Comments on the mode of action for metofluthrin-induced rat hepatocellular tumors and an evaluation of their human relevance.

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I am writing in support of the proposed decision by the UK Competent Authority (Chemicals Regulation Directorate) to not classify metofluthrin for carcinogenicity. Although metofluthrin induced a low incidence of liver tumors in male rats in a two-year bioassay, an extensive body of mechanistic information strongly indicates that this finding does not represent a relevant risk to humans (1-4).

As background, I have been involved in carcinogenesis research for over 50 years and have been an active member in the International Programme on Chemical Safety (IPCS) and the International Life Sciences Institute (ILSI)/US EPA/Health Canada efforts in developing a framework for evaluation for mode of action as well as examination of the human relevance of human findings. The efforts of this group have been published extensively and the framework continues to evolve (5-11).

Research over the past several decades have indicated that cancer is the result of an accumulation of multiple genetic events in a single stem cell (cancer is a clonal disease) (12,13). In addition, it has been well known since the 1950's that DNA repair is extremely precise, but errors occur every time DNA replicates. Based on these findings, there are only two fundamental ways that an agent can increase the risk of cancer: 1) directly damage DNA every time DNA replicates (DNA reactive, genotoxic); or 2) increase the number of DNA replications in the stem cell population of the target tissue (12,13). At high doses, DNA reactive carcinogens also produce cytotoxicity with consequent regenerative cell proliferation.

For the liver, extensive research over the past decades has indicated several specific modes of action for the development of liver tumors in rodents and humans (14,15). Hepatocellular carcinoma in humans is predominantly due to a variety of diseases involving hepatocyte damage with consequent regenerative proliferation accompanied by inflammation. In humans, such diseases include viral Hepatitis (Hepatitis B and C), ethanol-induced hepatitis, a variety of inherited disorders (α-1 antitrypsin disease, tyrosinemia, Wilson's disease, etc.), along with several acquired inflammatory disorders such as primary biliary cirrhosis, sclerosing cholangitis, and non-alcoholic steatohepatitis (NASH). In addition, a known genotoxic, DNA reactive carcinogen has been implicated in liver carcinogenesis in humans, aflatoxin, although this appears to be restricted to a specific location in China in which the contamination of peanut products is excessive.

In animal models, a variety of DNA reactive substances have been demonstrated to increase liver tumors, such as N-nitrosamines and aromatic amines (14, 15). In addition, a number of mechanisms have been identified which increase liver tumors in rodents by means of increasing cell proliferation (14, 15). These include receptor-mediated and non-receptor-mediated processes. Non-receptor-mediated processes include such mechanisms as cytotoxicity with

regenerative proliferation, infectious diseases, and iron accumulation with consequent cytotoxicity and regeneration. Receptor-mediated mechanisms include constitutive androstane receptor (CAR), pregnane X receptor (PXR), peroxisome proliferator activated receptor alpha (PPARα), estrogen, and statin-related lesions (15). For these receptor-mediated processes, the operative mechanism involves direct mitogenesis as a consequence of activation of these receptors rather than cytotoxicity and regeneration. Of interest, these receptor-mediated mechanisms do not appear to be relevant to humans (14, 15). Although the metabolic abnormalities associated with these various receptors usually occur in humans in a similar fashion as in rodents, there is not a corresponding increase in cell proliferation, one of the necessary key events in the mode of action of these chemicals for the induction of liver tumors (14, 15). Thus, the tumor findings in rodents are not relevant to human liver cancer risk.

In the mode of action framework developed by IPCS and others (5-11), there are several aspects which are evaluated in ascertaining whether a mode of action is applicable for a given chemical to induce a toxic event, including cancer. The framework includes an evaluation of necessary specific key events in the process along with identified associative events. For these events, temporality of the event in association with the ultimate toxic endpoint is evaluated, along with the parallel dose response for the key events to the toxicologic endpoint. Furthermore, consistency of the findings are evaluated along with their coherence and biological plausibility. An important aspect of the framework is the requirement for also evaluating other possible modes of action.

Based on an extensive array of mechanistic studies (1-4), the mode of action for metofluthrin clearly involves activation of CAR leading to an increase in hepatocellular proliferation with consequent ultimate formation of hepatocellular foci, adenomas, and ultimately carcinomas. The types of studies to demonstrate this association have followed closely those that were used to demonstrate this mode of action for the prototypic CAR activator, phenobarbital (16-19). Phenobarbital (or its sodium salt) was used as a positive control in these studies involving metofluthrin.

The temporality of the events is well defined. The dose response for metofluthrin for the various key events also follows closely the dose response for the development of tumors. CAR activation was demonstrated utilizing primarily the associated event of activation of CYP2B (1-4).

There have been several very pivotal experiments demonstrating the relationship of CAR in the events related to metofluthrin in hepatocytes (1-4). A pivotal study involved the evaluation of the effect of metofluthrin in rat hepatocytes employing a RNA interference technique to lower CAR mRNA levels by CAR-siRNA. Metofluthrin produced a significant induction of CYP2B1 mRNA levels in rat hepatocytes treated with control-siRNA. However, the lowering of CAR mRNA levels by CAR-siRNA resulted in a significant decrease in the magnitude of induction of CYP2B1 mRNA following treatment with metofluthrin, clearly indicating the importance of CAR activation in the mode of action of metofluthrin (1). Furthermore, treatment of rat hepatocytes *in vitro* induced CYP2B enzymes and increased proliferation, whereas human hepatocytes showed the

enzyme changes but not the increased proliferation response (2, 3). The ability of the human hepatocytes to respond to a mitogenic stimulus was confirmed using hepatocyte growth factor (HGF).

The relevance of this mode of action to humans has been extensively evaluated (16-19). I believe that the most noteworthy demonstration that the mode of action is not relevant to humans is the recent experiment involving administration of phenobarbital to chimeric mice, *i.e.* mice that have received a transplant of human hepatocytes (17). The range of replacement indices in chimeric mice used in the present study was 73–90%. Rodent hepatocytes in CD-1 mice and Wistar Hannover rats showed the usual metabolic changes in response to phenobarbital sodium salt and also showed a proliferative response. In contrast, the human hepatocytes in chimeric mice showed the metabolic response but did not show the proliferative response. Since proliferation is an essential key event in the mode of action, its absence in the human cells demonstrates that this mode of action does not translate to humans. This has been corroborated in recent investigations with metofluthrin in a similar chimeric mouse model (Yamada, unpublished observations). Epidemiology studies also strongly support a lack of cancer risk in humans with respect to administration of phenobarbital, even at doses which produce similar blood levels to those which are hepatocarcinogenic in rodents. (20).

With respect to metofluthrin, an examination of alternative modes of action has also been performed. There is no evidence of DNA reactivity (genotoxicity), eliminating that as a potential mode of action. Likewise, there is no evidence of cytotoxicity, either by examination of the histopathology or by an evaluation of liver enzymes in blood associated with hepatocellular damage. Furthermore, there is no activation of PPARα as evidenced by the lack of activation of CYP4A. Also, there is no evidence of iron deposition, no evidence of estrogen-like activity, and no evidence of statin-like effects on HMG (3-hydroxy-3-methyl-glutaryl)-CoA-reductase activity. Of interest, all statins produce high incidences of liver tumors in male and female rats and mice, but an extensive body of epidemiology literature, involving several hundred thousand individuals, shows that statins are not carcinogenic to humans, either to the liver or to any other tissues (15).

Thus, the mode of action for metofluthrin has been demonstrated to involve activation of CAR. It does not involve any of the other modes of action that have been identified for liver hepatocellular carcinogenesis, and involves a mode of action that is not relevant to human cancer risk. Based on these observations, I strongly support the conclusion of the UK Competent Authority (Chemicals Regulation Directorate) to not classify metofluthrin with regard to cancer.

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