Commentaar op conceptadvies *Methylisobutylketon*

Comments on draft advisory report *Methyl isobutyl ketone*

December 8, 2020

National Institute for Occupational Health and Safety, the USA

NIOSH Comments by Robert Streicher, Supervisory Research Chemist

SECTION & PARAGRAPH	COMMENT
General Comments	My background is chemistry, so my technical comments will be limited to the chemistry aspects of this document.
Specific Comments	
Pg. 10, line 3	Are all the synonyms intended to be English synonyms? All of them are except for "methylisobutylcetone." Also, the NIOSH Pocket Guide uses "Hexone" as its primary name for MIBK (reference 11 in the draft). This is not a good scientific name, but it suggests that it may be a very common synonym.
Pg. 10, line 7	Surface tension values for MIBK are available in the PubChem entry for MIBK: 23.6 dynes/cm = 0.0236 N/m at 20.0 °C. Link: https://pubchem.ncbi.nlm.nih.gov/compound/Methyl-isobutyl-ketone#section=Surface-Tension. PubChem cites another source as presumably the primary source of the data: http://cameochemicals.noaa.gov/. However, following the Cameo Chemicals link, and then the search that brings you to the methyl isobutyl ketone page (https://cameochemicals.noaa.gov/chemical/3943) I do not see the surface tension data. Perhaps the values provided on PubChem are not accurate? The surface tension given is quite reasonable for this compound, but the Cameo Chemicals source may be incorrect.
Pg. 11, line 1	The units for viscosity should be "mPa·s," not "mmPa.s."
Pg. 13, lines 6-7	NIOSH is the National Institute for (not of) Occupational Safety and Health. There is an additional ketones method in the latest (5th) edition of the NIOSH Manual of Analytical Methods: Method 2027. Link: https://www.cdc.gov/niosh/docs/2014-151/pdfs/methods/2027.pdf. We suggest that the other two methods should still be listed, just add 2027. The three methods (1300, 2555, and 2027) use different sorbents for collection of air samples (coconut charcoal, carbon molecular sieve, and silica gel, respectively).
Pg. 15, line 9	"…uptake via de dermal route…" should probably be "…uptake via the dermal route…"
Pg. 15, line 21	The word "in" is missing; suggest changing to "…detectable in the brain…"
Pg. 15, line 31	"The metabolite, MIBK,…". Should this be "MIBC?"
Pg. 24, line 30	Should be National Institute for (not of) Occupational Safety and Health.

NIOSH Comments by Bingbing Wu, ORISE Fellow

SECTION & PARAGRAPH	COMMENT
General Comments	The Committee's recommendations are appropriate.
Specific Comments	
Pg. 13, lines 9-10	No biological exposure monitoring data available for MIBK noted in this document. Suggest additional search for biological monitoring studies of MIBK and adding the data if applicable. An example for your reference: Kawai et al. [2003]. Methyl isobutyl ketone and methyl ethyl ketone in urine as biological markers of occupational exposure to these solvents at low levels. International Archives of Occupational and Environmental Health Vol 76(1):17–23. https://link.springer.com/article/10.1007/s00420-002-0374-9 Recommend listing the exclusion criteria for studies not included in the review process.
Pg. 15, line 24	Recommend collecting more information from different studies on MIBK elimination route. In this document, it is reported that "0.04% of the total dose was eliminated unchanged through the urine." However, Kawai et al. [2003] found that approximately 0.12% of MIBK absorbed in the lungs will be excreted in urine.
Pg. 21, line 23	Change "and" to "or" or "nor" in the sentence "not in female rats and in mice."
Pg. 19, lines 10-11	Suggest rephrasing this sentence to "a statistically significant increase of the incidence of hepatic adenomas and 10 carcinomas (combined) was observed at the highest exposure level."

MIBK REACH Consortium

J.M. Rijnkels, PhD

Subcommittee on the Classification of carcinogenic substances of the Dutch Expert Committee on Occupational Safety (DECOS) of the Health Council draftOSH@gr.nl

31 March 2020

Dear Mr Rijnkels,

On behalf of the MIBK REACH consortium members, the REACH consortium would like to respond to the public consultation on the draft advisory report on the carcinogenic and genotoxic properties of methyl isobutyl ketone (synonym, 4-methylpentan-2-one, MIBK). The current letter will mainly focus on the remaining uncertainties related to CAR/PXR MoA which seem to be the main concern according to the draft advisory report and the main reason for the proposal of classification as carcinogenic category 2.

We would also like to present more evidence related to the other uncertainties highlighted by the RAC opinion (CLH-O-0000001412-86-295/F). This new evidence was not presented to the RAC committee before but was shared recently with DG Environment (see enclosed letter). The enclosed letter addresses the three main uncertainties which were the basis for the classification as carcinogenic category 2 by RAC; namely 1) α2μ-globulin nephropathy MoA and relevance to human due to Chronic Progressive Nephropathy seen in female rats 2) low incidence of tubule Renal mesenchyme tumors (RMT) in female rats and 3) uncertainties related to CAR/PXR MoA (more elaboration on this point will be presented herein)

Remaining uncertainties related to CAR/PXR MOA

According to the Dutch draft report "*The Committee considered the suggestion that the liver tumours in mice could have been induced by CAR/PXR nuclear receptor activation, a nongenotoxic mode of action. However, whether this mode of action has indeed played a role in MIBK induced liver tumours in mice, has insufficiently been investigated. In addition, the relevance for humans has not been investigated. Until more data are available, the committee considers it possible that other mechanisms may have played a role, and thus that the findings in mice could be relevant to humans*."

To summarize: 1) the relevance for humans has not been investigated and 2) CAR/PXR MoA has insufficiently been investigated.

Regarding the first point: The MIBK Consortium acknowledges this data gap and therefore has initiated the performance of an in vitro experiment using Cryopreserved hepatocytes from both mice and human. Different endpoints characteristic to CAR/PXR MoA will be measured in response to MIBK exposure; (*i*) selected cytochrome P450 mRNA levels, (*ii*) cytochrome P450 enzymatic activities characteristic of the selected CYPs, (*iii*) cultures will also be incubated with BrdU to allow assessment of cell proliferation (S-phase staining), (*IV*) Cultures maintained with Epidermal Growth Factor (EGF) will be used as positive controls and some cultures will be dosed with the prototypical CAR/PXR activator, phenobarbital.

The study will be carried out with a laboratory recognized for its expertise in this area. Given limited availability in the laboratory, the in vitro study will be initiated in May and expected to take 12 weeks for completion. Given the required reporting on the study, the MIBK Consortium would

be able to report and submit the full study in October 2020. While the study is currently not recognized as an OECD test method, we would underline that ECHA have reviewed and accepted the results of this study on other substances being evaluated in ECHA. We can provide further information on this point, if required.

Regarding the second point: The Dutch report doesn't elaborate any further on the limitations of the CAR/PXR MOA investigation and therefore we would like to refer to the RAC opinion (CLH-O0000001412-86-295/F) which summarizes these limitations.

"*RAC agrees with the DS that the proposed MoA is plausible in male and female mice. Nevertheless, the MoA is not sufficiently investigated. Some limitations were noted in the studies and some uncertainties remain*":

i) Absence of dose-response data for CAR/PXR activation (single dose tested);

ii) No activation of CAR in high throughput assay data;

iii) No positive control in the in vivo Hughes et al., 2016 study; iv) Enzyme activity was not measured in the in vivo mice study (Hughes, 2016);

v) No in vivo CAR/PXR knock out animals were used to confirm the in vitro results;

vi) Increase in liver weight and hypertrophy in CAR KO mice indicates uncertainties whether CAR activation is the exclusive MOA.

vii) Human relevance has not been investigated (e.g. in vitro studies using human hepatocytes, humanized mice). Potential quantitative differences in the activation of CAR has thus not been investigated.

viii) In rats, 4-methylpentan-2-one has been showed to increase the total amount of CYP liver and (CYP2E, CYP1A, CYP2B) kidney enzymes according to the summary report from IARC. This increase has been associated with a potentiating effect of the substance on hepatotoxicant, neurotoxicant and nephrotoxicant. Hepatocellular hypertrophy and liver weight changes were also observed in rats. No tumour induction were observed in rats. Potential rodent species differences has not been investigated.

The REACH consortium would like to address each the above points below:

i) **Absence of dose-response data for CAR/PXR activation (single dose tested)**

The incidence of hepatocellular adenoma and carcinoma (combined) were only significantly increased in male and female mice exposed to 1800 ppm in the MIBK NTP study (Stout et al., 2008), the controls also had adenomas and carcinomas; these are frequent in B6C3F1 mice. Only the 1800 ppm was selected based on the findings from the NTP study, in which the 1800 ppm male mice exceeded the NTP historical control range for hepatocellular adenoma and carcinoma (combined).

ii) **No activation of CAR in high throughput assay data. High-throughput assay data for 4-methylpentan-2-one and its metabolites have been screened via TOXCAST7 (US EPA, 2018) with special focus on CAR and PXR. 4-methylpentan-2-one was positive for 1/4 assays on PXR and 4-hydroxy-4-methyl-2-pentanone for 1/5 assays on PXR**

The results of the TOXCAST7 are not in agreement with the Hughes et al., 2016 in vivo study. It is true that in the Hughes study the nuclear receptor activation (CAR/PXR) were not directly assessed. However, there was a clear biological signature of activation of CAR/PXR nuclear receptors, when measured at the gene expression level in both sexes in the B6C3F1 and C57BL/6 strains as demonstrated by the induction of Cyp2b10 and Cyp3a11 transcripts levels and no induction for the same transcripts in the knock out mice.

iii) **No positive control in the in vivo Hughes et al., 2016 study**

The study of Hughes et al., 2016 didn't include a positive control because previous internal data suggested the potential for a CAR/PXR nuclear activation MOA in B6C3F1 exposed to 1800 ppm for

7 days. The supporting evidence from the internal study includes increased liver weights, hepatocellular hypertrophy and increased transition of Cyp2b10. In addition, the NTP showed that MIBK induced changes in liver pathology and these adaptative changes were similar to CAR/PXR pathway. However, we acknowledge this data gap and therefore a positive control, namely, EGF will be included in our mechanistic in vitro hepatocytes study as noted previously.

iv) **Enzyme activity was not measured in the in vivo mice study (Hughes et al., 2016)**

Previous internal data for male mice treated at 1800 ppm for 7 days resulted in increased CYP 2b10 transcript levels and increase in enzyme activity (4-fold) was also observed. Also, slight hypertrophy and hepatocyte proliferation were noted. However, we acknowledge this data gap and therefore enzyme activity will be measured in our mechanistic in vitro hepatocytes study as noted previously.

v) **No in vivo CAR/PXR knock out animals were used to confirm the in vitro results;**

This comment is in conflict with other statements by RAC where RAC clearly referred to the Hughes et al., 2016 where KO mice were used. The use of KO mice was also indicated by RAC in the point below.

vi) **Increase in liver weight and hypertrophy in CAR KO mice indicates uncertainties whether CAR activation is the exclusive MOA.**

The reduced severity of hepatocyte hypertrophy (graded as very slight) and lack of hepatocellular proliferation in the MIBK exposed CAR/PXR KO mice, in contrast to the wild types, clearly demonstrates that the CAR/PXR nuclear receptor is necessary to induce these changes and such changes are consistent with the activation of the CAR/PXR receptors. Also, the reduced magnitude of the hepatocyte hypertrophic response (from slight to very slight in the wild type versus the CAR/PXR KO, respectively) as well as the decrease in liver weight increases in both male and females of the CAR/PXR KO group compared to the WT have also been observed for the prototypical CAR/PXR activator, phenobarbital (Ross et al., 2010; and Yamada et al., 2014); however, it is the difference in treatment-related hepatocellular proliferation in rodents that leads to a species specificity with regards to tumor formation similar to our observations with MIBK. Alternate findings such as increases in ALT and increased liver weight are not precursors to tumorigenesis and are most likely to represent adaptive responses. The observed increase in liver vacuolization in the CAR/PXR KO cannot be readily explained. However, CAR is involved in stimulating the transcription of Cyp2b genes that are involved in hepatic hydroxylation reactions. The KO mice may be less capable of metabolizing MIBK to 4-hydroxymethyl isobutyl ketone leading to increased toxicity by either the parent compound or the 4-methyl- 2-pentanol metabolites via the alcohol dehydrogenase/alcohol dehydrogenate ketone reductase pathways (Duguay and Plaa,1995; DiVincenzo et al., 1976; and Vezine et al., 1990).

vii) **Human relevance has not been investigated (e.g. in vitro studies using human hepatocytes, humanized mice). Potential quantitative differences in the activation of CAR has thus not been investigated.**

We acknowledge this data gap and therefore we have initiated the performance of a mechanistic in vitro experiment to demonstrate species difference in CAR/PXR MoA between mice and human in response to MIBK treatment as explained before.

viii) **In rats, 4-methylpentan-2-one has been showed to increase the total amount of CYP liver and (CYP2E, CYP1A, CYP2B) kidney enzymes according to the summary report from IARC. This increase has been associated with a potentiating effect of the substance on hepatotoxicant, neurotoxicant and nephrotoxicant. Hepatocellular hypertrophy and liver weight changes were also observed in rats. No tumour induction were observed in rats. Potential rodent species differences has not been investigated.**

We acknowledge that there are species differences in CAR/PXR activation which may hamper in some cases the extrapolation to human. However, we would like to share the following evidence:

a) According to our best knowledge mice is a better model compared to rats. Previous studies by Yao et al. (2010) show that there is more overlap in human and mouse ligands. This

study tested the effect of 30 food-derived phenolic compounds on human and mouse CAR activity. There was an overall similarity between the response profile between human and mouse CAR responses using a luciferase assay. A recent study by Niu et al. (2018) conducted in vivo genome-wide binding interactions of mouse and human CAR receptors and found similarities in genomic CAR binding profiles comparing activators of both mCAR and hCAR. Binding motifs resulting from direct- or indirect-activated CAR were largely equivalent for both mCAR and hCAR, indicating that different modes of receptor activation do not appear to alter CAR binding profiles. Structural conservation of CAR binding profiles of humans and mice suggests that a high degree of functional conservation likely exists among the receptors with respect to target gene interactions and subsequent regulation, indicating similar receptor activation between humans and mice.

b) Hughes et al. (2016) demonstrated comparable induction in Cyp2b10 (CARassociated) and Cyp3a11(PXR-associated) in the B6C3F1and C57BL/6 strains at 1800 ppm MIBK.

c) Our intended in vitro mechanistic study will reveal more of the species differences in CAR/PXR activation between mice and human in response to MIBK treatment.

d) The lack of response for Cyp2b10 in CAR/PXR KO mice in response to MIBK as a CAR activator was similar to other CAR activators (Ross et al., 2010; LeBaron et al., 2014). For example, phenobarbital (PN) has been determined to be a non-genotoxic carcinogen in mice and a tumor promoter which requires the activation of CAR to elicit a tumorigenic effect (Yamamoto et al., 2004). The key events in this MOA are well elucidated and include increased hepatocellular proliferation and an increase in CAR related gene transcripts (Elcombe et al., 2014) However, CAR activation in humans is not an inducer of DNA synthesis nor does it act as a mitogen. There is no association between PB use and increased tumor incidence from human epidemiological evidence (Elcombe et al., 2014, Friedman et al., 2009, Olson et al., 1989, LaVecchia & Negri., 2013). Furthermore, experiments with chimeric mice indicate that while the response to PB in human cells versus mice are similar with respect to hypertrophy and increased liver weight, albeit to a lesser extent in humans. The key difference is the proliferative response in mice while this same response is absent in human liver cells (Yamada et al., 2014). PB exposure and MIBK exposure in mice are similar in that they elicit these responses through the CAR/PXR nuclear receptor-mediated response. Therefore, non-genotoxic carcinogens eliciting tumor responses in rodents through a CAR nuclear receptor-mediated activation lack the key initiating events necessary for tumor formation in humans. As such, the CAR nuclear receptor-mediated MOA for MIBK lacks human relevance for risk assessment (Whysner et al., 1996; Holsapple et al., 2006; Elcombe et al., 2014).

We believe that we have addressed the remaining uncertainties related to CAR/PXR MoA for the liver tumours in mice in response to MIBK treatment and we hope that you agree that there is a substantial Weight of Evidence to support a CAR/PXR MoA.

Yours sincerely

Paula Diaz Rodriguez

References

1) DiVincenzo, G.D., Kaplan, C.J., Dedinas, J., 1976. Characterization of the metabolites of methyl n-butyl ketone, methyl isobutyl ketone, and methyl ethyl ketone in Guinea pig serum and their clearance. Toxicol. Appl. Pharmacol. 36, 511e522.

2) Duguay, A.B., Plaa, G.L., 1995. Tissue concentrations of methyl isobutyl ketone, methyl n-butyl ketone, and their metabolites after oral or inhalation exposure. Toxicol. Lett. 75, 51e58.

3) Elcombe, C.R., Peffer, R.C., Wolf, D.C., Bailey, J., Bars, R., Bell, D., et al., 2014. Mode of action and human relevance analysis for nuclear receptor-mediated liver toxicity: a case study with phenobarbital as a model constitutive androstane receptor (CAR) activator. Crit. Rev. Toxicol. 44, 64e82.

4) Friedman GD, Jiang SF, Udaltsova N, Quesenberry CP Jr, Chan J, Habel LA., 2009. Epidemiologic Evaluation of Pharmaceuticals With Limited Evidence of Carcinogenicity. Int J Cancer. 125 (9), 2173-8.

5) Holsapple, M.P., Pitot, H.C., Cohen, S.M., Boobis, A.R., Klaunig, J.E., Pastoor, T., et al., 2006. Mode of action in relevance of rodent liver tumors to human cancer risk. Toxicol. Sci. 89, 51e56.

6) Hughes, B.J, Thomas, J., Lynch, A.M., Borghoff, S.J., Green, S., Mensing, T., Sarang, S.S., LeBaron, MJ. 2016. Methyl isobutyl ketone-induced hepatocellular carcinogenesis in B6C3F1 mice: A constitutive androstane receptor (CAR)-mediated mode of action. *Regulatol. Toxicol. harmacol*, 81: 421-429.

7) LaVecchia C , Negri E. 2014. A Review of Epidemiological Data on Epilepsy, Phenobarbital, and Risk of Liver Cancer.Eur J Cancer Prev, 23 (1), 1-7

8) Olsen JH, Boice JD Jr, Jensen JP, Fraumeni JF Jr. Cancer among epileptic patients exposed to anticonvulsant drugs. J Natl Cancer Inst. 1989 May 10;81(10):803-8.

9) Ross, J., Plummer, S.M., Rode, A., Scheer, N., Bower, C.C., Vogel, O., Henderson, C.J., olf, C.R., Elcombe, C.R., 2010. Human constitutive androstane receptor (CAR) and pregnane X receptor (PXR) support the hypertrophic but not the hyperplastic response to murine nongenotoxic hepatocarcinogens phenobarbital and chlordane. Vivo. Toxicol. Sci. 116, 452e466.

10) Stout, M., Herbert, R., Kissling, G., Suarez, F., Roycroft, J., Chhabra, R., & Bucher, J. 2008: Toxicity and carcinogenicity of methyl isobutyl ketone in F344N rats and B6C3F1 mice following 2-year inhalation exposure (publication), Toxicology 244: 209-219.

11) Vezine, M., Kobusch, A.B., duSouich, P., Gresilin, E., Plaa, G.L., 1990. Potentiation of chloroform-induced hepatotoxicity by methyl isobutyl ketone and two metabolites. Can. J. Physiol. Pharmacol. 68, 1055e1061.

12) Whysner, J., Ross, P.M., Williams, G.M., 1996. Phenobarbital mechanistic data and risk assessment: enzyme induction, enhanced cell proliferation and tumor promotion. Pharmacol. Ther. 71, 153e191.

13) Yamada, T., Okuda, Y., Kushida, M., Sumida, K., Takeuchi, H., Nagahori, H., Fukuda, T., Lake, B., Cohen, S.M., Kawamura, S., 2014. Human hepatocytes support the hypertrophic but not the hyperplastic response to the murine nongenotoxic hepatocarcinogen sodium phenobarbital in an in vivo study using a chimeric mouse with humanized liver. Toxicol. Sci. 142 (1), 137e157.

14) Yamamoto, Y., Moore, R., Goldsworthy, T.L., Negishi, M., Maronpot, R.R., 2004. The orphan nuclear receptor constitutive active/androstane receptor is essential for liver tumor promotion by phenobarbital in mice. Cancer Res. 64, 7197e7200.

15) Yao R, Yasuoka A, Kamei A, Kitagawa Y, Tateishi N, Tsuruoka N. Dietary favonoids activite the constitutive androstane receptor CAR. *J Agric Food Chem*. 2010; 58(4): 2168-2173.

Bijlage bij brief MIBK REACH Consortium

Annex letter MIBK REACH Consortium

Sylvain BINTEIN
REACH and CLP Team leader
European Commission
Environment DG
Unit B2: Sustainable Chemicals
BU-9 05/34
B-1049 Brussels
Sylvain.BINTEIN@ec.europa.eu

5th March 2020

Dear Mr Bintein,

Proposal for Harmonised Classification and Labelling: isobutyl methyl ketone (MIBK)

Following our meeting on 20th February, I am now writing to you on behalf of the MIBK Consortium to provide additional information on the topics we discussed, related to the RAC opinion for classification of MIBK as a Category 2 carcinogen. As mentioned during the meeting, we ask your consideration of this information before making a decision to amend the relevant classifications in Annex VI of the CLR (Regulation (EC) 1272/2008).

Austria put forward its recommendation for the classification of MIBK based on three areas of concern. From the discussions in the RAC Committee, each issue appears to be a 'borderline' concern. Given the uncertainties in the evaluation, we would like to take the opportunity to provide you with further information on the three issues discussed:

1. Liver tumours in mice mediated by CAR/PXR MOA

The RAC opinion concluded that the evidence presented suggesting a CAR/PXR mediated mode of action (MOA) for the observed effects, which would not be considered relevant to humans, was lacking certain information that would eliminate the possibility that other modes of action may have also played a role and thus leaving some uncertainties on human relevance. Given the remaining uncertainty in the RAC evaluation, the MIBK Consortium initiated discussion on an in vitro experiment, which aims to demonstrate a CAR/PXR MOA for MIBK-related carcinogenicity, being a mechanism characteristic to rodent without relevance to human. Once results are reproduced in the mice in vitro liver cells, the experiment will be extended to human liver cells, to demonstrate the lack of proliferation in human cells as opposed to induction of proliferation in mice liver cells. Other relevant endpoints like cell survival, cytochrome P450 mRNAs and enzyme activity will also be assessed.

We believe that this will provide the mechanistic evidence in a human model, which was identified as the main data gap in the RAC opinion.

The study will be carried out with a laboratory recognized for its experience with this type of study. Given limited availability in the laboratory, the in vitro study will be initiated in May and will take 12 weeks for completion. Given the required reporting on the study, the MIBK Consortium would be able to report and submit the full study in October 2020. While the study is currently not recognized as an OECD test method, we would underline that ECHA has evaluated and accepted the result of this study on other substances being evaluated in ECHA[1]. We can provide further information on this point if required.

2. Kidney tumours in male rats mediated by α2μ-globulin MoA

While RAC opinion recognised non-relevant to humans, it was noted that there were some uncertainties regarding potential other Modes of Actions, with an increased severity of nephropathy effects in female rats. We would highlight however that the final RAC opinion referred to effects in female rats, while the Austrian evaluation mistakenly references kidney toxicity in female mice. Given the initial reference to female mice, the MIBK Consortium directed its efforts to address this issue. The MIBK Consortium, therefore, did not have the opportunity to share relevant literature, which does address the kidney toxicity seen in female rats. According to the data presented (NTP, 2007) and as cited in the CLH proposal (CLH-O-0000001412-86-295/F), the kidney toxicity seen in female rats exposed to MIBK is characterized as Chronic Progressive Nephropathy (CPN). RAC expressed concern about the increased incidence of CPN in female rats without a correlated increase in Renal Tubular Tumours (RTT), which could indicate that other MoA may be involved in kidney tumours in the male rats. We would summarize conclusions from three publications to address the uncertainties that were identified in the RAC opinion.

a) **Sex Differences in CPN**: Hard et al. (2012) re-examined the kidneys of all male and female control F344 rats in twenty-four chronic studies conducted by the NTP to record the presence of Chronic Progressive nephropathy (CPN) and its precursor, atypical tubule hyperplasia (ATH). This histopathological survey of 2,436 of the F344 strain showed that the severity of CPN for all studies was typically higher in males than in females. On the 0–8 grading scale, the mean severity grades for all studies was 6.4 (standard deviation 1.08) in males, and 5.0 (standard deviation 1.52) in females. This sex difference was highlighted by the fact that in the total study, only six females had developed end-stage (grade 8) CPN, whereas the number of males with end-stage CPN was 170. It is worth mentioning that in the MIBK carcinogenicity study (NTP, 2007), CPN was also observed in the female controls (19/50) compared to (42/50) in the male controls.

b) **CPN and Human relevance**: According to Hard et al. (2009), there are significant differences in pathology between CPN and human nephropathies. The histological characteristics in CPN include prominently dilated tubules filled with proteinaceous casts with consequent kidney enlargement, which contrasts with the shrunken kidneys found in end-stage human nephropathy. Unlike human nephropathy, CPN is devoid of vascular changes, it has no immunological or autoimmune basis, and inflammation is not a prominent feature. Various chemicals exacerbate CPN; no equivalent, chemical interactions are seen with human nephropathies. Humans are affected by several different nephropathies of known etiology, but generally, these are found much less frequently than CPN is found in the rat. Based on differences in biology and pathology, the analysis concluded that there is no clear human counterpart of CPN.

c) **CPN as a secondary MoA for tumor development**: According to Hard et al. (2013), CPN is a spontaneous renal disease of rats, which can be a serious confounder in toxicology. Extensive statistical analysis of National Toxicology Program studies shows a strong correlation between high-grade CPN, especially end-stage CPN, and renal tumor development. The importance of establishing a link between advanced CPN and RTT increase lies in the fact that many chemicals exacerbate the severity of this spontaneous disease process, and in so doing, they have the potential to produce a dose-related, sometimes statistically significant increase in RTT. In this regard, it is worth reminding that in the MIBK carcinogenicity study (NTP, 2007); a statistically significant increase in renal tubule adenoma and combined adenoma was only observed in the high dose group of treated male rats (at 1800 ppm). According to Hard et al., 2013, such results can be misinterpreted as indicating a direct causal relationship between the tumors and the test chemical. Not only is it important to avoid the inaccurate designation of chemicals as renal carcinogens, but chemical exacerbation of CPN represents a secondary MoA for tumor development. Such a mode of action has unlikely relevance for species extrapolation in risk assessment because biologically and histopathologically, there is no counterpart of rat CPN in humans (Hard et al., 2012). For some chemicals, there is the possibility of more than one MOA being operative in producing a given effect, including neoplasia. An example of this situation is provided by t-butyl alcohol. A re-evaluation of the renal histopathology of male rats in 13-week and 2-year drinking water exposure studies of this chemical found the evidence to be strongly compatible with the dual involvement of α2u-globulin nephropathy and exacerbation of CPN in the increased incidence of RTT ultimately observed in the longer-term study (Hard et al., 2013).

[1] Recent examples where the human hepatocyte studies had been accepted in ECHA evaluations include for pydiflumetofen (2019); fluxapyroxad (2019); silthiofam (2018) and penflufen (2018).

During our meeting, you requested that we provide you with the publications referenced here, and we have now listed the references at the end of this letter, with all related access links. Please let us know if you have any difficulties in accessing the studies.

3. Renal mesenchymal tumours RMT in female rats

The RAC opinion is based on the occurrence of two cases of renal mesenchymal tumours in female rats; these cases are considered relevant, especially given the view expressed that such tumours are rare and thus assumed to be substance related. The RAC opinion specifically states that[2]:

> *"Overall, the occurrence of 2 cases of renal mesenchymal tumours could be considered of concern due to their malignancy and their very rare occurrence."*

We have noted the reference made in the RAC opinion to a publication by *Hard et al., 2016*, in which the conclusions in the study do not appear to be consistent with the view expressed in the RAC opinion. We would in particular note that the publication concludes the following:

1. Renal mesenchymal tumours (RMT) was the most common spontaneous nonepithelial tumour in the rat kidney and thus not very rare. In this regard, it is worth mentioning that this tumour was given various names which could explain, at least partly, the conception of their rare occurrence,
2. More than 2 RMT occurred in carcinogenicity studies of ethylene thiourea (3 cases), nickel (II) oxide (3 cases), isobutene (3 cases), PCB 126 TEF evaluation (5 cases), and PCB 153 TEF evaluation (3 cases). Qualitatively, tumour occurrence was distributed evenly among control and treatment groups. For example, 3 rats in the isobutene study were diagnosed with RMT, 1 each in the control, low-dose, and high-dose groups.
3. Male and female rats were equally disposed to developing RMT
4. **Statistical analysis confirmed that their distribution among dose groups was random and demonstrated a lack of any relationship of these renal tumour types to test article administration in the NTP data bank.**
5. RMT has been induced only by genotoxic carcinogens (unlike MIBK, which is NOT genotoxic). However, they do occur sporadically across some 2-year carcinogenicity bioassays conducted in the National Cancer Institute (NCI)/NTP program for identifying chemical carcinogens.

As the RMT tumours in the MIBK NTP study were only slightly above the historical control, and only appearing in female rats together with the evidence presented above, the weight of these tumours type in the overall weight of evidence for classification can be considered to be very weak.

We would, therefore, welcome a review of this publication to ensure a common understanding of the issue that has been raised.

Timelines of discussion - RAC 50 meeting

During our meeting on 20th February, we briefly discussed the timing of the discussion on MIBK within the RAC. The evaluation of MIBK was discussed at the RAC 50 meeting only, with a vote and a decision being made during that meeting following an exchange of views between the attending experts. While the MIBK expert observer was given an opportunity to provide some input during the meeting, this opportunity was afforded only in the late stages of the discussion.

This is particularly unfortunate given the misunderstanding related to kidney toxicity in female mice, as the MIBK Consortium was only made aware during the process that it was the effects in female rats that were actually under consideration. The MIBK Consortium therefore did not have the opportunity to fully prepare and provide the relevant additional information for consideration by the RAC members.

In conclusion, the MIBK Consortium would request that the additional information referenced in this letter be taken into consideration prior to any decision to amend the classification of MIBK in Annex VI of the CLR Regulation. We understand that this issue will be discussed at the Caracal meeting in March; we would therefore request that this letter be made available to Caracal stakeholders, to support the discussion on the forthcoming ATP.

If you require any additional information, please do not hesitate to contact me.

Yours sincerely

Paula Diaz Rodriguez

[2] Last paragraph on page 19 of RAC opinion (CLH-O-0000001412-86-295/F)

Cc: Karin Kilian Karin.KILIAN@ec.europa.eu

An Jamers An.JAMERS@ec.europa.eu;

Dave Penney dave.penney@us.sasol.com;

Wasma Al-Husainy Wasma.Al-Husainy@shell.com

Euros Jones Euros.Jones@erm.com

References:

Hard GC, Johnson KJ, Cohen SM. 2009. A comparison of rat chronic progressive nephropathy with human renal disease-implications for human risk assessment. Crit Rev Toxicol 39(4):332–346. https://www.ncbi.nlm.nih.gov/pubmed/19514917

Hard GC, Betz LJ, Seely JC. 2012. Association of advanced chronic progressive nephropathy (CPN) with renal tubule tumors and precursor hyperplasia in control F344 rats from two-year carcinogenicity studies. Toxicol Pathol 40(3):473–481. https://www.ncbi.nlm.nih.gov/pubmed/22298794

Hard GC, Banton MI, Bretzlaff RS, Dekant W, Fowles JR, Mallett AK, McGregor DB, Roberts KM, Sielken RL Jr, Valdez-Flores C, Cohen SM. 2013. Consideration of rat chronic progressive nephropathy in regulatory evaluations for carcinogenicity. Toxicol Sci 132:268–27. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3595520/

Hard GC, Seely JC, Betz LJ. 2016. A survey of mesenchyme related tumours of the rat kidney in the national toxicology program Archives, with particular references to renal mesenchymal tumour. Toxicol Pathol 44(6):848-55. https://www.ncbi.nlm.nih.gov/pubmed/27169591