## Summary

To further investigate the correlation between the Coenzyme A (CoA) conjugation and Lysmeral induced testicular and sperm toxicity, new toxicological data on position isomers of tert-butyl benzoic acid (TBBA) have been generated in an *ex vivo* study using a 3D cell culture with primary seminiferous tubules from juvenile Sprague Dawley rats (Bio-Alter®). Cultures were incubated with para- or meta-TBBA at 2, 10 or 50 µM in DMSO respectively and cytotoxicity, blood-testis barrier functionality via trans-epithelial electrical resistance (TEER) measurements and cell numbers of different somatic and germ cell populations have been quantified. In addition, the content of TBBA conjugated with CoA has been assessed in cell culture lysates.

Clear differences between para- and meta-TBBA were observed when comparing endpoints for meiotic processes. In contrast to meta-TBBA, the presence of para-TBBA decreased the number of total germ cells, middle to late pachytene spermatocytes, secondary spermatocytes and round spermatids. At these effective concentrations of para-TBBA, the respective Coenzyme A conjugate para-TBBA-CoA was detectable, whereas no levels of CoA conjugated with the meta-substituted benzoic acid derivative (meta-TBBA-CoA) was found.

In conclusion, the data of the submitted *ex vivo* study on primary rat seminiferous tubule cultures (Bio-Alter®) indicate, that TBBA-CoA conjugates can also be formed in reproductive tissues. Concentrations of para-TBBA, that affect spermatogenic processes *in vitro* also lead to detectable CoA-conjugate levels, whereas meta-TBBA did not show these effects. The strong correlation between the formation of TBBA-CoA conjugates in rat hepatocytes, disruption of lipid synthesis and testicular toxicity – as outlined in the CLH report - is further supported by the new data of the submitted study. Accordingly, these data support the conjugation of TBBA with Coenzyme A as the mode of action for Lysmeral-induced testes toxicity and spermatotoxicity.

## Background

New toxicological data have recently been obtained for two position isomers of the tert-butyl benzoic acid (TBBA) in a mechanistic study using the Bio-Alter® technology. This technology involves a 3D cell culture which allows to assess rat spermatogenesis in culture. Since the metabolic formation of para-TBBA is a key event for Lysmeral (2-(4-tert butylbenzyl)propionaldehyde) induced testes toxicity in rats, these new data are considered of high relevance for the ongoing EU harmonized classification process, with stakeholder consultations taking place between 12 February – 13 April 2018.

## Methods

Maintenance of the blood-testis barrier as well as mitotic and meiotic phases of spermatogenesis and the initial steps of spermiogenesis have been studied in the Bio-Alter® test system in the presence of two isomers of TBBA (i.e. para-TBBA and meta-TBBA). Cultures of seminiferous tubules have been derived from pooled testes of juvenile (22-days-old) male Sprague Dawley rats.

The cultures were incubated with para- or meta-TBBA at 2, 10 or 50 µM in DMSO respectively. To confirm the absence of cytotoxicity at the concentrations to be tested in the Bio-Alter® test system, a cytotoxicity study was initially performed in the SerW3 rat Sertoli cell line. DMSO (0.1%) served as negative and 1,3-dinitrobenzene (DNB) at 10µg/ml as a positive control.

The blood-testis barrier functionality has been assessed in a 3-week culture (6 wells per testing condition) by using trans-epithelial electrical resistance (TEER) measurements at day 2 (before the addition of the compounds or vehicle), 3, 6, 8, 10, 13, 15, 17 and day 19. Total cell numbers

and the cell viability were determined in a 2-week culture via trypan blue exclusion on day 8 and day 15.
The cell numbers of different somatic and germ cell populations (spermatogonia, young spermatocytes I, middle to late pachytene spermatocytes I, secondary spermatocytes and round spermatids) have been measured using flow cytometry (3 wells per testing condition) on days 8 and 15. For determination of the respective populations, cells were immunolabeled with a monoclonal antibody against vimentin (i.e. an intermediate filament to identify somatic cells) and stained with Hoechst to assess their DNA content. Vimentin-positive somatic cells and vimentin-negative 4C, 2C and 1C germ cells were separated using the bivariate analysis of DNA content and vimentin.
Furthermore, cell culture lysates have been derived from 3 replicates (9 wells) per testing condition) at day 7 and day 14 to determine the content of TBBA conjugated with CoA. Decanoyl CoA was added as an internal standard, samples were purified by solid phase extraction and the CoA-conjugates were determined with high-resolution LC-MS.

## Results and conclusion

The presence of para-TBBA and meta-TBBA did neither affect cell viability in the SerW3 cell line nor in the primary culture of seminiferous tubules at any concentration tested.
A slight and transient increase of the TEER by 20 to 40% above respective controls was observed after incubation of para-TBBA and meta-TBBA. Both para-TBBA (up to 50% above control) and meta-TBBA (up to 100% above control) also increased the number of somatic cells at day 8. These changes indicated some disturbances of the blood testis-barrier, however, the potential effects were low and the biological significance of these slight modifications could not be ascertained beyond any doubt.

Para-TBBA and meta-TBBA slightly decreased the number of spermatogonia at day 15 by approx. 30% below controls, suggesting an adverse effect on the mitotic germ cell processes, although, the effects observed upon meta-TBBA treatment were less consistent.

Both compounds also decreased the number of round spermatids (day 8 and day 15), however, clear differences were observed between para-TBBA and meta-TBBA when comparing endpoints addressing meiotic processes:

- In contrast to meta-TBBA, para-TBBA decreased the number of total germ cells (day 15) at all tested concentrations by approx. 30% below control values.
- In contrast to meta-TBBA, para-TBBA (50µM) decreased the number of middle to late pachytene spermatocytes (day 8 and day 15) down to 60% below control values.
- In contrast to meta-TBBA, para-TBBA decreased the number of secondary spermatocytes at all tested concentrations (day 8 and day 15) down to 75% below control values.
- An evidently stronger decrease in the number of round spermatids was observed at day 8 and day 15 in the presence of para-TBBA (down to 90% below control numbers) when compared to meta-TBBA (approx. 40% below control numbers).

The CoA-conjugate para-tert-butyl-benzoyl-CoA (para-TBBA-CoA) was detectable both at 7 and 14 days in seminiferous tubules cultures dosed with 10 and 50 µM para-TBBA. In the mid test concentration, para-TBBA-CoA levels were close to the limit of detection and no para-TBBA-CoA conjugate was found in samples dosed with 2 µM para-TBBA. The level of the detected para-TBBA-CoA conjugate was in the range of 0.007 – 0.048 µM or 0.005 – 0.034 nmol/$10^6$ cells when corrected for the average recovery.

In contrast, no respective CoA-conjugate meta-tert-butyl-benzoyl-CoA (meta-TBBA-CoA) was detectable in the samples dosed with either 2, 10 or 50 µM of meta-TBBA collected after 7 or 14 days of exposure.

In conclusion, a clear difference exists in the potency of para-TBBA and meta-TBBA to affect spermatogenesis in the Bio-Alter® system. Para-TBBA showed a high potency to induce spermatotoxicity in the rat, whereas meta-TBBA did not exert such effects. Furthermore, TBBA-CoA conjugates were detected in the Bio-Alter® system indicating the capacity of reproductive tissues to form such conjugates. The differences between the position isomers (*para* vs. *meta*) have an impact on the formation on these conjugates which correlates with the ability to disturb spermatogenic processes.

**Relevance for the proposal for the harmonized classification of Lysmeral (2-(4-tert butylbenzyl)propionaldehyde)**

The data of the submitted *ex vivo* study on primary rat seminiferous tubule cultures (Bio-Alter®) indicate, that TBBA-CoA conjugates can also be formed in reproductive tissues. However, the levels of TBBA-CoA conjugates detected are approx.100 fold lower when compared to *in vitro* dosed liver hepatocytes. The same differences between the position isomers (*para* vs. *meta*) observed in hepatocytes *in vitro* have been detected in this *ex vivo* system on reproductive tissues as well. In contrast to meta-TBBA, concentrations of the para-substituted benzoic acid derivative para-TBBA affecting spermatogenic processes also lead to detectable CoA-conjugate levels.
The clear difference in the potency of para-TBBA and meta-TBBA to affect spermatogenesis in the Bio-Alter® system supports the findings from available *in vivo* data. Para-TBBA and para-Lysmeral (metabolized to para-TBBA) show a high potency to induce testes and spermatotoxicity in the rat, whereas the position isomer meta-Lysmeral (3-(3-*tert*-butylphenyl)-2-methylpropanal) does not show such effects.
As outlined in the CLH report, the formation of systemic TBBA is a key metabolic 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, which is further supported by the new data of the submitted *ex vivo* study on primary rat seminiferous tubule cultures. Accordingly, these data strengthen our conclusion, that the conjugation of TBBA with CoA represents the mode of action for Lysmeral-induced testes toxicity and spermatotoxicity.