Expert Statement on Metofluthrin (CLH Report, Version 2 of May 2015).
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Comments on the mode of action (MOA) for metofluthrin-induced rat liver tumour formation and human relevance

I am writing in support of the proposed decision by the UK Competent Authority (Chemical Regulation Directorate) to not classify metofluthrin for carcinogenicity. By way of background, I have been involved in research into the hepatocarcinogenicity of non-genotoxic chemicals in rodents for over forty years. My areas of expertise include investigations into the hepatic effects of compounds which induce cytochrome P450 (CYP) enzymes by activation of either the constitutive androstane receptor (CAR) or the peroxisome proliferator-activated receptor alpha (PPARα).

In a two year study metofluthrin was shown to increase the incidence of liver tumours in male Wistar rats at dietary levels of 900 and 1800 ppm and in female Wistar rats at a dietary level of 1800 ppm. Metofluthrin did not increase the incidence of liver tumours in male and female CD-1 mice exposed to metofluthrin at dietary levels up to 2500/1750 ppm for 78 weeks. Short term tests for genotoxic potential have demonstrated that metofluthrin is not a genotoxic agent. The formation of liver tumours by metofluthrin in the rat is thus due to a non-genotoxic mode of action (MOA).

The liver is a common site of tumour formation in rodent carcinogenicity studies. In recent years frameworks have been established for analysing the MOAs by which chemicals can produce tumours in the liver and other organs of rodents and the relevance of such tumours for human risk assessment (Boobis *et al.*, 2006; Holsapple *et al.*, 2006; Meek *et al.*, 2003, 2014). For non-genotoxic chemicals, established MOAs include activation of the aryl hydrocarbon receptor, activation of the CAR, activation of the PPARα, cytotoxicity, hormonal perturbation, immunosuppression and porphyria (Budinsky *et al.*, 2014; Cohen, 2010; Corton *et al*., 2014; Elcombe *et al.*, 2014; Holsapple *et al*., 2006; Lake, 2009; Meek *et al.*, 2003). Based on the results of a number of investigative *in vivo* and *in vitro* studies, the MOA for metofluthrin-induced rat liver tumour formation has been shown to be due to CAR activation (Deguchi *et al.*, 2009; Yamada *et al*., 2009). In a recent evaluation of this MOA for non-genotoxic rodent liver tumour formation (Elcombe *et al*., 2014), the key events were identified as:

- CAR activation
- altered gene expression specific to CAR activation
- increased cell proliferation
- clonal expansion leading to altered foci
- liver adenomas/carcinomas.

Associative events for this MOA included liver hypertrophy and induction of CYP enzymes, particularly of CYP2B subfamily enzymes. Phenobarbital (PB) is a model CAR activator which is known to promote liver tumours in rats and mice. In addition to the extensive data on the hepatic effects of PB and its sodium salt (sodium phenobarbital; NaPB) in rodents, much human data are available as a result of PB having been used in humans as a sedative, hypnotic and antiepileptic drug for many years.

The treatment of rats with metofluthrin results in an induction of hepatic CYP2B subfamily enzymes, thus providing evidence that metofluthrin is a CAR activator. Metofluthrin has also been shown to induce CYP2B enzymes in cultured rat hepatocytes. Additional evidence that metofluthrin is a CAR activator comes from studies in rat hepatocytes employing a RNA interference technique to lower CAR mRNA levels. The lowering of CAR mRNA levels resulted in a significant decrease in the magnitude of induction of CYP2B1 mRNA levels in rat hepatocytes following treatment with either metofluthrin or NaPB. The treatment of both male and female rats with metofluthrin results in liver hypertrophy and a transient induction of replicative DNA synthesis determined as the hepatocyte labelling index. Prolonged treatment with metofluthrin results in the appearance of altered hepatic foci and ultimately in the formation of liver tumours. The MOA was evaluated using the modified Bradford Hill considerations for causality and alternative MOAs were excluded. Overall, a robust MOA for metofluthrin-induced rat liver tumour formation has been established, this MOA being similar to that of PB and certain other non-genotoxic agents which are CAR activators in rodent liver. The hepatic effects of metofluthrin in the rat and subsequent liver tumour formation appear to be primarily mediated by CAR activation.

Some of the effects of CAR activators observed in rodent liver can also be demonstrated in human liver. For example, PB and other CAR activators can induce CYP enzymes in both rodent liver and in human liver (Elcombe *et al.,* 2014). However, in terms of the human relevance of a CAR activator MOA for rodent liver tumour formation, the key species difference is that while CAR activators are mitogenic agents in rodent hepatocytes, they do not appear to stimulate replicative DNA synthesis in human hepatocytes. The observation that CAR activators do not stimulate replicative DNA synthesis in human liver comes from *in vitro* studies with human hepatocytes and *in vivo* studies with humanised mice. For example, a number of studies have shown that while NaPB can stimulate replicative DNA synthesis in cultured rat hepatocytes, no such effects are observed in human hepatocytes (Hirose *et al*., 2009; Parzefall *et al*., 1991; Yamada *et al.,* 2015). In a recent *in vivo* study, the hepatic effects of NaPB were investigated in Wistar Rats, CD-1 mice and in chimeric mice having humanised livers. In contrast to the effects in Wistar rats and CD-1 mice, treatment with NaPB did not increase replicative DNA synthesis and did not increase the expression of cell proliferation related genes in the human hepatocytes of the chimeric mice (Yamada *et al*., 2014). The key role of increased cell proliferation in a CAR activator MOA for rodent liver tumour formation has been demonstrated in studies performed in mice lacking CAR. Thus in such CAR knockout mice, PB does not stimulate replicative DNA synthesis in hepatocytes and does not promote liver tumours after treatment with a genotoxic carcinogen (Huang *et al.,* 2005; Wei *et al.,* 2000; Yamamoto *et al.,* 2004).

To provide data to support the conclusion that the MOA for metofluthrin-induced rat liver tumour formation is not applicable to humans, the effect of metofluthrin and NaPB on CYP enzyme induction and replicative DNA synthesis in cultured rat and human hepatocytes was investigated (Hirose *et al.,* 2009; Yamada *et al.,* 2015). Treatment with metofluthrin induced CYP2B enzymes in rat hepatocytes also produced a weak effect in cultured human hepatocytes. However, while NaPB and metofluthrin stimulated replicative DNA synthesis in cultured rat hepatocytes, no increase in replicative DNA synthesis was observed in cultured human hepatocytes from six individual donors. Treatment with hepatocyte growth factor did produce an increase in replicative DNA synthesis, thus confirming the functional viability of the human

hepatocyte preparations used in these studies. In addition, the treatment of rat hepatocytes with metofluthrin increased mRNA levels of the cell proliferation related gene Ki-67, whereas no increase in Ki-67 mRNA levels was observed in four individual human hepatocyte preparations after treatment with either metofluthrin or NaPB. Recently, the effect of metofluthrin on replicative DNA synthesis has been investigated in human hepatocytes of chimeric mice (Yamada, unpublished observations). In three separate studies, chimeric mice with human hepatocytes from different donors were treated with 1800 ppm metofluthrin in the diet for 7 days. Treatment with metofluthrin did not result in any increase in replicative DNA synthesis. However, as a positive control, replicative DNA synthesis in human hepatocytes was increased when the chimeric mice were treated with epidermal growth factor.

The conclusion that the MOA for rodent liver tumour formation by a CAR activator is not relevant for humans is supported by available epidemiological data. For example, a recent evaluation of the literature for PB concluded that there was no evidence of a specific role of PB in human liver cancer risk (La Vecchia and Negri, 2014). Moreover, in such studies showing no evidence of increased cancer risk, the subjects received PB for many years at doses which produced similar plasma levels to those which are carcinogenic in mice (Monro, 1993).

In summary, a robust MOA for metofluthrin-induced rat liver tumour formation has been established. This MOA involves CAR activation and the stimulation of replicative DNA synthesis. However, in keeping with the properties of the model CAR activator PB, metofluthrin does not stimulate replicative DNA synthesis in cultured human hepatocytes and in human hepatocytes of chimeric mice. The MOA for metofluthrin-induced rat liver tumour formation is therefore qualitatively not plausible for humans. I therefore strongly support the conclusion of the UK Competent Authority (Chemicals Regulation Directorate) to not classify metofluthrin for carcinogenicity.

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