on the ECHA ED list for evaluation, and since then this has been completed and an ED MoA is now considered inconclusive (https://echa.europa.eu/de/substance-information/-/substanceinfo/100.001.173).

The RAC opinion on Lysmeral further stated that: *The biological* ***plausibility of the proposed MoA*** **[i.e. the tBBA-CoA dependent MoA]** ***is uncertain***. *Key event(s) leading from "disruption of the lipid synthesis" to "testicular toxicity" are not defined (e.g. not clear if conjugate accumulation occurs in liver or testis).* ***Importantly, alternative/multiple MoA(s) have not been considered.*** *In particular, an* ***endocrine disturbing (ED) MoA for Lysmeral cannot be ruled out***. The RAC opinion further summarised comments received: "E*ight comments were received [...]* ***All of them questioned the proposed MoA as other MoAs were not ruled out (e.g. endocrine disruption by binding of Lysmeral to the oestrogen receptor)."***

In the RAC opinion on *p*-tBBA (ECHA, 2010), no considerations of the mode of action were included, and no data on species differences were available at the time of the assessment. Because of the lack of such data, the CLP report on *p*-tBBA explicitly stated: "***Since no mechanistic data could plausible demonstrate a species specific effect****, all toxic effects observed in rats after repeated exposure to 4-tert-butylbenzoic acid were considered to be of toxicological significance to human health*." Thus, the decision was made **based on absence of mechanistic data and lack of data** on species specificity at the time of the assessment in the case of *p*-tBBA. The CLP report does not remedy those shortcomings.

The MoA was strongly contested in the previous assessments (Lysmeral) or not available at all (tBBA). This **situation has now changed**, and the **grouping used for the new CLH report is explicitly based on the common formation of the metabolite 4-iPBA** thereby confirming the view that formation of 4-iPBA is the first step determining toxicity. Therefore, previously available and new information on metabolite formation, the MoA with *p*-alkyl-benzoyl-CoA formation as initiating event and human relevance of this CoA-dependent biochemical MoA has to be assessed *ab initio*, rather than to base classification on prior decisions which were disregarding or questioning this MoA. Also, many questions on the MoA raised by RAC are now answered, *e.g.* the open question *"not clear if conjugate accumulation occurs in liver or testis"* has been settled by *ex vivo* and *in vivo* experiments.

Furthermore, it was argued that "if metabolism in humans *does not qualitatively differ* from rat metabolism, relevance to humans cannot be precluded". Indeed, CoA-conjugate formation can be observed in hepatocytes from all species and for reprotoxic and non-reprotoxic substances. However, this is not surprising **as transient CoA-conjugation is a general detoxifying metabolic pathway** in mammalian tissues: most acids are *transiently* conjugated to the energy-rich CoA which further transfers them to amino acids such as carnitine, glycine, glutamine or taurine (Figure 1). This is well described for e.g. Phenylacetic Acid as the key metabolite from catabolism of the amino acid Phenylalanine. Under physiological conditions, the CoA-conjugate is only a short-lived shuttle for the transfer of the acids (Figure 1). The observed **accumulation** of the CoA-conjugates of *p*-alkyl-benzoic acids in tissues and hepatocytes of rats is ***thus a quantitative, and not a qualitative exception***.

Scientifically, it is impossible to formally prove a MoA in humans (high dose intervention studies in humans are ethically unacceptable) – however, it is possible, based on a WoE determination, to conclude that a certain MoA is most likely (Figure 4). By grouping chemicals according to their ability to form the same *para*-substituted benzoic acid, the CLH report DS apparently confirms that these molecules have a common biochemical MoA with the formation of the benzoic acid as initiating event but **fails to properly discuss MoA** and only refers to previous RAC discussions.

5.2 Investigating the threshold of cellular effects: C8-CoA depletion in rat hepatocytes after treatment with 4-iPBA precursors is subject to a threshold

It had been argued, that for hazard assessment *quantitative* difference in metabolite formation between animal models and human cells are not relevant, as only *qualitative* differences in metabolite formation would be relevant for hazard assessment, postulating that the detectable presence of a metabolite is sufficient to postulate human relevance. However, this is a misrepresentation of the role of metabolites in apical toxicity. Thus, a biochemical MoA is subject to a threshold, if it is due to accumulation of a metabolite: Only above a certain threshold, the metabolite reaches a sufficient concentration in cells to cause an imbalance of the metabolic pathway. This is different from CMR endpoints caused by effects on the genetic material, for which a no-threshold assumption is correctly applied.

Therefore, in further studies in rat hepatocytes, the dose-response of formation of 4-iPBA-CoA and C8-CoA depletion was studied for both Cyclamen Aldehyde and *p*-cymene in more detail. For both test chemicals, there is a clear dose-dependent accumulation of the 4-iPBA-CoA conjugate. Only above a certain threshold of CoA-conjugate accumulation, a drop in endogenous C8-CoA is observed. (Figure 6, for details see attached report RCR 154'305). This indicates that there is a clear concentration threshold and above this concentration threshold, the accumulation of the xenobiotic-CoA leads to a loss of this key physiological CoA-conjugate in rat hepatocytes as a marker of effects on lipid homeostasis. This threshold was surpassed in rat hepatocytes at the 22 h timepoint, but not in rabbit and human hepatocytes (see reports RCR 154'270 and RCR 154'305 submitted with this document and Figure 3). This is a further indication that the biochemical MoA is subject to a threshold for metabolite accumulation, and the presence/absence of the metabolite itself is not predictive of the hazard. Thus, quantitative differences in metabolite formation are relevant. Since transient formation of a CoA-conjugate is a general detoxification mechanism (Figure 1), the detectable transient formation of a CoA-metabolite itself is not indicative of hazard.

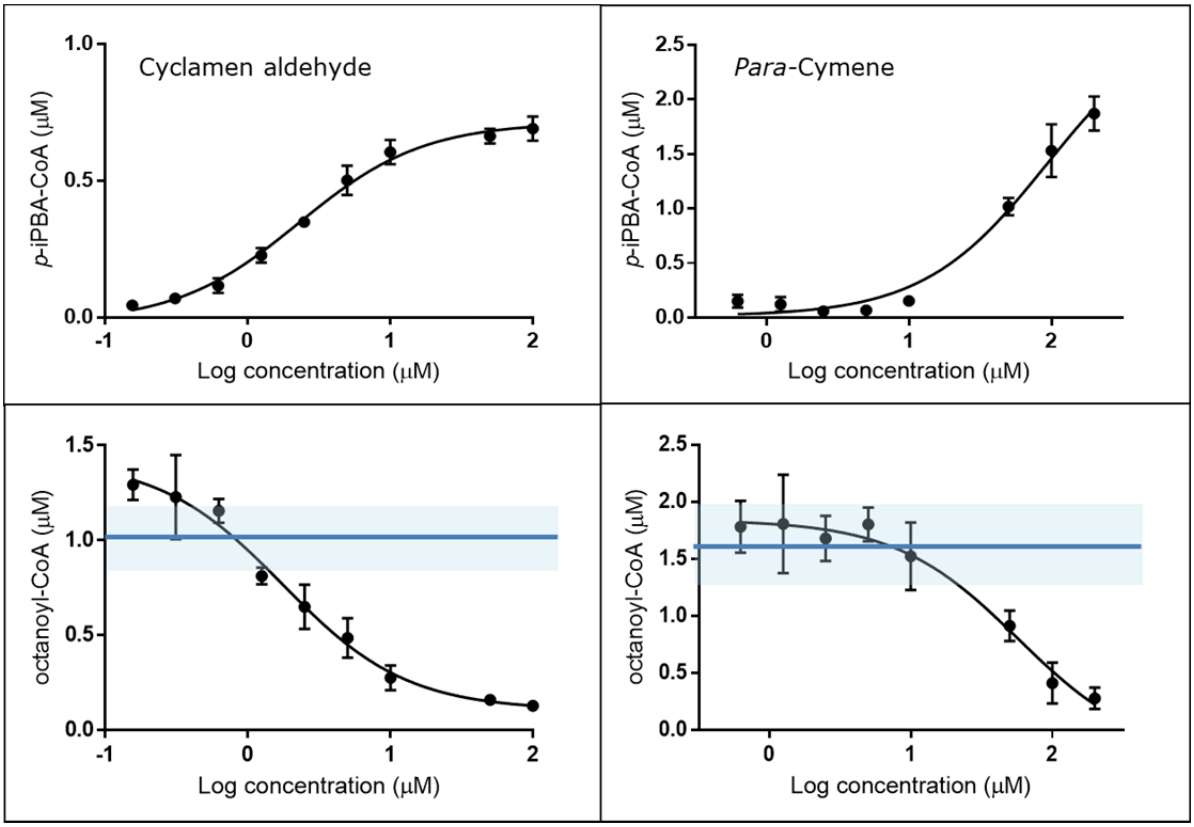

Figure 6. Dose-dependent accumulation of iPBA-CoA (top) in rat hepatocytes treated with Cyclamen Aldehyde and p-cymene for 22 h and concurrent drop of the key endogenous C8-conjugate (bottom). The blue line and light blue area indicates the average and standard deviation of levels of C8-CoA in the solvent treated cells in a given experiment.

(Note: as rapid losses of p-cymene were observed in the incubations due to either absorption to the cell culture plates and cover foil and/or due to evaporation, the bioavailable concentration is lower as compared to the nominal test dose plotted on the x-axis, for details on measured concentration, see test report RCR 154'305).

5.3. History of Human Use: Natural food and essential oil consumption of substances in this group that produce 4-iPBA, approved by JECFA and other world food Authorities

P-cymene is a central component of many naturals used for flavoring foods and as traditional medicine. Recently, the Flavor and Extract Manufacturers Association (FEMA) Expert Panel affirmed the generally recognized as safe (GRAS) status (Cohen et al, 2021) of Origanum Oil (FEMA 2828), Savory Summer Oil (FEMA 3013), Savory Summer Oleoresin (FEMA 3014), Savory Winter Oil (FEMA 3016), Savory Winter Oleoresin (FEMA 3017), Thyme Oil (FEMA 3064) and Thyme White Oil (FEMA 3065) (WHO, 2006). *P*-cymene was identified in all these naturals and ranged from 5 to 44%.

4-iPBA and *p*-cymene share the *p*-menthane skeleton with most monocyclic monoterpenes present in plants. Water abstraction, reduction/oxidation and/or aromatization can formally transform different monoterpenes into *p*-cymene. Thus, it is not surprising that *p*-cymene is a very common constituent in natural food products and essential

oils, which have been safely consumed by humans (and animals) over millennia indicating that there is a safe history of widespread use of p-cymene by humans which does not lead to any human-relevant toxicity.

The VCF database (https://www.vcf-online.nl/VcfHome.cfm) on published volatile compounds in natural (processed) food products lists 39 food sources in which over 3000 ppm (0.3%) of *p*-cymene had been analytically found including common foods such as citrus fruits, mint oils, origanum, rosemary, thyme, pepper, celery or cardamon among many others (See ANNEX I). Similarly, essential oils used for naturals-based consumer products widely contain *p*-cymene. Thus among 278 natural complex substances (NCS) most commonly used in the fragrance industry, 127 contain p-cymene, and the content is >0.3% in 87 (31%) of these NCS, which mainly comprise essential oils. These include widely different products such as tea tree oil, mandarin oil, lemon essence, cinnamon oil or pine oil.

The European Food Safety Authority (EFSA) estimated daily intake of *p*-cymene as 926µg/d/capita (EFSA, 2015). However, this is only based on flavours intentionally added to foods and it does not take into account the amounts naturally present in foods and the amounts coming from consumer products. Given the common occurrence of *p*-cymene in multiple foods and also in natural consumer products, the actual cumulative consumption is higher: In an older survey, Stoffberg and Grundschober *(Stoffberg, 1987)* estimated that actually *p*-cymene intake from food is much higher as compared to added flavorings.

The Research Institute for Fragrance Materials (RIFM) did further calculate aggregate exposure for the products in the CLH report with a standardized method based on use surveys and a probabilistic model (Safford et al., 2017) (Table 3). This indicates that in addition to *p*-cymene from food flavorings estimated by EFSA and *p*-cymene contained in natural foods, consumers are exposed to around 0.5 mg µg/d/capita of potential 4-iPBA precursors from fragrance use.

Table 3. RIFM estimate of aggregate exposure of the substances of the CLH report.

Index Number	Chemical name in the report	Synonym	EC number	CAS	Total (aggregate) Systemic Exposure (RIFM)
601-094-00-1	p-cymene; 1-isopropyl-4-methylbenzene	Para-cymene	202-796-7	99-87-6	0.00055 mg/kg/day (2019)
TBD	3-p-cumenyl-2-methylpropionaldehyde; 2-methyl-3-(4-isopropylphenyl)propanal [1]	Cyclamen Aldehyde	203-161-7	103-95-7	0.0055 mg/kg/day (2015)
TBD	3-(p-cumenyl)propionaldehyde; 3-(4-isopropylphenyl)propanal [2]	Cyclemax	231-885-3	7775-00-0	0.0014 mg/kg/d (2023)
TBD	4-isopropylbenzaldehyde; cuminic aldehyde [3]	Cuminic Aldehyde	122-03-2	204-516-9	0.00014 mg/kg/day (2017)
TBD	4-isopropylbenzoic acid; cuminic acid [4]	IPBA	208-642-5	536-66-3	not available

These analytical data on naturals indicate that humans have been exposed to *p*-cymene and hence 4-iPBA even long before the advent of the chemical industry. There is clearly an extensive history of safe food consumption in humans. This supports the mechanistic evidence showing the mode of action of the metabolic intermediate 4-iPBA is different in rodents compared to rabbits and humans.

The other side of this assessment is that a Repro 1B decision shall only be done based on clear evidence for human relevance, as, especially for the case of *p*-cymene widely present in natural products, this decision will have wide-ranging downstream consequences on use of a wide array of natural products which had been consumed by society with a long history of safe use. Implications of a Repr. 1B classification for p-cymene and related substances would result in the allocation of significant resources for risk assessment, which – in light of the clear species differences between rat and human cells - would be disproportionate to the intrinsic hazard.

5.4 Mechanistic information, metabolic data in human cells and human consumption from natural sources: Evidence that a Repr. 1B classification is not warranted

Based on all evidence presented, we conclude:

- 4-iPBA is the common metabolite of the group that has been firmly established and this is agreed by the DS;
- 4-iPBA is commonly produced from consumption of *p*-cymene and Cuminic Aldehyde in many natural foods, that show no evidence of concern for human health;
- Based on the detailed summary above and recent publications, accumulation of 4-iPBA-CoA in rat cells is a hallmark of the toxicity and the link of 4-iPBA-CoA accumulation to rat reprotoxicity is sufficiently proven;
- No other mechanism of action is known, and for such a low molecular weight organic acid no other key toxicity mechanisms are expected, since benzoic acid derivatives not leading to CoA-conjugate accumulation have low toxicity (Laue et al., 2020; Laue et al., 2017);
- Based on this MoA / AOP, in order **to assess relevance to humans, 4-iPBA-CoA-conjugate formation in human cells shall be specifically addressed**;
- Much lower CoA-conjugate formation (and concurrent C8-CoA depletion) is observed in human hepatocytes as compared to rat cells. More importantly, the kinetic over time is different compared to that in rat cells, whereby the initial formed benzoyl-CoA-conjugate is rapidly cleared by the cells indicating that human cells can further metabolize these CoA-conjugates (as is typical for CoA-conjugates in general);
- This is shown in detail for 4-iPBA formed from Cyclamen aldehyde (see (Natsch et al., 2021) and test reports RCR 154'270 and RCR 154'305), but has also been shown for related benzoic acids (Laue et al., 2020);
- This **human evidence is derived 'only' from *in vitro* data**, but mechanistic evidence in humans by definition must come from *in vitro* data, as CoA-conjugate measurement in the liver and testes of human subjects is an ethically unacceptable invasive measurement. Thus to assess relevance to humans for industrial chemicals, scientist have to rely on assessing the pattern of evidence from mechanistic studies

and *in vitro* studies with human tissue / cells. A clear species difference for the accumulation of the proven key toxicological metabolite is provided here, which is giving a most significant information on human relevance. Rejecting human *in vitro* evidence as insufficient to assess relevance to humans would be a regulatory non-sense, as per definition human intervention studies (left alone at test concentrations required from animal studies) are in general not feasible.

- Also, **this evidence is 'quantitative'** as CoA-conjugation is observed in human cells, too. As outlined above, CoA-conjugation is a general process for detoxification of acids, and hence no qualitative difference would be expected. Furthermore, as shown for the case of C8-CoA depletion, only quantitative accumulation above a certain threshold leads to effects on the lipid metabolism, and hence it is the quantitative difference which contributes to species-specific toxicity.

Based on all these considerations, including history of safe food consumption in humans, the established toxicity mechanism is of questionable relevance in humans and therefore the ECHA Guidance (ECHA, 2017) on the Application of the CLP Criteria should apply:

- "Where it is known that the mode or mechanism of action is not relevant for humans or is of doubtful relevance to humans, **this should be taken into account in the classification**.
- **In cases where *mechanistic information* shows a lower sensitivity in humans than in experimental animals, this may move substances which are close to the potency boundaries to a lower potency group**."

Thus, a Repr. 1B classification, as proposed by the DS, is not warranted.

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7. Glossary

C&L: Classification and Labelling

CA: Cyclamen Aldehyde

CMR: Carcinogenic Mutagenic and Reprotoxic

CoA: Coenzyme A

EFSA: European Food Safety Authority

GD: Gestation Day

IFRA: International Fragrance Association

LD: Lactation Day

MoA: Mode of Action

NCS: Natural Complex Substances

4-iPBA: para-Isopropyl Benzoic Acid

4-iPBA-CoA: Coenzyme A conjugate of 4-iPBA

RIFM: Research Institute for Fragrance Materials

SD: Study Day

tBBA: 4-tert-ButylBenzoic Acid

8. Annex I: 1-isopropyl-4-methylbenzene (=p-cymene): Occurrence in food products

Data from VCF database; https://www.vcf-online.nl/VcfHome.cfm; data extracted from 804 literature references. Only food items with reported levels > 3000 ppm (0.3%) are shown

Product	min-max	count
ALPINIA SPECIES	2700 — 16000 ppm	(n=2)
ANGELICA (Angelica archangelica L.)	7200 — 113000 ppm	(n=5)
ASAFOETIDA OIL	0 — 3680 ppm	(n=2)
ASHANTI PEPPER (Piper guineense Schum and Thom)	3400 ppm	(n=1)
CALABASH NUTMEG (Monodora myristica Dunal)	40000 — 171000 ppm	(n=2)
CALAMUS (sweet flag) (Acorus calamus L.)	<100 — 13800 ppm	(n=2)
CARDAMOM (Ellettaria cardamomum Maton.)	trace — 350000 ppm	(n=7)
CELERY (Apium graveolens L.)	0.02 — 7400 ppm	(n=4)
CINNAMOMUM SPECIES	trace — 11000 ppm	(n=10)
CITRUS FRUITS	trace — 81560 ppm	(n=110)
CORIANDER LEAF (=CILANTRO) (Coriandrum sativum L.)	11000 — 26000 ppm	(n=2)
CORIANDER SEED (Coriandrum sativum L.)	<1000 — 147000 ppm	(n=8)
CUMIN SEED (Cuminum cyminum L.)	46100 — 120100 ppm	(n=2)
CURRY (Bergera koenigii L.)	1000 — 17000 ppm	(n=5)
EUCALYPTUS OIL (Eucalyptus globulus Labill)	5000 — 27000 ppm	(n=3)
FENNEL (Foeniculum vulg., ssp. capillaceum; var.)	<100 — 17300 ppm	(n=17)
GINGER (Zingiber species)	trace — 21000 ppm	(n=6)
LAUREL (Laurus nobilis L.)	<100 — 25000 ppm	(n=5)
LOVAGE (Levisticum officinale Koch)	500 — 8000 ppm	(n=4)
MACE (Myristica fragrans Houttuyn)	14000 ppm	(n=1)
MASTIC (Pistacia lentiscus)	800 — 162200 ppm	(n=25)
MENTHA OILS	trace — 16000 ppm	(n=29)
MYRTLE (Myrtus communis L.)	2200 — 9300 ppm	(n=3)
NUTMEG (Myristica fragrans Houttuyn)	3000 — 31000 ppm	(n=5)
OCIMUM SPECIES	trace — 6500 ppm	(n=6)
ORIGANUM (Spanish) (Coridothymus cap.(L.) Rchb.)	6000 — 136000 ppm	(n=5)
PEPPER (Piper nigrum L.)	trace — 101000 ppm	(n=8)
PIMENTO (ALLSPICE) (Pimenta dioica L. Merr.)	400 — 14000 ppm	(n=8)
PISTACHIO OIL (Pistacia vera)	<1000 — 6000 ppm	(n=3)
PISTACIA ATLANTICA	<1000 — 100000 ppm	(n=6)
PISTACIA PALAESTINA (Pistacia terebinthus L.)	<1000 — 5000 ppm	(n=3)
ROSEMARY (Rosmarinus officinalis L.)	10200 — 42000 ppm	(n=7)
SALVIA SPECIES	trace — 19000 ppm	(n=15)
SATUREJA SPECIES	22000 — 163200 ppm	(n=6)
STAR ANISE	200 — 4000 ppm	(n=4)
THYME (Thymus species)	11800 — 301000 ppm	(n=12)
TURPENTINE OIL (Pistacia terebinthus)	<2000 — 273000 ppm	(n=5)
WILD MARJORAM (origanum vulgare L. ssp. vulgare)	1400 — 189200 ppm	(n=1)
WORMWOOD OIL (Artemisia absinthium L.)	0 — 67000 ppm	(n=7)
XYLOPIA SPECIES	18000 — 30000 ppm	(n=2)