Electronic Supplementary Material (ESI) for Toxicology Research.

Supplementary Table. Overview of IPCS requirements for analyzing cancer mode of action

	Metofluthrin [1]	Momfluorothrin [2]
A. Postulated mode of action (MOA) for the induction of tumour in experimental animals	**CAR-mediated MOA:** Involve activation of CAR, which results in a pleiotropic response including the stimulation of the cytochrome P450 (CYP) CYP2B subfamily enzymes, liver hypertrophy and increased cell proliferation. Prolonged treatment results in the formation of altered hepatic foci and liver tumours.	
B. Key events in experimental animals	Key 1. **CAR activation:** The treatment of Wistar rat hepatocytes with CAR-siRNA significantly reduced CAR mRNA in the presence of either NaPB and metofluthrin, resulting in a reduction in the magnitude of induction of CYP2B1/2 mRNA levels by both compounds [3]. Key 2. **Altered gene expression specific to CAR activation:** The altered genes by metofluthrin treatment at 1800 ppm for 7 days overlapped with genes altered after 7-day treatment with 1000 ppm NaPB [3]. Key 3. **Increased hepatocellular proliferation:** Hepatocyte cell replicative DNA synthesis (determined as BrdU-labeling indices) was significantly increased by 7-day treatment with 900 and 1800 ppm metofluthrin in males and 1000 ppm NaPB in both sexes. In females, labeling indices, although not statistically significant at the small sample sizes used in this study, showed tendencies for increases at 1800 and 3600 ppm metofluthrin [3]. Another investigation also demonstrated that metofluthrin could increase replicative DNA synthesis [4] and Ki-67 mRNA levels [5] in cultured Wistar rat hepatocytes. CAR KO rats were not available at that time. Key 4. **Clonal expansion leading to altered hepatic foci:** The chronic treatment of male and female rats with 1800 ppm metofluthrin also resulted in significant increases in eosinophilic hepatocellular foci or mixed cell foci [3]. Key 5. **Liver adenoma/carcinoma production:** treatment with metofluthrin in rats for 2 years produced hepatocellular tumours in males at 900 and 1800 ppm and in females at 1800 ppm. The no tumourigenic dose levels (no observed effect levels (NOEL) for tumours) in male and female rats were established at 200 ppm and 900 ppm, respectively [3].	Key 1. **CAR activation:** The treatment of Wistar rat hepatocytes with CAR-siRNA significantly reduced CAR mRNA in the presence of either NaPB and momfluorothrin, resulting in a reduction in the magnitude of induction of CYP2B1/2 mRNA levels by both compounds [2]. This was also strongly supported by the in vivo study in CAR KO rats; where 3000 ppm momfluorothrin increased CYP2B1/2 mRNA in Wild-type rats but not in CAR KO rats [2]. Key 2. **Altered gene expression specific to CAR activation:** The altered genes by momfluorothrin treatment at 3000 ppm for 14 days overlapped with genes altered after 14-day treatment with 1000 ppm NaPB. Those by momfluorothrin also overlapped with genes altered after 7-day treatment with a CAR activator metofluthrin (1800 ppm) or NaPB (1000 ppm). [2] Key 3. **Increased hepatocellular proliferation:** The treatment of male and female Wistar rats with 1500 and 3000 ppm momfluorothrin for 7 days resulted in significant increases in replicative DNA synthesis. [2] Another investigation also demonstrated that momfluorothrin could increase replicative DNA synthesis in cultured Wistar rat hepatocytes [6] Furthermore, momfluorothrin increased replicative DNA synthesis in Wild-type rats, but not in CAR KO rats, demonstrating that CAR activation is required for momfluorothrin-increased hepatocellular proliferation. [2] Key 4. **Clonal expansion leading to altered hepatic foci:** The chronic treatment of male and female rats with 3000 ppm momfluorothrin also resulted in significant increases in eosinophilic hepatocellular foci [2]. Key 5. **Liver adenoma/carcinoma production:** treatment with momfluorothrin in rats for 2 years produced hepatocellular tumours in males at 1500 and 3000 ppm and in females at 3000 ppm. The no tumourigenic dose levels (no observed effect levels (NOEL) for tumours) in male and female rats were established at 500 ppm and 1500 ppm, respectively [2].
C. Concordance of	● The effects of metofluthrin on CYP2B	● The effects of momfluorothrin on CYP2B

dose-response relationship	enzyme induction, hypertrophy (liver weight), and cell proliferation (replicative DNA synthesis) showed similar dose-dependency. In particular, in males at 900 and 1800 ppm, a key event cell proliferation was significantly increased and corresponded with the tumour producing doses. Associative events CYP2B1/2 mRNA and/or CYP2B protein were significantly increased or trended to increase at 900 and 1800 ppm [3]. ● In females at 1800 ppm, the increased incidence of liver tumour was observed but the cell proliferation effects were equivocal; labeling indices, although not statistically significant at the small sample sizes used in this study, showed tendencies for increases at 1800 (and 3600) ppm metofluthrin [3]. ● In females given 900 ppm metofluthrin, while the early events such as the increased hepatic CYP2B1/2 mRNA levels were observed, no relative liver weight and hepatocellular proliferation were observed after 7 days of treatment. Consequently, liver tumour formation was not increased [3].	enzyme induction, hypertrophy (liver weight), and cell proliferation (replicative DNA synthesis) showed similar dose-dependency. In particular, in males and females at 3000 ppm, those effects were significantly increased and corresponded with the tumour producing dose [2]. ● In males at 1500 ppm, the increased incidence of liver tumour was equivocal; not statistically significant, but equivalent to or higher than the maximum incidence of the historical background in male Wistar rats. Thus 1500 ppm in males appeared to be borderline for tumour production. The increased relative liver weights and hepatocellular proliferation observed in rats given 1500 ppm momfluorothrin correlated with the observed tumour formation [2]. ● In females given 1500 ppm momfluorothrin, while the early events such as the increased hepatic CYP2B activity, relative liver weight and hepatocellular proliferation were observed after 7 days of treatment, liver tumour formation was not increased [2].
D. Temporal association	● Data are available for the effect of treatment of male and female rats with metofluthrin or momfluorothrin at various time points ranging from 7 days to 2 years. ● Overall, there is a logical temporal sequence for all key and associative events in metofluthrin- or momfluorothrin-produced liver tumour, in which all key and associative events precede tumour formation.	
E. Strength, consistency, and specificity of association of tumour response with key events	● The effects of metofluthrin or momfluorothrin on key and associative events at early phase of treatment correlated with the dose-relationship for hepatocellular tumour formation. ● Furthermore there is a logical temporal sequence for all key and associative events in metofluthrin- or momfluorothrin-induced hepatocellular tumour formation, in which all key and associative events precede tumour formation. ● The effects of metofluthrin or momfluorothrin on liver weight, hepatocellular hypertrophy and CYP2B enzyme induction after 7 days of treatment were shown to be reversible after 7 days of cessation of treatment [2, 3]. Therefore, effects of short term treatment with metofluthrin or momfluorothrin on the liver are reversible, which is consistent with the known hepatic effects of other mitogenic rodent liver CAR activators [7-12].	
F. Biological plausibility and coherence	● Succession of key and associative events and tumour development in rodent liver in agreement with knowledge about biological processes [13, 14]. ● Succession of key and associative events similar to that of a well-known CAR activator phenobarbital which causes hepatocellular tumours in rodents via a similar MOA [13]. ● Succession of key and associative events similar to that of two close structural analogues metofluthrin and momfluorothrin which causes hepatocellular tumours in rodents via a similar MOA [1, 2].	
G. Other possible MOAs	● Metofluthrin and momfluorothrin are clearly not genotoxic, being negative in a variety of *in vivo* and *in vitro* genotoxicity assays (Data are unpublished but refereed to the CLH report) [15, 16] ● In the general toxicity studies with metofluthrin or momfluorothrin, utilizing both histopathology and electron microscopy techniques, there was no evidence of hepatocellular toxicity (e.g. necrosis, fatty liver), peroxisome proliferation, porphyria, statin-like alterations, increased iron deposition or any evidence of hormonal perturbation (Data are unpublished but refereed to the CLH report) [15, 16]. ● Unlike effects on CAR, gene expression profiling analysis studies demonstrated no marked alterations in either PPARα, AhR or pregnane X receptor (PXR) signaling [2, 3]. ● Although some oxidative stress related genes were increased in the global gene expression analysis, we have not examined the direct endpoints related to oxidative stress on momfluorothrin. However, there was no histopathological evidence indicating increased oxidative stress, such as degenerative findings like necrosis and fibrosis (Data are unpublished but refereed to the CLH report) [15]. For metofluthrin, no evidence was obtained for the involvement of oxidative stress in phenobarbital or metofluthrin-induced rat liver tumour formation [3].	

	● Statistically significant decreases in the distances of fluorescent dye transfer were noted in both sexes of the 1800 ppm metofluthrin and both sexes of the NaPB 1000 ppm groups. After the recovery period of 1 week, the distances in all treatment groups were comparable to the control group [3]. ● In both sexes, cytoplasmic histone-associated DNA fragments as an apoptosis marker were not changed in any groups with metofluthrin [3].	
H. Uncertainties, inconsistencies, and data gaps	● The CAR dependency for the stimulation of cell proliferation by metofluthrin has not been established. However, we do not believe that this evaluation is essential and we consider that this data gap does not alter the overall postulated MOA for metofluthrin-produced rat liver tumours based on weight of evidence. ● Owing to high values in the control group significant increases in the BrdU-labeling index were not observed in female rats treated with metofluthrin. However, compared to the BrdU-labeling index value of the female 200 ppm metofluthrin group (lowest dose level), the labeling index values of the 1800 ppm were higher [3]. ● Some of the parameters except for BrdU-labeling index were not statistically significantly increased but trended to increase in the males administered 900 ppm metofluthrin, even though that dose was weakly tumourigenic in the 2-year bioassay [3]. This could be due to the weak tumourigenic activity of metofluthrin (only 8 out of 50 animals [16%] had liver tumours in the 900 ppm males) and small size (five animals per dose per sex) of the MOA study. Importantly, there were changes in the BrdU labeling index in this and at the higher dose of 1800 ppm, these being the most specific measures of the postulated key events.	● No data have been obtained for effects of momfluorothrin treatment on apoptosis and inhibition of gap junctional intercellular communication, we consider that, since decreased apoptosis or inhibition of gap junctional intercellular communication are not key events [13], these data gaps do not alter the overall conclusion regarding the postulated MOA for momfluorothrin-produced rat liver tumours. ● The treatment of male Wistar rats with 1500 ppm momfluorothrin did not result in a significant increase in CYP2B enzyme activity, hence the dose response between tumour formation and CYP2B induction was not completely matched. However, there were 16-18 fold increases in CYP2B1/2 mRNA levels in male Wistar rats given 3000 ppm momfluorothrin for 7 and 14 days, which suggest that some increase in CYP2B1/2 mRNA levels would have been observed in male rats treated with 1500 ppm momfluorothrin. It should be recognize that hepatocellular tumour incidence at 1500 ppm was only marginally increased (not statistically significant) and increased incidences of eosinophilic hepatocellular foci were only observed at 3000 ppm in both sexes. The tumour incidence data suggests that the potency for CAR activation by momfluorothrin would be less at a dose level of 1500 ppm than at 3000 ppm [2].
I. Assessment of postulated mode of action	● As described above, the key and associative events in the postulated MOA for metofluthrin-produced liver tumour formation have been established, with a strong dose-response and temporal consistency. ● The postulated MOA is similar to that of certain other non-genotoxic mitogenic rodent liver carcinogenic agents which are CAR activators including that of a close structural analogue momfluorothrin [2, 7-12, 17, 18]. ● Alternative MOAs for metofluthrin -produced rat liver tumour formation have been excluded. ● Thus, we consider that the level of confidence in the postulated MOA is high.	● As described above, the key and associative events in the postulated MOA for momfluorothrin-produced liver tumour formation have been established, with a strong dose-response and temporal consistency. ● The postulated MOA is similar to that of certain other non-genotoxic mitogenic rodent liver carcinogenic agents which are CAR activators including that of a close structural analogue metofluthrin [1, 7-12, 17, 18]. ● Alternative MOAs for momfluorothrin-produced rat liver tumour formation have been excluded. ● Thus, we consider that the level of confidence in the postulated MOA is high.

References in Supplementary Table

1. T. Yamada, S. Uwagawa, Y. Okuno, S. M. Cohen and H. Kaneko, Case study: an evaluation of the human relevance of the synthetic pyrethroid metofluthrin-induced liver tumors in rats based on mode of action, *Toxicol. Sci.*, 2009, **108**, 59-68.

2. Y. Okuda, M. Kushida, K. Sumida, H. Nagahori, Y. Nakamura, H. Higuchi, S. Kawamura, B. G. Lake, S. M. Cohen and T. Yamada, Mode of action analysis for rat hepatocellular tumors produced by the synthetic pyrethroid momfluorothrin: evidence for activation of the constitutive androstane receptor (CAR) and mitogenicity in rat hepatocytes, *Toxicol. Sci.*, 2017, **158**, 412-430.

3. Y. Deguchi, T. Yamada, Y. Hirose, H. Nagahori, M. Kushida, K. Sumida, T. Sukata, Y. Tomigahara, K. Nishioka, S. Uwagawa, S. Kawamura and Y. Okuno, Mode of action analysis for the synthetic pyrethroid metofluthrin-induced rat liver tumors: evidence for hepatic CYP2B induction and hepatocyte proliferation, *Toxicol. Sci.*, 2009, **108**, 69-80.

4. Y. Hirose, H. Nagahori, T. Yamada, Y. Deguchi, Y. Tomigahara, K. Nishioka, S. Uwagawa, S. Kawamura, N. Isobe, B. G. Lake and Y. Okuno, Comparison of the effects of the synthetic pyrethroid Metofluthrin and phenobarbital on CYP2B form induction and replicative DNA synthesis in cultured rat and human hepatocytes, *Toxicology*, 2009, **258**, 64-69.

5. T. Yamada, H. Kikumoto, B. G. Lake and S. Kawamura, Lack of effect of metofluthrin and sodium phenobarbital on replicative DNA synthesis and Ki-67 mRNA expression in cultured human hepatocytes, *Toxicol. Res.*, 2015, **4**, 901-913.

6. Y. Okuda, M. Kushida, H. Kikumoto, Y. Nakamura, H. Higuchi, S. Kawamura, S. M. Cohen, B. G. Lake and T. Yamada, Evaluation of the human relevance of the constitutive androstane receptor-mediated mode of action for rat hepatocellular tumor formation by the synthetic pyrethroid momfluorothrin, *J. Toxicol. Sci.*, 2017, **42**, 773-788.

7. T. Yamada, Y. Okuda, M. Kushida, K. Sumida, H. Takeuchi, H. Nagahori, T. Fukuda, B. G. Lake, S. M. Cohen and S. Kawamura, 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.*, 2014, **142**, 137-157.

8. H. Tinwell, D. Rouquie, F. Schorsch, D. Geter, S. Wason and R. Bars, Liver tumor formation in female rat induced by fluopyram is mediated by CAR/PXR nuclear receptor activation, *Regul. Toxicol. Pharmacol.*, 2014, **70**, 648-658.

9. M. J. LeBaron, B. B. Gollapudi, C. Terry, R. Billington and R. J. Rasoulpour, Human relevance framework for rodent liver tumors induced by the insecticide sulfoxaflor, *Crit. Rev. Toxicol.*, 2014, **44 Suppl 2**, 15-24.

10. M. J. LeBaron, D. R. Geter, R. J. Rasoulpour, B. B. Gollapudi, J. Thomas, J. Murray, H. L. Kan, A. J. Wood, C. Elcombe, A. Vardy, J. McEwan, C. Terry and R. Billington, An integrated approach for prospectively investigating a mode-of-action for rodent liver effects, *Toxicol. Appl. Pharmacol.*, 2013, **270**, 164-173.

11. T. G. Osimitz and B. G. Lake, Mode-of-action analysis for induction of rat liver tumors by pyrethrins: relevance to human cancer risk, *Crit. Rev. Toxicol.*, 2009, **39**, 501-511.

12. J. L. LaRocca, R. J. Rasoulpour, B. B. Gollapudi, D. L. Eisenbrandt, L. A. Murphy and M. J. LeBaron, Integration of novel approaches demonstrates simultaneous metabolic inactivation and CAR-mediated hepatocarcinogenesis of a nitrification inhibitor, *Toxicol Rep*, 2017, **4**, 586-597.

13. C. R. Elcombe, R. C. Peffer, D. C. Wolf, J. Bailey, R. Bars, D. Bell, R. C. Cattley, S. S. Ferguson, D. Geter, A. Goetz, J. I. Goodman, S. Hester, A. Jacobs, C. J. Omiecinski, R. Schoeny, W. Xie and B. G. Lake, 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.*, 2014, **44**, 64-82.

14. S. M. Cohen and L. B. Ellwein, Genetic errors, cell proliferation, and carcinogenesis, *Cancer Res.*, 1991, **51**, 6493-6505.

15. ECHA, CLH report, Proposal for Harmonised Classification and Labelling Based on Regulation (EC) No 1272/2008 (CLP Regulation),Annex VI, Part 2.

Substance Name: 1R-trans-Z-momfluorothrin. https://echa.europa.eu/documents/10162/13626/clh_proposal_1r_trans_z_momfluorothrin_en.pdf/34bf8211-758e-4cf5-a4d9-bfe616464965, 2014, Accessed date: September 12, 2017.

16. ECHA, CLH report, Proposal for Harmonised Classification and Labelling Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2. Substance Name: Epsilon-metofluthrin. https://echa.europa.eu/harmonised-classification-and-labelling-previous-consultations/-/substance-rev/9502/term. Accesed date: August 17, 2017., 2015.

17. R. A. Currie, R. C. Peffer, A. K. Goetz, C. J. Omiecinski and J. I. Goodman, Phenobarbital and propiconazole toxicogenomic profiles in mice show major similarities consistent with the key role that constitutive androstane receptor (CAR) activation plays in their mode of action, *Toxicology*, 2014, **321**, 80-88.

18. B. G. Lake, R. J. Price and T. G. Osimitz, Mode of action analysis for pesticide-induced rodent liver tumours involving activation of the constitutive androstane receptor: relevance to human cancer risk, *Pest Manag Sci*, 2015, **71**, 829-834.

(completed)