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Human relevance of rodent liver tumors: Key insights from a Toxicology Forum workshop on nongenotoxic modes of action

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Abstract

The Toxicology Forum sponsored a workshop in October 2016, on the human relevance of rodent liver tumors occurring via nongenotoxic modes of action (MOAs). The workshop focused on two nuclear receptor-mediated MOAs (Constitutive Androstane Receptor (CAR) and Peroxisome Proliferator Activated Receptor–alpha (PPARα), and on cytotoxicity. The goal of the meeting was to review the state of the science to (1) identify areas of consensus and differences, data gaps and research needs; (2) identify reasons for inconsistencies in current regulatory positions; and (3) consider what data are needed to demonstrate a specific MOA, and when additional research is needed to rule out alternative possibilities. Implications for quantitative risk assessment approaches were discussed, as were implications of not considering MOA and dose in hazard characterization and labeling schemes. Most, but not all, participants considered the CAR and PPARα MOAs as not relevant to humans based on quantitative and qualitative differences. In contrast, cytotoxicity is clearly relevant to humans, but a threshold applies. Questions remain for all three MOAs concerning what data are necessary to determine the MOA and to what extent it is necessary to exclude other MOAs.

Keywords

Rodent liver tumors; Nongenotoxic; Mode of action; Constitutive Androstane Receptor; Peroxisome proliferator-activated receptor; alpha (PPARα)

1. Introduction

Rodent liver (hepatocellular) tumors are frequently the basis for classification of chemicals as carcinogens, with significant consequences. As the biological understanding of the etiology of those tumors has increased, so has our opportunity to better reflect that knowledge in the hazard characterization and dose-response portions of the risk assessment, and to consider these in the context of the human relevance framework (Boobis et al., 2008, 2006). The Toxicology Forum[1] held a workshop in the fall of 2016, with the goal of reviewing the state of the science and understanding regulatory use of the data for the chosen nongenotoxic rodent liver tumor modes of action (MOAs). Using chemical-specific case studies and broader evaluations, the workshop aimed to (1) identify areas of consensus and areas where differences remain, as well as data gaps and research needs; (2) identify and evaluate the reasons for inconsistencies in current regulatory positions regarding human relevance; and (3) consider what data are needed to demonstrate a specific MOA, and when additional research is needed to rule out alternative possibilities.

[1]The Toxicology Forum (http://dialogue.toxforum.org/) is “an international, nonprofit organization that is devoted to conducting open dialogues among various segments of society concerned with problems in toxicology”.

The current workshop followed a 2010 workshop that examined three receptor-mediated MOAs for rodent liver tumorigenesis (Budinsky et al., 2014; Corton et al., 2014; Elcombe et al., 2014). The 2016 workshop aimed to incorporate new research data, and to address differences in how the data related to these MOAs are interpreted and integrated into risk assessments. While an official workshop report will not be issued, this manuscript summarizes the key themes, conclusions, and issues addressed at the 2016 workshop, noting areas of general consensus and areas where additional work is needed. That said, no votes were taken on specific issues, and there was no attempt to reach consensus. The intent of the manuscript is not to provide an in-depth review of the material presented at the workshop, but rather to highlight the key themes that emerged from the workshop.

The induction of liver tumors in the rodent by chemical agents has been extensively studied and several well-established mechanisms of action have been defined, characterized, and evaluated using the human relevance framework (Holsapple et al., 2006; Boobis et al., 2008, 2006; Klaunig, 2012; Cohen, 2010). These include modes of action that can be broadly categorized into genotoxic and nongenotoxic, with the latter including receptor-mediated and cytotoxicity mechanisms. In the current workshop two receptor-mediated MOAs (CAR and PPARalpha) and cytotoxicity were examined in detail and updated based on the current state of the science.

2. Constitutive Androstane Receptor (CAR) MOA

2.1. Proposed mode of action for CAR-related liver tumors

An overview of the biology and physiology of CAR was presented, and has been summarized by Elcombe et al. (2014). Based largely on data for phenobarbital (PB), the previous workshop (Elcombe et al., 2014) identified the key events (i.e., the necessary elements of the MOA) for the CAR MOA as (1) activation of CAR, (2) altered gene expression specific to CAR activation, (3) increased cell proliferation, (4) clonal expansion leading to altered hepatic foci, and (5) liver tumors. Associative events included liver hypertrophy, induction of CYP enzymes (particularly CYP2B enzymes) and inhibition of apoptosis. In addition, functional Wnt-pathway signaling is required, as knockout studies of Ctnnb1 (encoding β-catenin, a central pathway molecule) have demonstrated (Rignall et al., 2011). Important species differences were identified, including a lack of cell proliferation in cultured human hepatocytes exposed to PB. Elcombe et al. (2014) concluded that “the MOA for PB induced rodent liver tumor formation was considered to be qualitatively not plausible for humans.” This conclusion is supported by data from a number of epidemiological studies conducted in human populations chronically exposed (e.g., for decades) to PB in which there is no evidence for increased liver tumor risk (La Vecchia and Negri, 2014). The 2016 workshop addressed several outstanding issues related to the proposed key events, but did not make any significant changes to the key events identified in the 2014 publication. Two speakers presented new data challenging the conclusion of Elcombe et al. (2014) that the CAR MOA is qualitatively not relevant to humans. However, these data were questioned and a number of individuals supported the conclusions of Elcombe et al. (2014). and, consistent with the earlier conclusions of Holsapple et al. (2006), all the speakers agreed that the

animal models should not be used for human health risk assessment, due to quantitative differences between rodents and humans.

In order to demonstrate the key and associative events in the CAR MOA, Lake et al. (2015) stated that studies are needed on: activation of CAR, induction of CYP enzymes, liver hypertrophy, replicative DNA synthesis, and possibly apoptosis and altered hepatic foci. Activation of CAR can be evaluated directly or it can be inferred from induction of CYP2B enzymes as CYP2B genes are specific transcriptional targets of CAR (Elcombe et al., 2014). The definitive test for the involvement of CAR is evaluating the postulated key events and associative events in CAR KO mice or rats. The standard approach for demonstrating the CAR MOA relies on in vivo assays, but a workshop participant proposed a novel “lite” testing approach, based on in vitro methods.

2.2. Human relevance of the CAR MOA

Knockout (KO) models are playing an important role in evaluating the CAR MOA and human relevance. An important step in demonstrating the importance of CAR in liver tumor induction was the finding that treatment of CAR KO mice with PB does not increase liver weight, DNA synthesis or result in enzyme induction, and increased liver tumors do not result. Furthermore, the CAR/PXR double-KO demonstrated that the CAR/PXR pathway is essential for the tumor response. Similarly, studies in KO rats have demonstrated that CAR is needed for induction of enzymes and replicative DNA synthesis; similar results were seen in CAR/PXR KO rats. Based on results in the KO rodent systems and in human hepatocytes and a chimeric mouse/human model (in which the mouse liver is replaced with human hepatocytes), the lack of replicative DNA synthesis is identified as the key species difference in response to CAR activators, leading to the difference in tumor response (Elcombe et al., 2014).

A counter-argument regarding the rodent-specificity of the MOA in inducing liver tumors via the CAR/PXR pathway was made citing data from the hCAR/hPXR double-humanized mouse model (Scheer and Wilson, 2016). In this model, stimulation of replicative DNA synthesis does occur and PB mediates liver tumor promotion, similar as in wildtype mice, despite the absence of rodent CAR/PXR proteins (Braeuning et al., 2014). This clearly shows that the human receptors are principally capable of mediating tumor promotional activity upon stimulation. Caution is appropriate, however, because the human receptors function in a mouse-based heterologous system where gene regulatory protein interactions may differ from human hepatocytes and human-specific protective mechanisms may be missing. The more recent results with human hepatocytes and a chimeric mouse/human model (Yamada et al., 2014) are considered more physiological, since the human genes are in a human cellular context. Since replicative DNA synthesis is not seen in response to CAR/PXR activation in these latter systems, it was suggested that the human receptor acts like the mouse receptor when it is in a mouse environment but not a human environment. Based on these considerations, a workshop speaker recommended that the humanized hCAR/hPXR mouse not be used for chemical risk evaluation, although it is a useful model for understanding why some responses are turned on when the human receptor is placed in the mouse. PB is not a potent (this refers to dose potency and not

the magnitude of down-stream effects) CAR activator in humans. An audience member noted that CITCO ((6-(4-chlorophenyl)imidazo(2,1-b)(1,3)thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime) and PB induced DNA synthesis in rat and mouse hepatocytes, but not in human hepatocytes (Soldatow et al., 2016). Another suggested study would be to evaluate whether CITCO, an activator of human but not rodent CAR, promotes tumorigenesis in human hepatocytes transplanted into mice. It was mentioned that a carcinogenicity study using a chimeric humanized mouse liver model has been conducted with CITCO, but the results are not yet available. A workshop speaker outlined new opportunities for elucidating the molecular basis of strain and species differences in CAR effector gene regulation based on integrated transcriptomic and epigenomic profiling of liver tissue.

It was noted that a recent report indicates CAR mediates the liver tumors induced by disruption of circadian homeostasis in a mouse model of “jet lag” and that a similar mechanism might operate in humans (Kettener et al., 2016). It was also reported by one speaker that PB-mediated effects can have both stimulatory and inhibitory effects on hepatocarcinogenesis in mice, and that PB treatment actually inhibits tumorigenesis in rodents in which the standard initiation-promotion paradigm is reversed. While the barbiturate promotes the selective outgrowth of eosinophilic Ctnnb1-mutated liver tumors (Aydinlik et al., 2001), it inhibits the outgrowth of their basophilic counterparts with constitutively activated MAP-kinase signaling (Lee et al., 1998; Moennikes et al., 2000). Importantly, the phenotype of the PB-promoted Ctnnb1-mutated mouse liver tumors strongly resembles that of the human CTNNB1-mutated human hepatocellular carcinomas (HCCs) indicating that this pathway might be relevant to humans (Stahl et al., 2005; Dong et al., 2015). About 30% of human HCCs are CTNNB1-mutated, while a larger fraction shows activated MAP kinase signaling. Additionally, the observation of a gene expression signature shared by mouse tumors resulting from combined pharmacologic and genetic activation of CAR and beta-catenin and the subset of human tumors with activating mutations in beta-catenin (Dong et al., 2015) supports the relevance of the mouse models. It was suggested that the absence of an effect of chronic PB exposure on liver cancer incidence in humans might reflect the combination of induction and inhibition resulting in a net zero change, rather than the absence of any impact on the relevant pathways. There was agreement regarding the conclusion that robust epidemiology data indicate that PB does not increase the cancer incidence in human populations treated with the drug for decades with doses yielding blood levels that are in the same range as seen in mice that are sensitive to PB-induced liver tumorigenesis. Thus, in comparing the human and rodent data, it is clear that humans are not like the sensitive rodent strains, and the data are not consistent with any suggestion that humans are genetically predisposed to the development of liver cancer.

2.3. Conclusions regarding CAR

Overall, although no formal attempt was made to reach consensus, the sense (though not unanimous) of the workshop was that the CAR MOA is not qualitatively relevant to humans, e.g., PB affects an increase in replicative DNA synthesis in rodent hepatocytes (believed to be a key event) in primary culture but not in primary cultures of human hepatocytes. This observation of an important species difference is reinforced by the experimental evidence indicating that PB treatment does not increase replicative DNA synthesis in

human hepatocytes in a chimeric humanized liver mouse model. Additionally, important quantitative differences exist. Some participants indicated that there should be more of a focus on the dose-response for CAR activators, not solely on whether the chemical is a CAR activator.

3. Peroxisome Proliferator-Activated Receptor alpha dependent mode of action for liver tumor induction

The Peroxisome Proliferator-Activated Receptor–alpha (PPARα) is a cellular receptor for fibrates, a class of drugs used in the treatment of dyslipidemia. Fibrates effectively lower serum triglycerides and raise serum HDL-cholesterol levels (Staels et al., 2008). In addition, PPARα has been identified as the target for a diverse class of rodent hepatocarcinogens that cause proliferation of peroxisomes (Hess et al., 1965; Reddy et al., 1980; O'Brien et al., 2005; Smith and Aitchison, 2013). Over or under activation of PPARα can lead to adverse effects. In the PPARα-null mouse, there is decreased activation of genes that control steatosis, steatohepatitis, and liver cancer (Howroyd et al., 2004). On the other hand, sustained activation leads to cellular growth in the liver and ultimately liver cancer in rodents.

3.1. Proposed mode of action for PPARα induced liver tumors

An overview of the biology and physiology of PPARα has been summarized by Corton et al. (2014). Extensive mechanistic studies in the rodent liver identified a number of key events that are required for PPARα activators to cause liver cancer. The key events are 1) activation of PPARα, 2) alteration of cell growth pathways, 3) alteration in hepatocyte fate including increased cell proliferation and decreases in apoptosis, and 4) clonal expansion leading to the apical endpoint of increases in hepatocellular adenomas and carcinomas (Klaunig et al., 2003; Corton et al., 2014). A large number of studies using structurally diverse hypolipidemic agents (WY-14,643, clofibrate, gemfibrozil) and environmentally relevant compounds (di-2-ethylhexyl phthalate (DEHP), perfluorooctanoic acid (PFOA)) have shown the consistency of the key event responses in the livers of both rats and mice. The alteration of cell growth pathways may be secondary to and/or influenced by an increase in oxidative stress including through activation of NF-κβ (modulating factor described in Corton et al., 2014).

The necessity of PPARα activation and downstream key events was established using the PPARα-null mouse model, in which the key events are blocked completely when exposed to PPARα activating compounds. Overall, there is a strong weight of evidence from many laboratories using multiple activators of PPARα that the key events as described above lead to liver tumor formation in rodent (rat/mice) models (Corton et al., 2014).

3.2. Apparent inconsistencies in the PPARα MOA

The majority of the mechanistic data from studies of PPARα activators are consistent with the MOA; however, the interpretation of two studies (Ito et al. (2007); Yang et al. (2008)) using genetically altered mouse models have been cited as evidence that either PPARα activation or downstream hepatocyte proliferation are not sufficient for liver tumor

induction and therefore the key events in the MOA are not operationally linked (Guyton et al., 2009). In the Yang et al. (2008) study, PPARα-mediated proliferation was apparently uncoupled from liver tumor induction. Yang et al. created a mouse model in which PPARα was constitutively activated by fusing the protein to the strong viral transactivation domain from the VP16 protein (the VP16PPARα mouse). In the absence of exposure to a chemical activator of PPARα, the liver exhibited a number of characteristics of exposure including activation of fatty acid beta-oxidation and increases in hepatocyte proliferation. When the mice were allowed to age, the VP16PPARα mice exhibited no increases in liver tumors despite 100% tumor incidence in wild-type mice exposed to a strong PPARα activator. However, there are key differences between the model and wild-type mice treated with PPARα activators. Whereas endogenous PPARα becomes transcriptionally active through a number of molecular events also observed with many nuclear receptors, the VP16PPARα fusion protein is activated through a viral transactivation domain that causes distinctly different protein-protein interactions with the transcriptional machinery (Hagmann et al., 1997) similar to other transcription factorVP16 fusion proteins that cannot induce all typical phenotypes observed when the transcription factor is activated through endogenous pathways (Schwarz et al., 1992). Furthermore, the mechanism of hepatocyte proliferation induced by VP16PPARα is not the same as that induced by WY in wild-type mice. Global transcriptional responses compared between wild-type and VP16PPARα mice treated with control vehicle or WY, revealed a class of genes linked to cell proliferation and DNA repair induced by WY but not the transgene (Qu et al., 2010). The basis for these differences likely lies in the fact that in the VP16PPARα mice only hepatocytes, but not other cell types (the nonparenchymal cells) in the liver, were proliferating, whereas treatment of wild-type mice with WY led to proliferation of both hepatocytes and nonparenchymal cells.

Ito et al. (2007) claimed that PPARα-null mice treated with DEHP exhibited increases in liver tumors in the absence of induction in the treated wild-type mice. A number of reviews and papers referencing this study have made the argument that because DEHP caused liver tumors in the null mice, DEHP does not function through the PPARα mode of action. One issue with the Ito et al. study was the lack of equivalent responses in the wild-type mice, which in addition to the differences in tumor frequency, included differences in gene induction and oxidative stress induction. However, given the low doses used, the lack of a tumor response in the wild-type mice was not surprising. In addition, to achieve significance in the knockout animals the authors combined hepatocellular adenomas, hepatocellular carcinomas and one hepatoblastoma, despite the fact that combining tumor types of different cellular origins is not a standard method for determining significance. The tumors seen in the null mice may reflect an increase in spontaneous liver tumor induction. When control PPARα-null mice were allowed to age, Howroyd et al. (2004) showed an increase in liver tumors in untreated mice compared to similarly aged wild-type mice that was likely secondary to increases in liver steatosis and inflammation. The role of steatohepatitis in increases in the background and chemical-induced liver tumor incidence in the PPARα-null mice is addressed in a recent review article (Corton et al., 2017). Overall, these issues raise serious doubts as to how much weight can be given to this study to provide evidence of the linkage of the key events in the PPARα MOA.

There is also a body of evidence suggesting that the liver tumors in the null mice originate by pathways not activated to the same levels in wild-type mice. Much of this evidence is based on microarray studies comparing global gene expression in the livers of treated wild-type vs. PPARα-null mice. For all chemicals examined > 75% of the changes require PPARα (Rosen et al., 2017). Many of the compounds activate CAR to greater extents in null mice than wild-type mice. DEHP (Ren et al., 2010) and four perfluorinated compounds (Rosen et al., 2017) were shown to activate CAR to greater extents in null mice compared to wild-type mice.

In summary, these studies show that activation of PPARα is the major determinant in mediating the effects of both perfluorinated compounds and DEHP, with many of the PPARα-independent targets likely regulated by sustained activation of CAR. Given the low level of CAR activation in wild-type mice, there is strong support that the DEHP-induced tumors in wild-type mice are PPARα-dependent.

3.3. Human relevance of the mode of action

PPARα activation occurs in both rodents and humans, but the downstream responses are unique to mice and rats. All test species including hamsters, guinea pigs, and monkeys as well as humans possess a functional PPARα, which, when activated, can regulate an overlapping set of lipid metabolizing enzymes, albeit to different extents. In rats and mice, this induction leads to increases in hepatocyte proliferation and liver weight, and under chronic exposure conditions, liver tumor formation. Syrian hamsters exhibit weak increases in cell proliferation and do not develop liver tumors upon long term exposures (summarized in Corton et al., 2014). Guinea pigs and Cynomolgus monkeys do not exhibit changes in cell proliferation or apoptosis. PPARα activation in humans does not lead to increases in liver to body weight ratios. Human primary hepatocytes are refractory to the increases in proliferation that are seen in parallel studies of rat primary hepatocytes (summarized in Corton et al., 2014).

To further address the relevance of PPARα responses to humans, two PPARα humanized mice were developed in which the human PPARα was expressed either from a liver-specific promoter (Cheung et al., 2004) or from the natural human promoter (Yang et al., 2008), both of which were expressed in the absence of a functional mouse PPARα. Treated humanized mice were refractory to responses typically observed in wild-type mice. Instead, humanized mice exhibited either no increases (Cheung et al., 2004; Morimura et al., 2006), or attenuated increases in cell proliferation compared to wild-type mice (Yang et al., 2008). The humanized mice did not exhibit increases in liver tumors after long-term treatment with Wy-14,643 (Morimura et al., 2006).

One critique of the humanized studies is that the compounds used are more potent for the mouse receptor than human receptor. To address the issue of potency, results were presented at the workshop in which humanized, PPARα-null, and wild-type animals were exposed to a high affinity human PPARα agonist (GW7647) (Foreman and Peters, unpublished data). Similar to the prior studies in the humanized mice, the studies with GW7647 showed responses that were consistently diminished compared to those in wild-type mice,

suggesting that the differences in responses were not due to receptor potency for test compound, but due to true species differences.

There are striking differences in species responses of the key events in the MOA. Due to differences in PPARα expression and activity, Syrian hamsters, guinea pigs and nonhuman primates are better human surrogates than mice and rats. While these test species exhibit PPARα activation and associated increases in genes and proteins involved in lipid homeostasis that underlie the universal hypolipidemic effects, they lack the activation of key events downstream of PPARα including alteration of cell growth pathways, hepatocyte proliferation, and liver cancer. Human hepatocytes in culture or in the context of humanized livers do not respond to exposure with a proliferative response. Epidemiological studies of large numbers of patients that have been prescribed hypolipidemic drugs for up to a decade do not show any increases in adverse liver effects or cancer (Corton et al., 2014). Taken together, the weight of evidence supports a previous conclusion by Corton et al. (2014) that the PPARα MOA is either “not relevant” or “unlikely to be relevant” in humans.

3.4. Future directions and conclusions

Overall there was agreement within the workshop that there is ample scientific support for a rodent cancer MOA and lack of human relevance for PPARα acting compounds. Alternate PPARα MOAs were considered and discussed including the impact on the proposed MOA of studies by Ito et al. (2007) and Yang et al. (2008) using genetically modified mice. Flaws in the experimental design and interpretation of the results of these studies were discussed, but it was agreed, they did not outweigh the overwhelming number of studies that supports the currently accepted PPARα MOA (Klaunig et al., 2003; Corton et al., 2014).

Additional experiments were suggested that would be helpful in further supporting the MOA. For example, the livers from PPARα-null mice treated with DEHP could be analyzed further to determine if there were augmented increases in the background steatosis and steatohepatitis, which may contribute to liver cancer induction. It would also be useful to further characterize PPARα-independent MOAs either by microarray analysis or by targeted assessment of marker genes. Although epidemiology results have consistently shown no linkage between PPARα activator exposure and liver cancer in humans, the epidemiology data has been criticized as incomplete (Guyton et al., 2009). Given advances in the ability to easily measure gene expression in the livers from formalin fixed paraffin embedded livers (Rooney et al., 2017), gene expression analysis of biopsies from the exposed patient population may help to address whether key events in the PPARα MOA were modulated. It was also suggested that the statistical power to detect small changes in liver effects in the epidemiology studies should be reviewed to address whether a signal at the potency predicted from the animal data could be detected in humans.

4. Cytotoxicity as a MOA for rodent liver tumors

Cytotoxicity, followed by regenerative cell proliferation, is a widely recognized, well characterized nongenotoxic MOA. This MOA is considered relevant to humans, but is widely recognized as having a threshold dose-response. Therefore, it does not fit well with

many hazard characterization schemes, since the carcinogenic potential of chemicals that act via this MOA varies with exposure.

Workshop presenters identified criteria for establishing a cytotoxicity MOA, which include: 1) the chemical is not DNA reactive; 2) clear evidence of cytotoxicity by histopathology, such as presence of necrosis and/or increased apoptosis; 3) evidence of toxicity by increased serum enzymes that are relevant to humans; 4) presence of increased cell proliferation as evidenced by increased labeling index and/or increased number of hepatocytes; 5) demonstration of a parallel dose response for cytotoxicity and formation of tumors; and 6) reversibility (ideally).

During the organization of the Workshop, a number of case studies were considered for nongenotoxic rodent liver carcinogens that might be acting by a MOA involving cytotoxicity and regenerative hyperplasia. Chloroform is an example of a well-studied chemical that acts through a cytotoxicity MOA (e.g., U.S. EPA, 2001). Key events in its MOA include generation of the metabolite phosgene by CYP2E1 metabolism and cytotoxicity. However, while regeneration/proliferation leading to tumor formation is known to occur in rodents, there is a lack of adequate data for these key events in human liver formation. The lack of data in humans leads to the following implications in risk assessment 1) while the cytotoxicity MOA is possible in humans, it is a high dose phenomenon; 2) the effect is threshold mediated; and 3) sustained exposure is required (Golden et al., 1997). However, finding other case studies that meet all the criteria described above, while at the same time ruling out other modes of action, was a challenge. In a review of mechanisms of non-genotoxic carcinogens, Hernandez et al. (2009) listed 18 chemicals as having a MOA involving cytotoxicity and regenerative hyperplasia. Each of these also had evidence of other contributing MOAs including endocrine modification, mitogen/tumor promotion, hyper/hypomethylation, inhibition of gap junction intercellular communication (GJICs), immunosuppression, inflammation, and induction of reactive oxygen species.

Similarly, there are many examples of nongenotoxic carcinogens for which quantitative risk assessments are based on a data supporting the assumption of a threshold, typically by application of uncertainty factors to a NOAEL (e.g., Butterworth et al., 2007 assessment of 1,4-dichlorobenzene). Many of these are based on a lack of genotoxicity and overall weight-of-evidence to conclude that linear extrapolation is not an appropriate approach to establish a risk value for these chemicals. While cytotoxicity is likely a factor for many nongenotoxic hepatocarcinogens tested in rodent bioassays at high doses, data to identify this as the MOA are often insufficient. While a threshold-based approach is accepted by some regulatory agencies, others require more data to definitively establish the MOA and it is not uncommon for nongenotoxic chemicals to be assessed using the default of linear low-dose extrapolation. This has significant implications for the determination of a human exposure limit that is considered to be acceptable.

5. Regulatory considerations

Presentations by scientists from regulatory agencies in the United States, Canada and Europe highlighted a variety of different approaches to evaluation of MOA, weighting of

uncertainties, and decision making. One key difference was between the perspective and mandate of agencies regulating pharmaceuticals and those regulating other exposures (e.g., agricultural, industrial, consumer products, food additives). Although exposures are much higher (often by many orders of magnitude) in the pharmaceutical context, the role of pharmaceuticals in treating disease underscores the goal to be predictive, not just protective in their evaluations. Many beneficial pharmaceuticals are tumorigenic in rodents, and so more detailed mechanistic research is needed for these drugs to determine whether the rodent tumors are relevant to humans. Automatically excluding such drugs could result in a net decrement to human health, due to the loss of useful treatment options. In contrast, default assumptions regarding human relevance of rodent tumors have been utilized for chemicals in other sectors.

Another difference between regulation of pharmaceuticals and other chemicals is that interaction with the regulated community is expected for pharmaceutical development and regulation, with mechanisms in place to ensure engagement. This interaction in the pharmaceutical world allows for earlier uptake in regulatory settings of advancements in animal testing that are developed by the pharmaceutical industry. For example, the ICH guidelines on carcinogenicity (ICH, 2016) currently account for the duration of exposure, causes for concern, and the nature of the patient population and clinical indication as part of a determination of whether 2-year rodent studies are necessary. A working group of the ICH, consisting of regulators and the regulated community, is now discussing expanding these considerations to include the potential for tumorigenicity in humans (and rodents), and the level of certainty regarding the determination in humans, as part of a determination of whether a 2-year rat study would add value.

Differences were noted in how MOA questions are framed, and the implications of the answers. For example, for the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) to use a threshold approach for a risk assessment, it is sufficient to conclude that a chemical is definitively shown to be not genotoxic, even if a specific MOA has not been definitively demonstrated. In contrast, other agencies, require affirmative identification of the MOA, such that the lack of genotoxicity is not sufficient to support a nonlinear or threshold-based assessment (e.g., U.S. EPA, 2005). Frameworks for evaluation of MOA have aided in making such determinations, but it is very difficult in some regulatory contexts, and very little specific guidance is available on what is needed to establish a MOA and rule out alternatives. To address this concern, there is a movement towards conducting comparative weight of evidence evaluations, rather than needing to conclusively demonstrate a specific MOA (Meek et al., 2014). An intermediate approach used by Health Canada is that one needs to show that a chemical is not DNA reactive, and to have some data supporting a potential non-genotoxic MOA, even if a specific MOA among several alternatives has not been definitively identified. EPA allows for the presentation of alternative approaches when there is support for more than one MOA (U.S. EPA, 2005); however, without additional guidance, risk managers generally default to using the most conservative approach. Several participants also raised concerns about the utility of hazard classification systems in general (e.g., Boobis et al., 2016). Classification systems typically focus on identification of potential hazards and often do not consider factors such as dose-response, human exposure levels, and MOA, all of which are important for risk assessment and risk management.

As a result, hazard classification systems can hamper effective communication with the public regarding the implications of exposures to various agents, and can present barriers to effective prioritization and risk management.

6. Risk communication

Several workshop participants noted challenges in communication, and key messages to communicate to regulatory scientists and to the public. It was noted that there is a lot of public concern about chemical exposure, which is exacerbated by the current approach of classifying chemicals as carcinogens without taking into account exposure and human relevance. Information shared with the public about chemical risk has generally not provided information about the general use of conservative assumptions, and the resulting health-protectiveness of current regulatory regimes. For example, the public is typically not aware of the large amount of testing that pesticides and pharmaceuticals undergo. Although there is scientific debate about details of the approach, including human relevance of specific endpoints, the general approaches are health protective and there is a general consensus that the current methods are protecting the public. More concerning, a focus on labeling based solely on hazard characterization is counter-productive and may cause unmerited concern from the public.

With regard to regulatory agencies, opportunities for improvement in communications in both directions were noted. Participants noted the need for regulatory agencies to be transparent on the basis for their decisions. This transparency would provide an accumulated body of experience and case studies that can help to inform decisions made by the regulated communities. Regulatory scientists noted the need for the regulated community to share data to enable analyses of the potential for use of alternative testing strategies to reduce animal use and evaluate the MOA. It was also emphasized that incorporating MOA and dose-response considerations into classification systems is critical for these systems to provide meaningful information regarding risk to humans.

7. Evolving approaches to chemical testing

A recurring theme throughout the meeting was the set of challenges presented by the current model of chemical toxicity testing, and potential ways to improve the approach to testing. A fundamental issue relates to the *goal* of chemical testing, with many current testing approaches designed to inform hazard classification systems. Several speakers and workshop participants noted the poor predictivity of the current testing paradigm, both between rodent test species and between rodents and humans, and questioned the relevance to humans of effects observed at high doses in rodent bioassays. In particular, participants noted the potential for substantial nonlinearities at high doses, due to factors such as nonlinearities in kinetics or the overwhelming of defense mechanisms. Consequences of testing to high doses include the abandonment of chemicals that might offer a benefit to society, or the need for significant follow-up to investigate the qualitative and quantitative human relevance of the observed effects, requiring additional cost in time, money and use of animals. Recommendations were made to explicitly consider the potential for human exposure and kinetics in study design to minimize the generation of non-relevant data. This

is done for pharmaceuticals, for example, by comparing effect levels with human exposures. As tools improve for estimating human exposure to other chemicals, similar approaches might be appropriate for environmental and/or consumer product exposure, although careful consideration would be needed for the tails of the exposure distribution. In considering the data needed to establish an MOA and reach a regulatory conclusion, participants noted the substantial additional work that may be needed to rule out alternative MOAs; further discussion would be useful on the amount of additional research that is needed for other potential MOAs, as well as whether such research and testing is needed for every chemical.

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Consistent with the long-standing philosophy of the Toxicology Forum, the workshop aimed to promote dialogue, and for the participants to share data. No attempt was made to bring discussions to consensus, but areas of agreement were noted, as well as priority areas for additional research. We thank the Toxicology Forum for sponsoring this Workshop, and for supporting in part the contributions to the manuscript by Lynne Haber and Angela Lynch, as well as the assistance of Toxicology Forum staff (Marguerite Leishman, Kevin Merritt and Amy Willis) for handling the meeting logistics. The authors also extend a special thanks to Dr. Todd Bourcier for his contributions to the manuscript and its review. The authors thank the Workshop Organizing Committee members (listed below) for their input in the workshop, and gratefully acknowledge the contribution of all of the presenters.

References

Aydinlik H, Nguyen TD, Moennikes O, Buchmann A, Schwarz M, 2001. Selective pressure during tumor promotion by phenobarbital leads to clonal outgrowth of beta-catenin-mutated mouse liver tumors. Oncogene 20, 7812–7816. [PubMed: 11753661]

Boobis AR, Cohen SM, Dellarco V, McGregor D, Meek M.E. (Bette), Vickers C, Willcocks D, Farland W, 2006. IPCS framework for analyzing the relevance of a cancer mode of action for humans. Crit. Rev. Toxicol. 36, 781–792. 10.1080/10408440600977677. [PubMed: 17118728]

Boobis AR, Doe JE, Heinrich-Hirsch B, Meek MEB, Munn S, Ruchirawat M, Schlatter J, Seed J, Vickers C, 2008. IPCS framework for analyzing the relevance of a noncancer mode of action for humans. Crit. Rev. Toxicol. 38, 87–96. 10.1080/10408440701749421. [PubMed: 18259981]

Boobis AR, Cohen SM, Dellarco VL, Doe JE, Fenner-Crisp PA, Moretto A, Pastoor TP, Schoeny RS, Seed JG, Wolf DC, 2016. Classification schemes for carcinogenicity based on hazard-identification have become outmoded and serve neither science nor society. Regul. Toxicol. Pharmacol. 82, 158–166. 10.1016/j.yrtph.2016.10.014. [PubMed: 27780763]

Braeuning A, Gavrilov A, Brown S, Wolf CR, Henderson CJ, Schwarz M, 2014. Phenobarbital-mediated tumor promotion in transgenic mice with humanized CAR and PXR. Toxicol. Sci. 140 (2), 259–270. [PubMed: 24863967]

Budinsky RA, Schrenk D, Simon T, Van den Berg M, Reichard JF, Silkworth JB, Aylward LL, Brix A, Gasiewicz T, Kaminski N, Perdew G, Starr TB, Walker NJ, Rowlands JC, 2014. Mode of action and dose-response framework analysis for receptor-mediated toxicity: the aryl hydrocarbon receptor as a case study. Crit. Rev. Toxicol. 44, 83–119. 10.3109/10408444.2013.835787. [PubMed: 24245878]

Butterworth BE, Aylward LL, Hays SM, 2007. A mechanism-based cancer risk assessment for 1,4-dichlorobenzene. Regul. Toxicol. Pharmacol. 49, 138–148. 10.1016/j.yrtph.2007.06.004. [PubMed: 17688981]

Cheung C, Akiyama TE, Ward JM, Nicol CJ, Feigenbaum L, Vinson C, Gonzalez FJ, 2004. Diminished hepatocellular proliferation in mice humanized for the nuclear receptor peroxisome proliferator-activated receptor alpha. Cancer Res. 64, 3849–3854. 10.1158/0008-5472.CAN-04-0322. [PubMed: 15172993]

Cohen SM, 2010. Evaluation of possible carcinogenic risk to humans based on liver tumors in rodent assays: the two-year bioassay is no longer necessary. Toxicol. Pathol. 38, 487–501. 10.1177/0192623310363813. [PubMed: 20215581]

Corton JC, Peters Jeffrey M., Klaunig James E., 2017. The PPARα-dependent rodent liver tumor response is not relevant to humans: addressing misconceptions. Archives Toxicol (in press).

Corton JC, Cunningham ML, Hummer BT, Lau C, Meek B, Peters JM, Popp JA, Rhomberg L, Seed J, Klaunig JE, 2014. Mode of action framework analysis for receptor-mediated toxicity: the peroxisome proliferator-activated receptor alpha (PPARα) as a case study. Crit. Rev. Toxicol. 44, 1–49. 10.3109/10408444.2013.835784.

Dong B, Lee J-S, Park Y-Y, Yang F, Xu G, Huang W, Finegold MJ, Moore DD, 2015. Activating CAR and β-catenin induces uncontrolled liver growth and tumorigenesis. Nat. Commun. 6, 5944. 10.1038/ncomms6944. [PubMed: 25661872]

Elcombe CR, Peffer RC, Wolf DC, Bailey J, Bars R, Bell D, Cattley RC, Ferguson SS, Geter D, Goetz A, Goodman JI, Hester S, Jacobs A, Omiecinski CJ, Schoeny R, Xie W, Lake BG, 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, 64–82. 10.3109/10408444.2013.835786. [PubMed: 24180433]

Foreman J and Peters J Unpublished data.

Golden RJ, Holm SE, Robinson DE, Julkunen PH, Reese EA, 1997. Chloroform mode of action: implications for cancer risk assessment. Regul. Toxicol. Pharmacol. 26, 142–155. 10.1006/rtph.1997.1161. [PubMed: 9356278]

Guyton KZ, Chiu WA, Bateson TF, Jinot J, Scott CS, Brown RC, Caldwell JC, 2009. A reexamination of the PPAR-alpha activation mode of action as a basis for assessing human cancer risks of environmental contaminants. Environ. Health Perspect. 117, 1664–1672. 10.1289/ehp.0900758. [PubMed: 20049115]

Hagmann M, Georgiev O, Schaffner W, 1997. The VP16 paradox: herpes simplex virus VP16 contains a long-range activation domain but within the natural multiprotein complex activates only from promoter-proximal positions. J. Virol. 71 (8), 5952–5962 [PubMed: 9223485]

Hernandez LG, van Steeg H, Luijten M, van Benthem J, 2009. Mechanisms of non-genotoxic carcinogens and importance of a weight of evidence approach. Mutat. Res. 682, 94–109. 10.1016/j.mrrev.2009.07.002. [PubMed: 19631282]

Hess R, Stäubli W, Riess W, 1965. Nature of the hepatomegalic effect produced by ethyl-chlorophenoxy-isobutyrate in the rat. Nature 208, 856–858. [PubMed: 5870099]

Holsapple MP, Pitot HC, Cohen SM, Cohen SH, Boobis AR, Klaunig JE, Pastoor T, Dellarco VL, Dragan YP, 2006. Mode of action in relevance of rodent liver tumors to human cancer risk. Toxicol. Sci. 89, 51–56. 10.1093/toxsci/kfj001. [PubMed: 16221960]

Howroyd P, Swanson C, Dunn C, Cattley RC, Corton JC, 2004. Decreased long-evity and enhancement of age-dependent lesions in mice lacking the nuclear receptor peroxisome proliferator-activated receptor alpha (PPARα). Toxicol. Pathol. 32, 591–599. 10.1080/01926230490515283. [PubMed: 15603543]

ICH, 2016. ICH guideline S1: regulatory notice on changes to core guideline on rodent carcinogenicity testing of pharmaceuticals. ICH guideline S1. EMA/CHMP/ICH/536328/2013 Rev. 1. In: International Conference on Harmonisation (ICH). Committee for Human Medicinal Products, European Medicines Agency (EMA) Available at: http://www.ema.europa.eu/docs/en_GB/document_library/Regulatory_and_procedural_guideline/2012/12/WC500136405.pdf.

Ito Y, Yamanoshita O, Asaeda N, Tagawa Y, Lee C-H, Aoyama T, Ichihara G, Furuhashi K, Kamijima M, Gonzalez FJ, Nakajima T, 2007. Di(2-ethylhexyl) phthalate induces hepatic tumorigenesis through a peroxisome proliferator-activated receptor alpha-independent pathway. J. Occup. Health 49, 172–182. [PubMed: 17575397]

Kettener NM, Voicu H, Finegold MJ, Coarfa C, Sreekumar A, Putluri N, Katchy CA, Lee C, Moore DD, Fu L, 2016. Circadian Homeostasis of liver metabolism suppresses hepatocarcinogenesis. Cancer Cell 30 (6), 909–924. 10.1016/j.ccell.2016.10.007. Epub 2016 Nov 23. [PubMed: 27889186]

Klaunig JE, Babich MA, Baetcke KP, Cook JC, Corton JC, David RM, DeLuca JG, Lai DY, McKee RH, Peters JM, Roberts RA, Fenner-Crisp PA, 2003. PPARα agonist-induced rodent tumors: modes of action and human relevance. Crit. Rev. Toxicol. 33, 655–780. 10.1080/713608372. [PubMed: 14727734]

Klaunig JE, 2012. Chapter 8: chemical carcinogenesis. In: Klaassen (Ed.), Casarett & Doull's Toxicology: the Basic Science of Poisons, eighth ed. McGraw-Hill, New York, pp. 330–390.

La Vecchia 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. 10.1097/CEJ.0b013e32836014c8. [PubMed: 23492954]

Lake BG, Price RJ, Osimitz TG, 2015. 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. 71, 829–834. 10.1002/ps.3854. [PubMed: 25045103]

Lee GH, Ooasa T, Osanai M, 1998. Mechanism of the paradoxical, inhibitory effect of phenobarbital on hepatocarcinogenesis initiated in infant B6C3F1 mice with diethylnitrosamine. Cancer Res. 58 (8), 1665–1669. [PubMed: 9563480]

Meek MEB, Palermo CM, Bachman AN, North CM, Jeffrey Lewis R, 2014. Mode of action human relevance (species concordance) framework: evolution of the Bradford Hill considerations and comparative analysis of weight of evidence. J. Appl. Toxicol. 34, 595–606. 10.1002/jat.2984. [PubMed: 24777878]

Moennikes O, Buchmann A, Romualdi A, Ott T, Werringloer J, Willecke K, Schwarz M, 2000. Lack of phenobarbital-mediated promotion of hepatocarcinogenesis in connexin32-null mice. Cancer Res. 60, 5087–5091. [PubMed: 11016633]

Morimura K, Cheung C, Ward JM, Reddy JK, Gonzalez FJ, 2006. Differential susceptibility of mice humanized for peroxisome proliferator-activated receptor alpha to Wy-14,643-induced liver tumorigenesis. Carcinogenesis 27, 1074–1080. 10.1093/carcin/bgi329. [PubMed: 16377806]

O'Brien ML, Spear BT, Glauert HP, 2005. Role of oxidative stress in peroxisome proliferator-mediated carcinogenesis. Crit. Rev. Toxicol. 35, 61–88. [PubMed: 15742903]

Qu A, Shah YM, Matsubara T, Yang Q, Gonzalez FJ, 2010. PPARalpha-dependent activation of cell cycle control and DNA repair genes in hepatic nonparenchymal cells. Toxicol. Sci. 118, 404–410. [PubMed: 20813756]

Reddy JK, Azarnoff DL, Hignite CE, 1980. Hypolipidaemic hepatic peroxisome proliferators form a novel class of chemical carcinogens. Nature 283, 397–398. [PubMed: 6766207]

Ren H, Aleksunes LM, Wood C, Vallanat B, George MH, Klaassen CD, Corton JC, 2010. Characterization of peroxisome proliferator-activated receptor alpha–independent effects of PPARalpha activators in the rodent liver: di-(2-ethylhexyl) phthalate also activates the constitutive-activated receptor. Toxicol. Sci. 113, 45–59. [PubMed: 19850644]

Rignall B, Braeuning A, Buchmann A, Schwarz M, 2011. Tumor formation in liver of conditional beta-catenin-deficient mice exposed to a diethylnitrosamine/phenobarbital tumor promotion regimen. Carcinogenesis 32, 52–57. [PubMed: 21047994]

Rooney JP, Ryan N, Chorley BN, Hester SD, Kenyon EM, Schmid JE, George BJ, Hughes MF, Sey YM, Tennant A, MacMillan DK, Simmons JE, McQueen CA, Pandiri A, Wood CE, Corton JC, 2017. Genomic effects of androstenedione and sex-specific liver cancer susceptibility in mice. Toxicol. Sci. 160, 15–29. [PubMed: 28973534]

Rosen MB, Das KP, Rooney J, Abbott B, Lau C, Corton JC, 2017. PPARα-independent transcriptional targets of perfluoroalkyl acids revealed by transcript profiling. Toxicology. 10.1016/j.tox.2017.05.013. May 27. pii: S0300–483X(17)30153–1 [Epub ahead of print].

Scheer N, Wilson ID, 2016. A comparison between genetically humanized and chimeric liver humanized mouse models for studies in drug metabolism and toxicity. Drug Discov. Today 21 (2), 250–263. [PubMed: 26360054]

Schwarz JJ, Chakraborty T, Martin J, Zhou JM, Olson EN, 1992. The basic region of myogenin cooperates with two transcription activation domains to induce muscle-specific transcription. Mol. Cell Biol. 12, 266–275. [PubMed: 1309591]

Smith JJ, Aitchison JD, 2013. Peroxisomes take shape. Nat. Rev. Mol. Cell Biol. 14, 803–817. 10.1038/nrm3700. [PubMed: 24263361]

Soldatow V, Peffer RC, Trask OJ, Cowie DE, Andersen ME, LeCluyse E, Deisenroth C, 2016. Development of an in vitro high content imaging assay for quantitative assessment of CAR-dependent mouse, rat, and human primary hepatocyte proliferation. Toxicol. Vitro 36, 224–237. 10.1016/j.tiv.2016.08.006.

Staels B, Maes M, Zambon A, 2008. Peroxisome Fibrates and future PPARα agonists in the treatment of cardiovascular disease. Nat. Clin. Pract.Cardiovasc. Med. 5, 545–553.

Stahl S, Ittrich C, Marx-Stoelting P, Koehle C, Altug-Teber O, Riess O, Bonin M, Jobst J, Kaiser S, Buchmann A, Schwarz M, 2005. Genotype-phenotype relationships in hepatocellular tumors from mice and man. Hepatology 42, 353–361. [PubMed: 15965925]

U.S. EPA, 2005. Guidelines for Carcinogen Risk Assessment. EPA/630/P-03/001B. March 2005 United States Environmental Protection Agency (U.S. EPA) Available at: http://www.epa.gov/ncea/iris/backgr-d.htm.

U.S. EPA, 2001. Toxicological Review of Chloroform (CAS No. 67–66-3). United States Environmental Protection Agency (U.S. EPA), Washington, DC Available at: https://cfpub.epa.gov/ncea/iris/iris_documents/documents/toxreviews/0025tr.pdf.

Yamada T, Okuda Y, Kushida M, Sumida K, Takeuchi H, Nagahori H, Fukuda T, Lake BG, Cohen SM, 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), 137–157. [PubMed: 25145657]

Yang Q, Nagano T, Shah Y, Cheung C, Ito S, Gonzalez FJ, 2008. The PPAR alpha-humanized mouse: a model to investigate species differences in liver toxicity mediated by PPAR alpha. Toxicol. Sci. 101, 132–139. 10.1093/toxsci/kfm206. [PubMed: 17690133]