Critical Review of CLH report "Proposal for Harmonised Classification and Labelling of 2-(4-tert-butylbenzyl)propionaldehyde"

As a reproductive toxicologist I critically read the above mentioned CLH report submitted by BASF SE. Applying a weight of evidence approach in the interpretation of all available data I endorse the applicant's suggestion to classify 2-(4-tert-butylbenzyl)propionaldehyde (Lysmeral) as Repr. 2, H361f. A detailed rationale for this will be given below.

1. Introduction

Lysmeral has been shown to induce testicular toxicity and spermatotoxicity when administered orally to rats and at higher dose levels to dogs. Infertility in rats due to adverse effects of orally administered Lysmeral on the male reproductive system has been confirmed in feeding one-generation range-finding studies. Based on clear evidences from experimental animals, it is considered appropriate to classify Lysmeral for reproductive toxicity, with special regards to adverse effects on fertility applying a weight of evidence approach analyzing the available data.

2. Summary of fertility studies

Numerous repeated dose studies on male rats, one-generation range finding studies and an extended one-generation reproductive toxicity study (EOGRTS) on the compound are available. Furthermore, repeated dose studies on dogs, mice, guinea pigs, rabbits and primates were performed for the assessment of reproductive toxicity of Lysmeral.
The present report correctly concludes that these studies "provide evidence for adverse effects on male reproductive organs in rats after oral Lysmeral (2-(4-tert-butylbenzyl) propionaldehyde) administration. These effects were observed concomitantly with signs of general toxicity and adverse effects on the liver. The subchronic repeated dose toxicity study provides a NOAEL for testicular toxicity effects after oral administration at 25 mg/kg bw/day, and according to the findings from further repeated dose and reproductive toxicity studies, these effects can be expected to occur at doses above this NOAEL. This effect level was found to be independent from treatment duration. Adverse testicular findings were observed even after a single oral administration. These data support the conclusion for a clear dose threshold for the induction of testicular toxicity in rats independent of dose duration.
Accordingly, impairment of male fertility combined with signs of general toxicity and changes in clinical parameters of the liver was observed in the one-generation range finding studies in the rat after oral administration of Lysmeral. Due to the obvious testicular and spermatotoxic effects of Lysmeral, the relation between the observed lack of pregnancies, lack of delivered offspring and impairment of male fertility is clearly indicated. These findings were obtained at comparable dose levels also used in repeated dose studies. In contrast, dermal administration on rats led to no testicular toxicity except for dose levels above the limit dose. In the EOGRTS, oral administration of Lysmeral via feed did not affect male or female fertility and reproductive performance of parents and offspring at doses up to 10 mg/ kg bw/d nominal (approx. 15 mg/kg bw/d ingested). In dogs, general adverse effects together with liver and testicular toxicity were observed after oral administration, however, adverse testes effects occurred at higher dose levels than in the rat.

Considering the findings from the available studies in dogs, a NOAEL for testicular toxicity is set at 44.6 mg/kg bw/day. No testicular toxicity was observed in the mouse, guinea pig, rabbit and primates.
Identical adverse testicular effects and species specificity has been observed after oral administration of p-tert-benzaldehyde (TBB) and p-tert-butyltoluene (TBT). The rat has been found to be the most sensitive species for TBB and TBT induced testicular toxicity. In analogy to Lysmeral, systemic formation of p-tert-butylbenzoic acid (TBBA) has been observed after oral administration of TBB and TBT. Clear evidence of adverse testicular and spermatotoxic effects - identical in quality to Lysmeral - have been observed for the metabolite TBBA as well. Based on the lowest adverse effect level for testicular toxicity, TBBA application in rats revealed the highest potency and is included in Annex VI of the CLP regulation with a classification as Repr. 1B (H360F; Index No. 607-698-00-1). TBB and TBT showed lower potencies in exerting comparable testes effects. Lysmeral showed the lowest potency in testes toxicity when compared to TBB, TBT and especially to TBBA. Testes toxicity potencies correlated well with systemically formed urinary TBBA amounts. Therefore TBB, TBT and Lysmeral all share TBBA as common metabolite and the formation of the systemic TBBA intermediate represents a metabolic key event for Lysmeral induced testicular toxicity.
A strong correlation between the formation of TBBA-CoA conjugates in rat hepatocytes, disruption of lipid synthesis and testicular toxicity has been found. Complex lipids are present in high amounts in mammalian sperm and play an important role for spermatogenesis. Their synthesis depends on an intracellular process, that requires a sufficient pool of available CoA. Lysmeral treatment was found to disrupt fatty acid/ lipid synthesis and induced testes toxicity is always observed in the presence of liver toxicity. Other chemicals potentially transformed to benzoic acid metabolites show a strong correlation between sustained formation of benzoyl-CoA complexes in hepatocytes and spermatoxic/testicular toxicity in rats.
Taken together, the comparable pattern of testicular effects, the species dependencies and the observed differences in potencies substantiate, that the formation of systemic TBBA is a metabolic key event for Lysmeral and TBB/TBT induced testicular toxicity. Furthermore, the conjugation of TBBA with CoA represents the mode of action for Lysmeral induced testes toxicity and spermatotoxicity."
I support the authors' conclusions about the equivalence of effects of Lysmeral and the assumed active metabolite, TBBA. I am also in line with the authors that there is a clear species specificity in the observed testicular toxicity.

3. Species specificity of fertility effects

As outlined in the previous chapter, there is clear evidence about a species specificity of the observed testicular toxicity. The differences are so striking to even allow to classify different investigated species in responder and non-responder species. Rats and dogs were found to be responder species, while rabbits, guinea pigs and primates turned out to be non-responders. The most relevant question now was to extrapolate these data to the human situation in order to conclude whether humans can be expected to be responders or non-responders. In order to do so, it was important to evaluate toxicokinetics in different animal species and compare them to the available data in humans.

4. Toxicokinetics

Based on the available data, the provided report correctly summarizes that “quantitative data on the toxicokinetics of Lysmeral are available from rat, mouse, rabbit, guinea pig, dog and rhesus monkey and humans. Based on its physico-chemical properties, Lysmeral is considered to have a high bioavailability via the oral route and a limited bioavailability via the inhalation route. After acute and repeated oral and dermal administration of Lysmeral to experimental animals and humans there is clear evidence of systemic absorption. However, in humans only limited percutaneous absorption of Lysmeral is observed especially when compared to the rat. Distribution predominately to the liver and rapid urinary excretion has been observed in rats after dermal administration and can be assumed for the oral route as well. A detailed in vivo study on the metabolism of Lysmeral is not available.
Comparative assessment of the urinary metabolites in different laboratory animal species reveal species specific differences in the urinary excretion of p-tert-butylbenzoic acid (TBBA) and p-tertbutyl-hippuric acid (TBHA). Furthermore, these data substantiate, that TBBA is formed as common metabolite after administration of Lysmeral, p-tert-butyltoluene (TBT) or p-tert-butylbenzaldehyde (TBB) and their potency for testes toxicity correlates with systemically formed urinary TBBA levels.
On the basis of a qualitative and quantitative evaluation of metabolic profiles for different species in an in vitro metabolism study, a predominant formation of TBBA levels in rat hepatocytes was found when compared to other rodent, non-rodent animal or human hepatocytes. The TBBA levels observed in the model using human hepatocytes were found to be approx. 4-fold lower compared to rat hepatocytes at corresponding incubation concentrations, which reflect plasma levels obtained after oral administration of Lysmeral doses below and above the lowest adverse testicular effect level.
Furthermore, the TBBA levels formed in human hepatocytes after incubation of Lysmeral concentrations related to adverse testicular effect doses were comparable to TBBA levels found in the rabbit, a species not sensitive to testicular toxicity.
In rat hepatocytes, Lysmeral and the metabolite TBBA is rapidly transformed to TBBA-CoA, which leads to an accumulation of stable levels of this conjugate. TBBA - once conjugated to CoA - is not quantitatively transferred to secondary acceptors such as glycine to form TBHA. The observed decrease of physiological CoA conjugate levels in these hepatocytes indicates a competitive inhibition of other CoA dependent cellular processes, leading to cellular toxicity. In human hepatocytes a fundamentally different kinetics was observed in TBBA-CoA formation, since no accumulation of stable conjugate levels were detectable.
Overall, species specific differences in the formation of metabolites have been clearly identified both in vitro and in vivo between responder (e.g. rat) and non-responder species (e.g. mouse, rabbit) with respect to reproductive toxicity. The species-specific organ toxicity after repeated oral application of Lysmeral can be attributed to the toxic metabolite TBBA. In vitro studies show significantly lower production of TBBA in humans than in rats, with human TBBA production similar to that observed in rabbits at toxicologically relevant doses. Furthermore, the intracellular formation of stable levels of TBBA coenzyme A complexes is a rat specific effect and does not appear in human cells.”
I fully endorse the conclusions of the authors of the report. The observed species specificity of testicular (and other forms of) toxicity can be fully explained by species specific toxicokinetics. There is also enough data available to conclude that human toxicokinetics resemble those of non-responder species, clearly showing that humans are expected to be non-responders too.

5. Proposed mechanism of action

Taking together the above outlined data, the following mechanism of action can be derived and is highly plausible:
The observed testicular toxicity is not a direct effect of the compound under consideration, Lysmeral. It could be shown that formation of TBBA is responsible for inducing the observed testicular toxicity, since TBBA administered itself leads to the same effects on testes. It could also be shown that, if TBBA conjugates to CoA, this complex will be more stable and less subject to metabolism/excretion, thus increasing systemically bioavailable concentrations of TBBA. These processes could be confirmed to take place in studies in rats, the most sensitive species for testicular (and other forms of) toxicity. In contrast to this, non-responder species (mice, guinea pigs, primates) show a significantly lower production of TBBA, thus preventing the formation of high enough systemic levels to induce testicular toxicity, for which a clear threshold was shown. All available human data clearly show that human belongs to non-responder species. Not only does this proposed mechanism of action plausibly underline the sequence of events needed to exert the observed testicular effects, it also shows that rat data cannot be simply extrapolated to the human situation due to fundamental species differences and that in this particular case only non-responder species data are relevant for the human situation.

6. Exposure routes

Though CLP is exclusively based on the intrinsic hazard of a compound under consideration, the exposure hazard needs to be taken into account. In the human situation skin absorption will be the main route of exposure. In animal studies it could be shown that Lysmeral is highly bioavailable via the oral route, while bioavailability after dermal exposure is much lower. In the most responsible responder species, rat, a clear threshold level of 50 mg/kg for inducing testicular (and other) toxicity was found with a NOAEL of 25 mg/kg after oral treatment. Compared to oral studies, dermal administration of Lysmeral in rats led to testicular toxicity only at an excessive dose level, clearly above the limit dose, whereas at 1000 mg/kg body weight, no adverse testicular effects were observed. When compared to doses leading to rat testicular toxicity, a prolonged human uptake of Lysmeral doses inducing systemic toxicity (testes toxicity or spermatotoxic effects) is highly unlikely. In humans, dermal penetration is even lower than in rats: while in rats a dermal absorption rate of approx. 19 % was found, the maximum absorption in humans was determined at 7 %.
Taking this altogether, assuming the most relevant, dermal, route of exposure in humans, the formation of systemically available levels above the threshold for induction of testicular effects can be excluded.

7. Developmental toxicity

An OECD 414 study was performed in rats. The present report correctly summarizes the findings of this study: “High dose dams (41 mg/kg bw/d) showed clinical signs (transient salivation), transient reduction of mean food consumption and body weight loss on day 6-8 p.c. Mean body weight gain was decreased over the entire treatment phase resulting in lower mean body weights on day 13 - 20 p.c. and net body weight gain compared to controls. Increased levels of alanine aminotransferase and glutamate dehydrogenase, decreases serum cholinesterase levels and organ weight changes (increased liver weights, reduced uterus weights) were noted.

In mid dose dams (13 mg/kg bw/d) body weight gains were transiently decreased on day 6-8 p.c. Furthermore, alanine aminotransferase levels were increased, serum cholinesterase levels were decreased and increased liver weights were found. These findings reflect a Lysmeral induced general systemic and liver toxicity for high dose and less pronounced for mid dose dams.
The number of mainly early resorptions was increased due to postimplantation losses in the high dose group whereas gestational parameters were not significantly influenced in lower dose groups (5, 15 mg/kg bw/d). Subsequently, the number of fetuses and live fetuses per dam was found to be slightly below the respective historical control range in the high dose group. Concomitantly, prenatal developmental toxicity in terms of reduced fetal body weights was observed in the mid and high dose groups. These findings coincided with significant maternal toxicity at the same dose levels.
Sporadic malformations were observed, which lacked a consistent pattern, occurred in very few of the large number of examined fetuses and there incidences were found within the respective historical control ranges. External variations were not observed and soft tissue variations occurred in a dose independent manner in all test groups including control animals.
In contrast, an overall incidence of skeletal variations was statistically significantly increased in mid and high dose animals. These variations represented mainly delays and minor disturbances in ossification processes of the skull, sternebrae and pubic girdle. Supernumerary (14th) ribs were found in control and dosed animals at high incidences, and structural variations like a supernumerary thoracic vertebra (14th) or a misshapen sacral vertebra (1st sacral arch) were found to be increased evidently in the high dose group fetuses. The observed skeletal variations are well correlated to statistically significantly decreases in mean fetal body weights and evident maternal toxicity in the respective dose groups. Clustering of incidences for a supernumerary or misshapen vertebra in single litters was observed, and a maternal predisposition which affects the respective offspring in situations of maternal stress conditions could be hypothesized here.
Supernumary ribs and delays of ossification in rodent offspring are among the common endpoints related to chemical exposure stress. Delays in ossification are by definition transitory, occur in conjunction with decreased fetal weights and represent an indicator for adverse effects on fetal maturation rather than a teratogenic potential.
Overall, the increased numbers of fetuses with common skeletal variations are considered an embryo-/fetotoxic effect due to fetal growth retardations, representing a manifestation of a nonspecific stress on the dams and not a teratogenic effect of Lysmeral. Increased early resorptions and the subsequent decrease in number of fetuses are further manifestations of the non–specific maternal stress induced by Lysmeral administration.
The findings of the one-generation range finder studies are largely consistent with the effects observed in the present key teratogenicity study. Slight, non-significant and dose independent increases in postimplantation losses were found in dose groups having offspring. A slight reduction in the number of delivered pups has been observed at doses not affecting fertility indices.
Furthermore, a significant reduction in birth weights, pup weights at weaning and pup weight gain has been seen when compared to controls. These findings coincided with adverse systemic effects to the dams. No effects on the gestation and live birth indices were observed due to the absence of any stillborn in the dosed animals. Whereas effects on early pup survival occurred, lactation indices were not significantly affected and no test substance related findings in pup necropsy have been found.

Furthermore, the highest Lysmeral dose tested in the EOGRTS (in the range of the LOAEL of the developmental toxicity study) resulted in pup body weight reductions of the F1 and F2 offspring and was associated with adverse maternal liver and general systemic effects. Lysmeral did not have a consistent impact on the number of postimplantation losses, delivered pups and pup survival up to this dose. Further developmental toxicity endpoints including developmental neurotoxicity and immunotoxicity were not affected by treatment with Lysmeral.
Taken together, developmental toxicity has been observed at doses leading to evident maternal toxicity and is considered to be a secondary non-specific consequence of general systemic toxicity in the dams. Therefore, these findings do not warrant a classification with respect to developmental toxicity."
I fully agree with the assessment of these data by the authors of the report and endorse their conclusion that the available data do not warrant a classification with respect to developmental toxicity.

8. Summary and conclusions

Based on these data and applying weight of evidence in interpreting them, I conclude that the observed **testicular toxicity** is a species-specific phenomenon with **very limited relevance for humans**, as shown by the following facts:
1. Testicular toxicity in animal studies is **only observed at dose levels causing other forms of toxicity**, e.g. hepatotoxicity.
2. There are striking **inter-species differences** in terms of testicular toxicity, with the **rat** being **the most sensitive species**.
3. Mechanistic studies clearly indicate that testicular toxicity is highly **correlated with the formation of para-tert-butylbenzoic acid (TBBA)**, representing a key metabolite for Lysmeral induced testicular toxicity.
4. It could be shown that direct TBBA administration leads to the same testicular effects as observed for Lysmeral, thus underlining that **TBBA is the relevant metabolite**.
5. There is good evidence to show that specifically formation of **TBBA-CoA** is critical in terms of testicular toxicity.
6. Both in vivo and in vitro studies on toxicokinetics show that the **rat** is the species producing the **highest levels of both TBBA and TBBA-CoA.**
7. This strongly supports the hypothesis that the formation of these two metabolites is a key event for the induction of testicular toxicity and can explain the species specificity of the observed effect, allowing to distinguish between **responder and non-responder species**.
8. Studies on **testicular toxicity** showed that **rats**, and to a lesser degree, **dogs** were **responder species**, while **mice, guinea pigs, Rhesus monkeys and rabbits** belonged to the **non-responder species**.
9. Even in the most responsible responder species, rat, **a clear threshold level of 50 mg/kg for inducing testicular (and other) toxicity** was found with a NOAEL of 25 mg/kg.
10. Based on toxicokinetics, it could be shown that the **human metabolism is closest to the rabbit situation** and, consequently, the formation of **TBBA or TBBA-CoA levels above the threshold to induce testicular toxicity in humans** is **most unlikely**.
11. Therefore, the assumption is well justified that **humans belong to non-responder species**.

12. The **most likely human route of exposure is skin absorption**, a route that only induced testicular toxicity even in the most sensitive species, rat, in **doses higher than the limit dose of 1000 mg/kg even in the most sensitive species, rat**.
13. **Dermal absorption in humans is significantly lower than in rats**. Consequently, the formation of systemic levels capable to induce **testicular toxicity in humans is most unlikely**.
14. In summary, the available data form an **excellent pattern** which, based on the mode of action and underlying toxicokinetics, explains the observed **species-specific effects regarding testicular toxicity and plausibly shows that humans belong to the non-responder species**.
15. The available data strongly support the conclusion that **Lysmeral does not have "an intrinsic property to produce an adverse effect on reproduction" in humans**.
16. Consequently, the proposal to classify **Lysmeral in Category 2 for fertility effects** is scientifically well justified and no higher classification is warranted.
17. **Sporadic effects in fetuses and pups** are considered variations and developmental retardations observed only in the presence of evident maternal toxicity and **do not warrant any classification for developmental toxicity**.

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