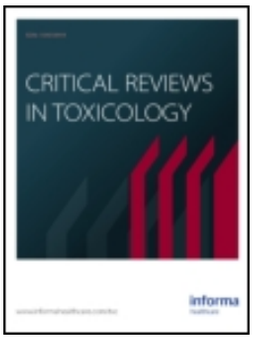

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REVIEW ARTICLE

Human relevance framework for rodent liver tumors induced by the insecticide sulfoxaflor

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Abstract

Sulfoxaflor, a novel active substance that targets sap-feeding insects, induced rodent hepatotoxicity when administered at high dietary doses. Specifically, hepatocellular adenomas and carcinomas increased after 18 months in male and female CD-1 mice at 750 and 1250 ppm, respectively, and hepatocellular adenomas increased after 2 years in male F344 rats at 500 ppm. Studies to determine the mode of action (MoA) for these liver tumors were performed in an integrated and prospective manner as part of the standard battery of toxicology studies such that the MoA data were available prior to, or by the time of, the completion of the carcinogenicity studies. Sulfoxaflor is not genotoxic and the MoA data support the following key events in the etiology of the rodent liver tumors: (1) CAR nuclear receptor activation and (2) hepatocellular proliferation. The MoA data were evaluated in a weight of evidence approach using the Bradford Hill criteria for causation and were found to align with dose and temporal concordance, biological plausibility, coherence, strength, consistency, and specificity for a CAR-mediated MoA while excluding other alternate MoAs. The available data include: activation of CAR, *Cyp2b* induction, hepatocellular hypertrophy and hyperplasia, absence of liver effects in KO mice, absence of proliferation in humanized mice, and exclusion of other possible mechanisms (e.g., genotoxicity, cytotoxicity, AhR, or PPAR activation), and indicate that the identified rodent liver tumor MoA for sulfoxaflor would not occur in humans. In this case, sulfoxaflor is considered not to be a potential human liver carcinogen.

Keywords

agrochemical, CAR, cytochrome p450, HRF, MoA, PB, pesticide, phenobarbital-like, plant protection product, rodent liver tumor

History

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Introduction

Introduction of new plant protection products (PPPs) and the active substance(s) contained within them into commerce requires a defined set of toxicology studies to assess their safety to humans and environment. The battery of mammalian toxicology studies includes acute oral/dermal toxicity, skin and eye irritation, skin sensitization, genetic toxicity, systemic toxicity, developmental and reproductive toxicity, neurotoxicity, and carcinogenicity. In addition, further studies may be undertaken to more fully evaluate a given chemical, including mode of action (MoA) and other specific studies or integrated end points. The purpose of this evaluation is to characterize the MoA in the rodent and identify the potential hazards to human health, which are the basis for human risk assessment and ultimately determine the use profile for the given PPP.

In recent years, there has been a strong movement toward a more relevant toxicity testing strategy (e.g., International Life Sciences Institute/Health and Environmental Sciences Institute—Agricultural Chemical Safety Assessment Technical

Committee [ILSI/HESI-ACSA] and new European Union Directives [1107/2009 EC]). Generally, these initiatives have been aimed at reducing the unnecessary toxicity testing; however, it is also becoming more common for PPPs to be tested beyond the "standard" requirements and incorporate investigative studies such as MoA to facilitate better characterization of the underlying hazard characteristics to inform the risk assessment and risk management decisions. The MoA/Human Relevance Framework (HRF) developed by the International Programme on Chemical Safety of the World Health Organization (Boobis et al. 2006, 2008, Sonich-Mullin et al. 2001) and ILSI (Meek et al. 2003, Seed et al. 2005) can be used as a template upon which to elucidate the human relevance of effects observed in animals. This paper, along with the companion papers (Terry et al. 2014, Rasoulpour et al. 2014, Ellis-Hutchings et al. 2014), discusses the application of the MoA/HRF approach to a recently registered active substance, sulfoxaflor (CAS# 946578-00-3; XDE-208, X11422208, XR-208, [1-(6-Trifluormethylpyridin-3-yl)ethyl)](methyl)-oxido-l^4-sulfanylidenecyanamide). The compound has broad spectrum insecticidal properties in sap-feeding pests mediated via its agonism at the highly abundant insect nicotinic acetylcholine receptor (Zhu et al. 2011). Sulfoxaflor belongs to the new sulfoximine class of compounds that are chemically and functionally distinct from the neonicotinioid class of insecticides (Watson et al. 2011).

In a series of investigative studies, interspecies comparisons of mice, rats, rabbits, and dogs indicated a similar profile of negligible metabolism of sulfoxaflor and high bioavailability when administered orally. During the conduct of initial repeat-dose toxicology studies in rodents given sulfoxaflor, treatment-related liver effects were evident, including liver hypertrophy during the initial rat palatability screen to establish the acceptable doses for future repeat-dose toxicity tests. As a result, a proactive MoA investigation was undertaken by adding molecular and apical end points into the standard toxicity testing battery, years before the liver carcinogenicity potential of sulfoxaflor was determined. In total, data from mouse and rat short-term (28-day), sub-chronic (90-day), carcinogenicity (18 months in the mouse and 24 months in the rat), as well as several short-term ($\leq$ 7 days) MoA studies were integrated and analyzed. As predicted from the knowledge gained from the MoA data, the chronic/carcinogenicity dietary studies in mice and rats identified increased incidence of hepatocellular tumors (adenomas and carcinomas). The detailed MoA evaluation responsible for the rodent liver effects, including tumors, was recently published and is the source of the primary data (LeBaron et al. 2013), although a summary of the relevant toxicity and MoA data are presented below. In this paper, the relevance to humans of the proposed MoA for rodent liver tumors was analyzed using the HRF approach (Boobis et al. 2006), and the advantages and disadvantages of this proactive MoA investigation are discussed.

Modes of action for rodent hepatic carcinogens

Most hepatocarcinogens can be classified based on their MoAs through mechanistic studies, and this information can be used in a rational evaluation in terms of their relevance to humans (Cohen 2010, Elcombe et al. 2014). The key events that lead to the development of liver cancer in rodents can be usually identified in short-term assays (i.e., 13 weeks or less) (Cohen 2010). Hence, these short-term assays can provide a detailed dose–response and mechanistic basis for the tumorigenic effect in rodents and form the basis for a rational extrapolation to possible human effects. Several MoAs have been identified for liver carcinogenesis and those applicable to the rodent model are listed in the seminal publications by Cohen (2010) and Klaunig et al. (2012). These include DNA reactive and non-DNA reactive mechanisms. Non-DNA reactive MoAs include cytotoxicity, oxidative stress/damage, inflammation, infection, or receptor-mediated. The receptor-mediated MoAs include hormone-mediated (including estrogen) or other nuclear receptor-mediated effects such as aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR), or peroxisome proliferator-activated receptor-alpha (PPAR-α).

Key events for chemicals acting through the CAR

Activation of nuclear receptors in the rodent liver, which has been traditionally associated with induction of cytochrome p450 (i.e., Cyp) enzymes, is a well-known MoA for rodent hepatocarcinogenesis with the non-genotoxic tumor promoter phenobarbital (PB) as a standard example (Whysner et al. 1996, Holsapple et al. 2006). More specifically, the MoA for CAR-mediated effects includes the following key events: (1) activation of CAR leading to Cyp isozyme induction, (2) increased hepatocellular proliferation, and (3) subsequent induction of proliferative lesions in the liver including hepatocellular foci, adenomas, and carcinomas (Cohen 2010, Elcombe et al. 2014). There is uncertainty as to whether the Cyp induction is a critical step (necessary) or an indicator of chemical activity that is secondary to activation of CAR (associative). Activation of rodent CAR produces a cascade of alterations in the liver including gene transcription and increased hepatocellular proliferation in rodents, a critical event in the development of liver tumors (Whysner et al. 1996, Cohen 2010, Elcombe et al. 2014). In humans, PB results in activation of CAR and PXR leading to the induction of Cyp enzymes as in rodents; however, a different response is induced in humans compared to that of rodents (Lambert et al. 2009) and, importantly, there is no evidence of increased hepatocellular proliferation. Extensive human epidemiologic studies at PB exposure levels similar to those used in rodent bioassays did not reveal increased cancer risks (Whysner et al. 1996, Lamminpaa et al. 2002). Based on this assessment, PB is not considered to be a hepatocarcinogen in humans. Therefore, a non-genotoxic hepatocarcinogenic response in rodents due to a CAR-mediated MoA is considered to be not relevant to humans (Holsapple et al. 2006, Elcombe et al. 2014, Cohen 2010).

The important role of CAR in PB-induced liver tumor formation in rodents has been demonstrated in genetically engineered mice lacking this nuclear receptor (reviewed in Lake 2009). In CAR knockout (CARKO) mice, PB exposure does not induce Cyp2b forms or liver enzyme activity, increase liver weight, or stimulate replicative DNA synthesis. Also, no liver tumors were observed in CARKO mice following initiation with diethylnitrosamine (DEN) and promotion with PB (Yamamoto et al. 2004, Huang et al. 2005). The data requirements

for assessment of the CAR activation MoA (Cohen 2010, Elcombe et al. 2014) are described in detail below and are also summarized in Table 1.

Key event #1: CAR receptor activation. Activation of CAR has been shown to occur by two independent mechanisms: (1) direct agonism by a ligand such as TCPOBOP (1,4-bis [2-(3,5-dichloropyridyloxy)]benzene), or (2) indirectly by compounds such as PB that activate CAR through a phosphorylation-dependent mechanism (Rencurel et al. 2006). Regardless of the means of CAR activation, the key events related to the MoA are identical. Therefore CAR activation, and not necessarily direct binding, is the most appropriate first key event.

A sensitive, specific, and appropriate biomarker of CAR activation is the induction of *Cyp2b*-family transcript (i.e., *Cyp2b10* in mice and *Cyp2b1* in rats) (Ueda et al. 2002). Associative, supportive evidence that can aid in substantiation of CAR activation (and hence a CAR-mediated MoA) includes liver weight increases accompanied with microscopic hepatocellular hypertrophy and is commonly seen following exposure to PB-like compounds. PB-induced liver hypertrophy is normally observed in the centrilobular region of the liver lobule, although some related compounds may produce either a diffuse hypertrophy or hypertrophy in other regions of the liver lobule (Lake 2009). Specifically, morphological features of enzyme induction in mice and rats can be characterized using light microscopy and/or increased smooth endoplasmic reticulum using electron microscopy. These changes are readily reversible upon discontinuance of administration of the chemical.

Key event #2: increased hepatocellular proliferation. The second key event is demonstration of an increase in proliferation of hepatocytes, often measured using increased replicative DNA synthesis. Typically for most CAR-activating compounds, the increase in hepatocyte labeling index appears very quickly, usually within 1–2 weeks of treatment initiation and the index returns to normal by four weeks of administration; however, a PB-induced increase in hepatocellular proliferation in mice was more prolonged than in rats (Kolaja et al. 1996a). Although the hepatocyte-labeling index returns to control levels even with sustained PB treatment, the livers of treated animals remain enlarged and stereologic studies indicate that hepatocellular proliferation is still enhanced due to the increase in the total number of hepatocytes per animal (Lake 2009).

Apical end point/Key event #3: hepatocellular tumors. A large number of chemicals have been shown to induce rodent liver tumors (reviewed in [Lake 2009, Cohen 2010], and references therein) and while the biological effect (i.e., hepatic adenomas and/or carcinomas) is equivalent, detailed mechanistic investigations have established genotoxic and non-genotoxic MoAs that can be applied to the pathogenesis of hepatocellular tumors.

Other potential key events associated with CAR activation.

Hepatocellular foci. Increased hepatocellular proliferation is a key event for a CAR-mediated MoA for hepatocellular carcinogens. The increased hepatocellular proliferation leads to the induction of proliferative lesions in the liver, including foci, adenomas, and carcinomas (Cohen 2010). The progression from foci of altered cells (preneoplastic foci) to hepatocellular adenomas/carcinomas following CAR activation is well documented in rodents (Whysner et al. 1996). For example, PB administration results in a dose-dependent increase in cell proliferation within foci that is associated with the ability of foci to progress to hepatocellular adenomas (Klaunig 1993). Although development of altered hepatic foci is sometimes listed as a key event for CAR-mediated MoAs, the foci are a reflection of the hepatocellular proliferation that is the actual key event that leads to selective clonal expansion resulting in the formation of microscopic hepatocellular foci and the subsequent development of adenomas and/or carcinomas. The data for sulfoxaflor-related hepatic foci in mice indicated eosinophilic foci were increased only at the carcinogenic dose of 750 ppm (i.e., 3, 2, 3, and 10 out of 50 animals in the controls, 25, 100, and 750 ppm groups, respectively), but the incidence of foci was not altered in rats (data not shown). The recent publication by Cohen (2010) does not include hepatocellular foci as a specific key event in the data necessary to support a CAR-mediated MoA, although it is characterized as a key event in Elcombe et al. (2014).

Inhibition of apoptosis. Inhibition of apoptosis, which is sometimes listed as a separate key event for a CAR-mediated MoA, primarily pertains to the tumorigenic progression of preneoplastic hepatocytes within foci of altered cells rather than other liver tissue (non-focal hepatocytes) (Schulte-Hermann et al. 1989, 1990, Kolaja et al. 1996b, Whysner et al. 1996, Lake 2009). The data for inhibition of apoptosis in foci of altered cells are primarily derived from initiation-promotion experiments rather than from studies with promoters such as PB alone. PB promotes growth of foci through inhibition of cell loss due to apoptosis and to phenotypic remodeling (Schulte-Hermann et al. 1990). The frequency of apoptosis in foci is enhanced after PB withdrawal. Although Goldsworthy and Fransson-Steen (2002) identified occasional extrafocal apoptotic bodies (i.e., not within foci of altered cells) in mice treated with DEN and/or PB, these apoptotic bodies were limited to the adjacent area surrounding foci of altered cells. Thus,

Table 1. Key events of rodent CAR-mediated liver tumor MoA.

Key event*	Biomarkers and associated supportive evidence*
(1) CAR activation	Induction of liver Cyp genes/proteins Increased liver weight Microscopic hepatocellular hypertrophy/cytoplasmic eosinophilia Reversibility after cessation of treatment
(2) Increased hepatocellular proliferation	Reversibility after cessation of treatment
(3) Hepatocellular tumors	

*Adapted from (Holsapple et al. 2006, Cohen 2010, Elcombe et al. 2014).

there should be no expectation that standard regulatory toxicity studies in rodents would detect CAR-mediated inhibition of apoptosis unless a specific analysis of foci, which typically develop relatively late in long-term studies, was conducted. Also, short-term mechanistic studies in mice with PB or CAR-associated compounds typically do not develop hepatocellular foci for months (Goldsworthy and Fransson-Steen 2002). Studies in mice initiated with DEN followed by promotion with PB suggest that cell proliferation has a major role in foci growth and that inhibition of apoptosis is only a minor determinant of tumor promotion (Goldsworthy and Fransson-Steen 2002, Bursch et al. 2005). While inhibition of apoptosis may contribute to the tumorigenic process in the liver, along with other key events, characterizing the biological significance of this relatively rare event (by current methodologies) is challenging (Elcombe et al. 2014). The recent publication by Cohen (2010) does not include inhibition of apoptosis as a specific key event in the data necessary to support a CAR-mediated MoA, and consistent with that, Elcombe et al. (2014) identifies the role of inhibition of apoptosis as an associated event in CAR-mediated liver toxicity, but not as a key event.

Discussion

Summary of sulfoxaflor rodent liver tumor MoA data

The relevant experimental data for evaluation of the sulfoxaflor rodent liver tumor MoA and human relevance includes standard, repeat-dose mouse and rat studies (4- and 13-week; 18- and 24-month), as well as specifically designed rat and mouse MoA studies. These studies are presented in detail in LeBaron et al. (2013) and are briefly summarized here. Although liver tumors were seen in both sexes of mice and in male rats, the data presented will be limited to males, as male mice and rats were more sensitive to the liver effects of sulfoxaflor. Furthermore, a mechanistic evaluation using genetically engineered mouse models (i.e., knock-out [CARKO/PXRKO] and humanized, knock-in CAR/PXR [hCAR/hPXR]) was performed in male mice to analyze the molecular response in those animals.

The MoA for sulfoxaflor-induced rodent liver tumors is comparable to the MoA for CAR-mediated rodent liver tumors. The relevant molecular and pathological end points for sulfoxaflor-induced liver effects in mice and rats are summarized in Tables 2 and 3, respectively. The tables are organized such that the metrics are consistent with the established key events and framework for characterizing the MoA of nuclear receptor-mediated rodent hepatocarcinogenesis (Lake 2009, Cohen 2010, Elcombe et al. 2014). The data presented herein represent a practical application (i.e., a case-study) of the previously constructed frameworks using sulfoxaflor-mediated rodent liver effects.

Key event #1 for the sulfoxaflor-induced liver tumor MoA is defined as activation of the CAR nuclear receptor, which is surrogately measured using liver-specific induction of the specific Cyp2b cytochrome biomarker (*Cyp2b10* in mice and *Cyp2b1* in rats) gene, protein, or correlative 7-Pentoxy-Resofufin-O-Deethylation (PROD)/7-Benzyloxy-Resorufin-O-Deethylation (BROD) enzymatic metabolic activity. As detailed in LeBaron et al. (2013) and qualitatively summarized in Table 2, in mice exposed to a tumorigenic dose of 750 ppm liver gene expression analysis indicated a clear, CAR-mediated response to sulfoxaflor (*Cyp2b10*; 54.8-fold or 56.5-fold after 7 or 90 days of treatment, respectively), with a minor contribution of PXR (*Cyp3a11*; 2.7-fold or 2.8-fold after 7 or 90 days of treatment, respectively). Similar CAR activation was noted at 500 ppm after 7 days (42.1-fold) and 300 ppm after 28 days (61.7-fold). In CARKO/PXRKO animals treated with the same carcinogenic dietary concentration of sulfoxaflor (750 ppm), no evidence of CAR (or PXR) activation was noted, either through gene expression or liver enzyme activity. Importantly, as detailed in LeBaron et al. (2013), a molecular comparison of the CD-1 and C57/Bl6 mouse strains demonstrated similar responsiveness to sulfoxaflor as these strains are known to be differentially responsive to CAR activators. In animals with hCAR/hPXR, sulfoxaflor-treatment induced quantitatively less CAR activation compared to wild-type mice; however, the PXR-associated response in hCAR/hPXR

Table 2. Sulfoxaflor: temporality and dose response for MoA key events related to CD-1 male mouse liver tumors.

		Temporal →			
		Key event #1 CAR activation		Key event #2	
	Dose (ppm)	Biomarker Cyp2b10 transcripts and protein 7–90 days	Associated event: increased liver weights/hypertrophy 7–90 days	Hepatocellular proliferation 7 days	Increased hepatocellular tumors 18 months
Dose	25				–
	100		–		–
	300	+	–		
	500	+	–	+	
	750	+	+	+	+
	≥ 1250*		+		
	750 KO	–	–	–	
	750 HU	+	+	–	

KO – CAR/PXR knock-out C57Bl6 mouse model, HU – CAR/PXR humanized knock-in C57Bl6 mouse model.
+ indicates effect present.
– indicates effect absent at indicated duration of treatment.
Blank cell = no data.
*Includes similar results from 1500 ppm and 3500 ppm.

Table 3. Sulfoxaflor: temporality, dose response, and reversibility for MoA key events related to male F344 rat liver tumors.

		Temporal →				
		Key event #1 CAR activation		Key event #2		
	Dose (ppm)	Biomarker Cyp2b10 transcripts and protein 3–7 days	Associated increased liver weights/hypertrophy 7–90 days	Hepatocellular proliferation 7 days	Associated liver hypertrophy after recovery 90 days Plus 28 days recovery	Increased hepatocellular tumors 2 years
Dose	25					–
	100	–	–	–		–
	500					+
	750	+	+	+		
	1000		+			
	1500	+	+	+	–	
	2000		+			

+ indicates effect present.
− indicates effect absent at indicated duration of treatment.
Blank cell = no data.

mice was equal to or greater than the induction noted in wild-type mice.

As in mice, rats showed a similar CAR-mediated response after dietary administration of sulfoxaflor. At dietary concentrations equivalent to a non-hepatotumorigenic dose (100 ppm), no significant induction of CAR-mediated, Cyp2b-associated response was observed; however, at doses at or above 750 ppm, clear induction of CAR-mediated transcription was seen (Table 3). This response occurred at concentrations only slightly higher than the rat liver carcinogenic dietary concentration of 500 ppm. In summary, the data for sulfoxaflor-induced, CAR-mediated liver effects in both mice and rats were consistent with the response seen with other CAR-inducers and are consistent with the first key event in CAR-mediated liver tumorigenesis.

Although liver weight increases accompanied with microscopic hepatocellular hypertrophy do not constitute direct evidence of causality of CAR-mediated hepatic effects, it can provide associative support of a CAR-mediated MoA and is commonly seen following exposure to PB-like xenobiotic compounds. In mice exposed to non-tumorigenic concentrations of sulfoxaflor, no increase in liver weight or histopathologic identification of centrilobular hypertrophy was identified (Table 2). Conversely, when sulfoxaflor was administered at and above dietary concentrations that caused liver tumors ($\geq$750 ppm), a clear dose-responsive increase in liver weight and hypertrophy were identified. These hepatic hypertrophic effects were also seen in the hCAR/hPXR mice, but not in the CARKO/PXRKO mice, further supporting the molecular and phenotypic basis for CAR-mediated, liver effects of sulfoxaflor.

In rats, similar to mice, no hypertrophic effects of the liver were noted in animals treated with less than a tumorigenic dietary concentration of sulfoxaflor (500 ppm). At doses above 500 ppm, however, a dose-responsive increase in liver weight and hypertrophy were demonstrated. Importantly, reversibility of hepatic hypertrophy (both liver weight and microscopic) was demonstrated in rats by a 90-day treatment with 1500 ppm sulfoxaflor, followed by a 28-day recovery period on control diet. The hepatic hypertrophic effects seen in both mice and rats following sulfoxaflor treatment are considered associative, supporting effects of the initial key event of CAR receptor activation for the MoA of CAR-mediated rodent liver tumorigenesis.

Key event #2 is an increase in hepatocellular proliferation and with sulfoxaflor this effect was seen in mice (both CD-1 and C57Bl6 strains) and rats. In the CD-1 male mouse, clear increases in hepatocellular proliferation were noted at the tumorigenic concentration of 750 ppm sulfoxaflor after 7 days of administration, along with a similar response at a slightly lower concentration (500 ppm). Consistent with the known proliferative profile of PB (reviewed in Cohen 2010), prolonged administration (28–90 days) of tumorigenic concentrations of sulfoxaflor resulted in a return to "normal" levels of hepatocellular proliferation as measured by a labeling index (LeBaron et al. 2013). Important mechanistic information was elicited with the use of genetically engineered mouse models in that no sulfoxaflor-induced hepatocellular proliferation was identified in either the CARKO/PXRKO or hCAR/hPXR mice (Table 2).

In the rat, 7 days of sulfoxaflor treatment induced a dose-responsive increase in hepatocellular proliferation, as measured by Ki-67 staining. While administration of the non-tumorigenic concentration of 100 ppm did not result in a significant increase in proliferation, a tumorigenic concentration of 750 or 1500 ppm in the diet clearly increased the proliferative response of hepatocytes (Table 3).

When analyzed in total, the key events for sulfoxaflor show clear, thresholded, dose-responsive alterations and provide informative, temporal-specific characterization of sulfoxaflor-induced liver effects. Importantly, this also includes the induction of rodent hepatic tumors. These key events are consistent with a CAR-mediated MoA and provide convincing evidence that the key events for a CAR-mediated MoA do not occur at sub-hepatotumorigenic doses of sulfoxaflor in the rodent, but are supported at the hepatotumorigenic doses of 750 ppm (time weighted average of 79.6 mg/kg body weight/day (mkd) in the 18-month study) in the mouse or 500 ppm (time weighted average of 21.3 mkd in the 24-month study) in the rat.

Strength, consistency, and specificity of association of effects with key events

Activation of nuclear receptors in the rodent liver, which is commonly associated with subsequent induction of

cytochrome p450 enzymes, is a well-known MoA for rodent hepatocarcinogens, and PB is a standard example of a CAR-mediated cytochrome inducer. The key events for this MoA are: (1) CAR activation, with associated Cyp isozyme induction and (2) an increase in hepatocellular proliferation that results in subsequent foci, adenomas, and carcinomas. In addition to these key events in the pathogenesis of hepatocellular tumors in rodents, reversibility of hepatic effects upon discontinuance of treatment is considered as the necessary data to support this MoA.

When taken together the MoA and repeat-dose toxicity studies for both mice and rats described herein clearly demonstrate a robust and dose-related, sulfoxaflor-mediated increase in the CAR-associated biomarkers (i.e., specific *Cyp2b* transcripts and associated increases in Cyp2b protein [*Cyp2b10* in mice and *Cyp2b1* in rats] and enzymatic activity [PROD/BROD]). These results are consistent with the activation of the CAR nuclear receptor. In addition, analysis of hepatocellular proliferation indicates a clear, thresholded, dose-related induction of S-phase DNA synthesis. Both of these key events were demonstrated to be dependent upon the activation of the CAR nuclear receptor using the genetically modified mouse models (i.e., CARKO/PXRKO), where no CAR activation (as measured using the biomarkers of gene or protein expression of Cyp2b10) or increase in hepatocellular proliferation was noted at a carcinogenic dose level of 750 ppm. Furthermore, the gross and microscopic hypertrophic effects of sulfoxaflor on the liver were reversible upon removal of sulfoxaflor administration; however, a more complete evaluation of the reversibility (e.g., apical and molecular characterization of the short-term alterations and reversibility in the MoA studies) could have been undertaken. Lastly, the *Cyp2b*/CAR-associated gene expression and protein data from these MoA experiments in both mice and rats define a very specific sulfoxaflor MoA while simultaneously rule out other nuclear receptor-mediated MoAs for rodent hepatic carcinogens such as PPAR-α or AhR activation (as shown in LeBaron et al. 2013).

As previously summarized in the analysis of the MoA, sulfoxaflor repeat-dose dietary studies in mice and rats over a range of study durations and dose levels demonstrate a consistent dose and time association to the key events based on the CAR-mediated biomarkers, liver weights, microscopic hepatocellular hypertrophy, and hepatocellular proliferation data. Ultimately, taking into consideration both the mouse and rat cancer studies, as well as the short-term studies, it is clear that non-tumorigenic doses of sulfoxaflor were not associated with CAR activation, hypertrophy, hyperplasia, or hepatocellular tumors while higher dose levels (>100 ppm in the mouse and rat) resulted in the relevant key events and eventual hepatocellular tumors. Overall the studies conducted with sulfoxaflor provide a strong and consistent association with sulfoxaflor-induced key events and a specific MoA that results in rodent hepatocellular tumors.

Biological plausibility and coherence

Dietary administration of sulfoxaflor to mice and rats results in the early key events (CAR activation and hepatocellular proliferation) and eventually result in hepatocellular tumors after prolonged exposure to high dose levels of sulfoxaflor. The early key events associated with hepatocellular hypertrophy are reversible upon cessation of treatment with sulfoxaflor. The MoA demonstrated for sulfoxaflor is consistent with the well-known MoA for CAR activation in rodents and the MoA is consistent with current understanding of cancer biology and nuclear receptor-mediated carcinogenesis.

In addition, the specificity for the MoA was demonstrated for sulfoxaflor using genetically engineered mouse models. As previously described, the CARKO/PXRKO mice were refractory to the sulfoxaflor-induced hepatic effects. Moreover, and most importantly, humanized (hCAR/hPXR) mice demonstrated a similar, although quantitatively less, response for most end points directly associated with CAR activation, but no increase in hepatocellular proliferation was noted. These data are consistent with the known MoA for CAR activators, and is considered to be supportive of why humans are refractory to the hepatotumorigenic effects of PB.

Other possible MoAs for sulfoxaflor-induced rodent liver tumors

As previously discussed, the MoAs for hepatocellular carcinogenesis are broadly categorized as DNA reactivity or increased cell proliferation (i.e., mitogenic, which can be subcategorized as either receptor- or non-receptor–mediated) (Cohen 2010). These alternative MoA are summarized in Table 4 with respect

Table 4. Summary evaluation for alternative rodent liver MoAs.

	DNA reactivity	AhR or PPARα activation	Cytotoxicity (28–90 days)	Increased apoptosis	Estrogens, statins, metals, infectious
Strength of association	− Ames − *in vitro* mammalian chrom. abs and gene mutation − *in vivo* micronuclei	− Targeted gene expression	+ Ind. cell necrosis, ↑ AST, ALT in some studies	− No histopath evidence	−
Consistency of association	−	−	−	−	−
Specificity of association	−	−	−	−	−
Dose-response concordance	− No tumors at lower doses in mice or rats	−	+/− Ind. cell necrosis at high doses, but not at tumorigenic levels	−	−
Temporal relationship	− Late onset tumors	−	−	−	−
Coherence and plausibility	Not plausible; − Coherence	Not plausible; − Coherence	Plausible; − Coherence	Not plausible; − Coherence	Not plausible; − Coherence

+ indicates attribute present.
− indicates attribute absent.
+/− indicates equivocal.
MoA adapted from (Holsapple et al. 2006, Cohen 2010, Elcombe et al. 2014).

to strength, consistency, and specificity of association, dose–response concordance, temporal relationship, and coherence and plausibility.

DNA reactivity

DNA reactivity is a broad category of a MoA for hepatocellular carcinogens. A battery of *in vitro* genotoxicity studies demonstrated that sulfoxaflor does not cause gene mutations or chromosome aberrations (summarized in Table 4; i.e., the bacterial reverse mutation test [Ames test], an *in vitro* mammalian cell gene mutation test, and an *in vitro* mammalian cell chromosome aberration test, all conducted both in the absence and presence of a metabolic activation system [rat liver S9]). Additionally, an *in vivo* mouse micronucleus assay demonstrated that sulfoxaflor does not induce micronuclei in somatic cells. An evaluation of the genetic toxicity data for sulfoxaflor unequivocally supports no DNA reactivity and hence is not a potential MoA for the induction of hepatocellular tumors in mice and rats. A thorough summary of the genetic toxicity data is presented in the companion manuscript (Rasoulpour 2014). The lack of genotoxicity for sulfoxaflor administration was also supported by the regulatory reviews, including: "...no evidence from a comprehensive battery of genotoxicity assays of any mutagenic, clastogenic, aneugenic or DNA reactive activity of sulfoxaflor. Based on the lack of genotoxicity in the available studies, a mutagenic MoA is not supported..." (USEPA 2012). The Joint Meeting (FAO/WHO) on Pesticide Residues indicated no genotoxic potential was demonstrated for sulfoxaflor and the available evidence indicates that genotoxicity is not an alternative MoA (JMPR 2011). The EU DAR concluded, "...a complete lack of genotoxicity seen with sulfoxaflor in *in vitro* and *in vivo* studies..." (EU 2012).

Receptor-mediated, increased cell proliferation

MoAs for hepatocellular carcinogens that cause receptor-mediated hepatocellular proliferation include AhR, CAR, PXR, and PPAR-α activation, as well as estrogens and statins (Cohen 2010). The MoA studies in mice and rats with sulfoxaflor clearly demonstrate a specific, dose-related increase in the biomarkers associated with CAR activation. Furthermore, CARKO/PXRKO animals further supported specificity for the sulfoxaflor-induced activation of the CAR/PXR receptor. Taken together, these findings are consistent with activation of the CAR/PXR nuclear receptors in the liver by sulfoxaflor. At the same time, the MoA studies in mice and rats ruled out AhR- and PPAR-α-mediated nuclear receptor-mediated MoAs (LeBaron et al. 2013).

Estrogens have a specific receptor-mediated MoA that results in cell proliferation in tissues including the liver; however, the carcinogenic activity may be due to an interaction of DNA adduct formation with increased cell proliferation (dual MoA) (Cohen 2010). Sulfoxaflor is not likely to have an estrogenic MoA based on structural dissimilarity to estrogens and, in addition, there was no evidence of estrogenic activity from a definitive two-generation toxicity study in the rat or *in vitro* estrogen binding or transcriptional activation assays (reviewed in the companion manuscript Rasoulpour 2014). This conclusion was supported by the conclusions of JMPR (2011) and EU (2012).

Statins act through inhibition of a specific enzyme, HMG-CoA-reductase, which leads to marked reduction in cholesterol production in humans (Endo et al. 1979). Statins increase hepatocellular proliferation and hepatocellular tumors in rodents (MacDonald et al. 1988, MacDonald and Halleck 2004); however, statins do not decrease serum cholesterol in rodents, and the MoA for statins is presumably due to an actual increase in liver HMG-CoA-reductase. Epidemiologic evidence in human patients demonstrates that statins are not associated with an increase in liver or other tumors (Farwell et al. 2008). Thus, a statin MoA in rodents appears to be irrelevant to human carcinogenesis based on understanding the mechanism and extensive epidemiologic evidence (Cohen 2010). Serum clinical chemistry values in rodents indicated that treatment with high doses of sulfoxaflor increased cholesterol. Although various classes of statins act differently, a common response is an increase in *Cyp2b* and *Cyp4a* gene expression and protein levels in the rodent liver (Kocarek and Reddy 1996), whereas sulfoxaflor did not exhibit any induction in *Cyp4a* transcript levels, and in many cases it decreased (LeBaron et al. 2013). Overall, the lack of concordant localized periportal atypia and bile duct hyperplasia indicate the hepatic effects seen with sulfoxaflor administration are not consistent with known effects of statins in the rodent liver.

Non-receptor–mediated, increased cell proliferation

MoAs for hepatocellular carcinogens that cause non-receptor–mediated increased cell proliferation include cytotoxicity, infection, iron (copper) overload, and increased apoptosis (Cohen 2010). Cytotoxicity is unlikely to be a relevant MoA for sulfoxaflor as relevant toxicity data from numerous repeat-dose toxicity studies indicated a lack of treatment-related necrosis and necrosis-related end points (e.g., alanine aminotransferase [ALT], aspartate aminotransferase [AST], alkaline phosphatase [ALP]; data not shown). Moreover, in the studies that did demonstrate notable individual cell necrosis and appreciable elevations in ALT/AST, the dose levels, where these effects occurred, were at much higher levels than those that were associated with liver tumorigenesis. Furthermore, elevations in ALT/AST were associated with the 28- and 90-day studies in mice, whereas ALT/AST elevations were not noted in the rat repeat-dose studies. Taken together, the weight of evidence does not support a consistent association of cytotoxicity/necrosis in sulfoxaflor-treated animals with eventual hepatocellular tumors.

As hepatic cytotoxicity was not evident, several specific regenerative proliferative MoA can be excluded. An infectious MoA is not relevant for sulfoxaflor based on biological and histopathological evaluations, nor did sulfoxaflor increase iron or copper accumulation in the liver (based on standard histopathological hematoxylin and eosin evaluation). Although no specific investigation of metal content of the liver was undertaken, the lack of concordance of measurable cytotoxicity at the tumorigenic dose levels indicates that regenerative proliferation (via infection or metal accumulation) is not the operant MoA. In addition, sulfoxaflor did not increase apoptosis in any of the previously described toxicity studies, although no direct

measure was undertaken beyond the standard histopathological analysis. Thus, the category of increased apoptosis resulting in hepatotumorigenesis for sulfoxaflor is not likely.

A summary evaluation for other possible MoAs for the sulfoxaflor-induced rodent liver tumors is presented in Table 4. Evaluation of the data for sulfoxaflor indicates these alternative possible MoAs are not likely to be relevant.

Identification of data gaps

Male mice and rats were more sensitive to the hepatic effects of sulfoxaflor and, hence, most of the mechanistic evaluations were performed in male mice and rats, including the studies with genetically engineered mouse models. Accordingly, the MoA/HRF evaluation described herein and in LeBaron et al. (2013) focused on the evaluation of the MoA in male mice and rats, although hepatocellular tumors were also identified in female mice treated with a dietary concentration of 1250 ppm for 18 months. Histopathological examination of the liver of female mice at dose levels with hepatocellular tumors (and of liver tissue in the shorter duration studies) revealed a phenotype consistent with that identified in males. While inclusion of females in the MoA studies and MoA evaluation may have been informative, the MoA data provide compelling evidence that the sulfoxaflor liver tumor MoA is not sex specific. In addition, restricting the MoA investigations to the more sensitive sex significantly reduced the number of animals used for the MoA studies.

Reversibility of sulfoxaflor-induced hepatic effects was investigated in a standard, repeat-dose 90-day rat toxicity study. In that study, animals administered the top dietary concentration of 1500 ppm (i.e., 3-fold greater than the hepatocellular carcinogenic dose level in the 2-year rat study) for 90 days had a relative liver weight increase of 41% with clear microscopic hepatocellular hypertrophy identified (LeBaron et al. 2013). A subset of these animals were then subsequently switched to control diet for an additional 28 days and the data indicated those animals did not have significantly increased relative liver weights or microscopic hepatocellular hypertrophy compared to control. As previously described, a complete evaluation of the molecular reversibility for sulfoxaflor-induced hepatic effects across all MoA studies was not undertaken in an effort to restrict animal usage, as the more definitive experiment for specificity of sulfoxaflor-induced liver effects was demonstrated with the use of CARKO/PXRKO (knockout) and hCAR/hPXR (humanized) mice.

In mice, a further characterization of the biomarkers of CAR activation and proliferation (i.e., the early key events) could have been undertaken at non-tumorigenic doses of 25 and 100 ppm (Table 2). While the data from the CARKO/PXRKO mice clearly linked the hepatic effect with CAR activation, further demonstration of the threshold nature of CAR activation would have supported the tumor response data. Likewise, key event characterization of the carcinogenic dose in rats (500 ppm) would have more explicitly defined the MoA versus the analysis performed at 750 ppm (Table 3). In both of these instances, the prospective nature of the integrated MoA investigation for sulfoxaflor-related hepatic effects and efficient utilization of animals influenced the study design and MoA analysis. The investigative studies into the MoA were preformed prior to completion of, and hence identification of, hepatic tumors in the bioassays.

Assessment of postulated sulfoxaflor mouse and rat liver tumor MoA

The data for sulfoxaflor support a direct, threshold-based, dose-responsive MoA for hepatocellular adenomas and carcinomas in mice and rats. The MoA demonstrated for sulfoxaflor is consistent with the well-known MoA for CAR activation in rodents and the MoA is consistent with the current understanding of cancer biology and nuclear receptor-mediated carcinogenesis. These MoA data for sulfoxaflor are judged with a high degree of confidence to adequately explain the development of hepatocellular tumors in mice and rats following chronic dietary administration of sulfoxaflor. The sulfoxaflor MoA analysis is summarized in Table 5 in terms of the criteria for the HRF.

Sulfoxaflor rodent liver tumor HRF

Question 1. Is the weight of evidence sufficient to establish the MoA in animals?

As described throughout and in the MoA publication (LeBaron et al. 2013), the data support a CAR-mediated MoA for sulfoxaflor-induced mouse and rat liver tumors, and is compatible with that described for PB-induced rodent liver tumors. The relevant molecular and pathological end points for sulfoxaflor-induced liver effects in mice and rats are supported by sulfoxaflor repeat-dose mouse and rat studies, and there is good correlation for the dose–response between the MoA data and the hepatocellular tumors. Critically, the MoA of sulfoxaflor-mediated hepatic effects and key events demonstrated dependence on rodent CAR/PXR involvement, as CARKO/PXRKO or hCAR/hPXR animals did not respond similarly to sulfoxaflor as wild type mice. When all the mechanistic and standard studies for sulfoxaflor are analyzed, the key events show clear, thresholded, dose-responsive alterations and are consistent with a CAR-mediated MoA. In addition, other possible MoAs were examined and evaluated to be unlikely based on analysis of the relevant data for sulfoxaflor.

Question 2. Can human relevance of the MoA be reasonably excluded based on fundamental qualitative differences in key events between experimental animals and humans?

The answer is yes. Activation of the CAR nuclear receptor in the rodent liver is a well-known and accepted

Table 5. Concordance analysis of sulfoxaflor rodent liver tumor CAR MoA.

Key event #1: CAR receptor activation Cyp2b biomarker induction Associated liver hypertrophy Key event #2: Hepatocellular proliferation Reversible effects (including specific knockout and humanized mouse data) Increased hepatocellular tumors	
Strength of association	+
Consistency of association	+
Specificity of association	+
Dose-response concordance	+
Temporal relationship	+
Coherence and plausibility	+ Plausible; + Coherence

MoA reviewed in Holsapple et al. (2006), Cohen (2010), Elcombe et al. (2014) and primary references therein.

MoA for rodent hepatocarcinogenesis. The key events in CAR-mediated hepatocellular carcinogenesis include activation of CAR (as measured using induction of Cyp2b isoforms), leading to increased hepatocellular proliferation with subsequent induction of proliferative lesions in the liver including foci, adenomas, and carcinomas. On the other hand, although PB in humans results in activation of CAR and PXR leading to the induction of Cyp enzymes, a different response is induced in humans compared to that of rodents (Lambert et al. 2009) and, importantly, there is no evidence of increased hepatocellular proliferation in humans or primary human hepatocytes *in vitro* (reviewed in Lake 2009, Elcombe et al. 2014). This finding was reinforced in the course of these studies with sulfoxaflor, where humanized CAR/PXR knock-in mice were refractory to the hepatocellular proliferative effect of sulfoxaflor, whereas wild-type mice demonstrated increased proliferation. Extensive epidemiologic studies in humans exposed to levels of PB comparable to those in rodent bioassays did not find an increased risk of cancer (Whysner et al. 1996, Lamminpaa et al. 2002). Based on the MoA assessment, PB is not a hepatocarcinogen in humans. Furthermore, a hepatocarcinogenic response in rodents for compounds that have data to support a CAR-mediated, PB-like, MoA is not relevant to humans. On this basis, the rodent liver tumors associated with administration of high dose levels of sulfoxaflor would not pose a cancer hazard to humans.

Question 3. Can human relevance of the MoA be reasonably excluded based on quantitative differences in either kinetic or dynamic factors between experimental animals and humans? As human relevance of the experimental animal MoA can be reasonably excluded on the basis of qualitative differences in key events (Question 2), a quantitative assessment of kinetic or dynamic factors is not necessary. Regardless, as shown in studies with sulfoxaflor, hCAR/hPXR mice did not have significant increases in hepatocellular proliferation at doses that did cause increases in WT (both CD-1 and C57Bl6) mice and eventual hepatocellular tumors in CD-1 mice. Furthermore, in a series of investigative studies, interspecies comparisons of mice, rats, rabbits, and dogs indicated a similar profile of negligible metabolism of sulfoxaflor and high bioavailability when given orally. These data indicate that the most critical key event in response to CAR and PXR activation in the development of hepatic tumors, that is, hepatocellular proliferation only occurs in the rodent via the CAR/PXR receptor.

Conclusions

Statement of confidence in the evaluation

This HRF evaluation for sulfoxaflor-induced hepatocellular tumors in mice and rats follows the guideline established for this process (Sonich-Mullin et al. 2001, Cohen et al. 2003, Meek et al. 2003, USEPA 2005, Holsapple et al. 2006, Boobis et al. 2006). The extensive toxicological database for sulfoxaflor, including several focused MoA studies in both mice and rats, as well as a study in genetically engineered (knockout and humanized) mice provide the necessary data to establish the CAR-mediated MoA for sulfoxaflor-induced rodent liver tumors. The key events for sulfoxaflor show clear, thresholded, dose-responsive alterations and provide informative, temporal-specific characterization of sulfoxaflor-induced liver effects. The concordance analysis points out clear differences for a CAR-mediated MoA in rodents as compared to humans. A hepatocarcinogenic response in rodents for compounds that have data to support a CAR-mediated MoA, such as sulfoxaflor, is not relevant to humans (Holsapple et al. 2006).

Other possible MoAs for hepatocellular carcinogenesis have been evaluated with respect to sulfoxaflor and have been dismissed because they lack plausibility and coherence or, in the case of cytotoxicity, because of the lack of coherence when the dose–response for cytotoxicity is compared to the hepatocellular tumor dose–response.

The increased incidence of sulfoxaflor-related liver tumors and relevance to human health risk assessment has been considered by global regulatory authorities. The Joint (FAO/WHO) Meeting on Pesticide Residues (JMPR 2011) concluded that, "... for the liver tumours in both mice and rats, there was sufficient evidence to support the proposed PB-like MoA. In particular, sulfoxaflor exhibited clearly higher activity towards rodent CAR than towards human CAR. The marked qualitative and quantitative species differences in the key events in the MoA for neoplasia in response to CAR activation allowed for the conclusion that the sulfoxaflor-induced liver tumours in rats and mice are not relevant to humans..." The USEPA (2012) concluded, "The hypothesized MoA (CAR mediated, mitogenic) for liver tumors was adequately supported by studies that clearly identified the sequence of key events, dose–response concordance and temporal relationship to the tumor type. There is convincing evidence that the hepatocarcinogenic effects are not likely to occur below a defined dose range." The MoA data met the criteria established by the Agency to enable this effect to be considered not relevant to human risk assessment. Furthermore, the effect was considered treatment-related but was not considered relevant to humans by the European regulators, "...based on mechanistic data, the Committee agreed with the dossier submitter not to classify this substance for carcinogenicity [or reproductive toxicity]" (ECHA 2013).

Implications for risk assessment

There is convincing evidence that the MoA for sulfoxaflor-induced hepatocarcinogenic effects in the mouse and rat liver do not occur below a defined dose level. Specifically, the key events for the CAR-mediated MoA only occur at dietary concentrations greater than 100 ppm in the mouse and rat and tumors were noted at 750 and 500 ppm, respectively. Furthermore, a hepatocarcinogenic response in rodents for compounds that have data to support a CAR-mediated MoA, such as sulfoxaflor, is not relevant to humans (Holsapple et al. 2006). On this basis, the mouse and rat liver tumors associated with administration of higher dose levels of sulfoxaflor would not pose a cancer hazard to humans. Based on this hazard assessment for the sulfoxaflor-induced mouse and rat liver tumors, a margin of exposure risk assessment based on the reference dose would be protective of human health.

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Declaration of interest

The authors are employed by The Dow Chemical Company, the developer and producer of sulfoxaflor. The authors have sole responsibility for the writing and content of the paper.

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