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A human relevance investigation of PPARα-mediated key events in the hepatocarcinogenic mode of action of propaquizafop in rats

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ABSTRACT

Propaquizafop is an herbicide with demonstrated hepatocarcinogenic activity in rodents. A rodent-specific mode of action (MOA) in the liver via activation of peroxisome proliferator-activated receptor α (PPARα) has been postulated based on existing data. Experience with PPARα-inducing pharmaceuticals indicates a lack of human relevance of this MOA. The objective of the present investigation was to evaluate the dependency of early key events leading to liver tumors on PPARα activation in wildtype (WT) compared to PPARα-knockout (KO) rats following 2 weeks exposure to 75, 500 and 1000 ppm propaquizafop in the diet. In WT rats, both WY-14643 (50 mg/kg bw/day) and propaquizafop (dose-dependently) induced marked increases in liver weights, correlating with liver enlargement and hepatocellular hypertrophy, along with increased CYP4A and acyl-CoA oxidase mRNA expression and enzyme activities *versus* controls, while in KO rats liver weight was mildly increased only at the high dose with minimal microscopic correlates and without any changes in liver peroxisomal or CYP4A activities. In addition, BrdU labeling resulted in higher numbers and density of positive hepatocytes *versus* controls in WT but not in KO rats, indicating increased mitotic activity and cell proliferation only in WT rats, thus confirming the PPARα-dependency of the biochemical and histological changes in the liver. Based on an assessment of the results of this investigation, together with existing propaquizafop data according to the MOA-Human Relevance Framework, we conclude that liver tumors observed in rodents after dietary administration of propaquizafop do not pose a relevant health risk to humans.

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are a family of steroid hormone receptors that control gene expression by co-localizing onto nuclear DNA together with other nuclear receptor proteins, following initial ligand binding. Three sub-types of PPARs have been identified, including PPAR-α, γ and β/δ. PPARs mediate a wide range of biological activity, including lipid metabolism, insulin sensitivity, hormone biosynthesis and certain aspects of immune function such as inflammation (Yang et al., 2008; Zhang and Young, 2002). As nuclear receptor transactivation factors, PPARs can lead to stimulation or inhibition of gene expression by binding with a variety of endogenous ligands, including certain xenobiotics known as peroxisome proliferators. PPARα is highly present in the liver in rodents, and its activation by peroxisome proliferating agents results in the induction of the microsomal and peroxisomal enzymes responsible for beta-oxidation of fatty acids (Peters et al., 1996).

In rodents, peroxisome proliferators are considered as non-genotoxic

Abbreviations: ACO, Acyl-CoA oxidase; bw, body weight; BrdU, bromodeoxyuridine; CAT, catalase; CYP, cytochrome P450; DNA, deoxyribonucleic acid; GSH-Px, glutathione peroxidase; GSH, reduced glutathione content; GSSG, oxidized glutathione content; H&E, hematoxylin-eosin; HRF, human relevance framework; IARC, International Agency for Research on Cancer; ILSI-RSI, International Life Sciences Institute-Risk Sciences Institute; IPCS, International Programme on Chemical Safety; KO, PPAR-α knockout rats; MOA, mode of action; mRNA, messenger ribonucleic acid; MRM, Multiple-Reaction-Monitoring mode; GSSG, oxidized glutathione content; PPARα, peroxisome proliferator-activated receptor α; PBS, phosphate buffered saline; PROD, pentoxyresorufin deethylase; GSH, reduced glutathione; RIN, RNA Integrity Number; SOD, superoxide dismutase; SD, standard deviation; USEPA, United States Environmental Protection Agency; WOE, weight of evidence; WT, wildtype sprague dawley rats; WHO, World Health Organization

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hepatocarcinogens following long-term exposure, while in humans they have much lower levels of induction/promoter activity and have not been associated with increased cancer risk (IARC Technical Report No.24, Lyon, 1995). It is reported that PPARα-DNA binding activity is lower when compared to other DNA binding proteins in untreated human liver samples and is much lower than the PPARα-DNA binding activity in untreated mouse liver samples (Palmer et al., 1998).

The hypothesized MOA (mode of action) for PPARα activator-induced hepatocarcinogenesis in rodents was recently reviewed in detail and considered for human relevance using the MOA/HRF (human relevance framework) (Corton et al., 2014, 2018). The expert panel (Corton et al., 2014) determined that the collective data support the temporal and dose-response relationships of key events linked with many activators of PPARα, such as gemfibrozil (a fibrate drug) and phthalates. It was concluded that while the key events were biologically plausible in humans, there were significant quantitative differences in the PPARα activator-induced responses among the species studied that made the rodent MOA essentially non-relevant in humans. Four principal key events have been identified: 1) ligand-activation of PPARα; 2) induced gene transcription; 3) peroxisome proliferation, leading to liver enlargement and hepatocellular hypertrophy; and 4) suppression of apoptosis and stimulation of cell proliferation, leading finally to adenoma (Klaunig et al., 2003; Corton et al., 2014).

Propaquizafop has been found to induce liver tumors in mice and rats (see Table 1) following long-term (rats: 104 and mice: 80 weeks) dietary exposure (DAR, 2006), and mechanistic investigations in male rats demonstrated strong, dose-dependent increases in peroxisomal fatty acid beta-oxidation, and induction of CYP4A mRNA and enzyme activity, as well as extensive proliferation of peroxisomes in liver hepatocytes (DAR, 2006). *In vitro* studies in cultured primary hepatocytes demonstrated that CYP4A activity was largely increased in mouse and rat, but not in guinea pig, marmoset or human hepatocytes following treatment with propaquizafop (DAR, 2006).

The present investigation aimed to demonstrate the dependency on PPARα for triggering a series of key events in rat liver after dietary administration of propaquizafop by comparing responses in wildtype (WT) and genetically modified male rats lacking PPARα (KO) at tumorigenic dose levels. The data were evaluated according to the MOA/HRF developed by the World Health Organization International Programme on Chemical Safety (IPCS) (Meek et al., 2014a and b) and previous work on this mode of action (Corton et al., 2014).

2. Materials and methods

2.1. Animals

Wildtype (WT) male Sprague Dawley rats (RjHan:SD; 10–11 weeks old) were obtained from Janvier (Le Genest-Saint-Isle, France). PPAR-α KO male Sprague Dawley rats (PPAR-α tm1sage; 11–12 weeks old) were obtained from SAGE Labs (Boyertown PA, USA). These rats have a bi-allelic deletion within the peroxisome proliferator-activated receptor alpha gene (GACCTTGTGCATGGGtgagaaGACGCTTGTGGCCAAGATG). The founder line #24(M) 4-23-10 was used and homozygous knockouts exhibited complete loss of the protein via Western blot (SAGE Labs production, from Horizon Discovery - St. Louis, 2033 Westport Center Drive, Saint Louis, MO 63146, USA).

2.2. Reagents

Propaquizafop (propaquizafop technical, 93.19% purity, batch No. 191) was provided by ADAMA Agan Ltd. (Northern Industrial Zone, Ashdod 7710201, Israel). Powdered maintenance diet (reference No. A04 C-10, batch No. 15294) was purchased from Safe (Augy, France). Antibodies for immunohistochemistry were purchased from Abcam (Paris, France). WY-14643 (reference No. C7081), bromodesoxyuridine (BrdU; reference No. B5002-1G) and the other reagents were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France), except where noted.

2.3. Study design

2.3.1. Preliminary study: dietary administration of propaquizafop in wildtype male rats

The *in vivo* study was performed at CiToxLAB, France (Evreux, France). One group of five male rats was exposed to propaquizafop (1500 ppm) in powdered maintenance diet (oral route), daily for two weeks. One control group of five male rats received untreated powdered maintenance diet under the same experimental conditions. Each animal was checked at least once daily for mortality and clinical signs. Body weight was recorded once before the beginning of the treatment period, and twice a week thereafter. Food consumption was measured once a week during the treatment period. BrdU was intraperitoneally injected in phosphate buffered saline (PBS) on Days 3, 11 and 14 to allow for evaluation of hepatocellular proliferation (Biegel et al., 1999). On completion of the treatment period, all animals were sacrificed and subjected to a full macroscopic post-mortem examination. After weighing, a 3–4 g liver sample was preserved in 10% buffered formalin, embedded in paraffin wax, sectioned at a thickness of approximately 4 μm and stained with H&E (Morawietz et al., 2004).

2.3.1.1. Immunohistochemistry and image analysis. Additional liver sections were prepared at CiToxLAB (Evreux, France) to investigate hepatocellular proliferation (via BrdU detection) and hepatocellular apoptosis (via TUNEL assay). BrdU labeling assessment was performed using a Ventana Discover Ultra automat. After the

Table 1
Liver tumor incidence in rats and mice following long-term dietary exposure to propaquizafop (summarized from DAR, 2006).

Tumor	Sex	Dose (mg/kg bw/d)									
		0	0	5	25	30	50	75	100	150	300
Mice											
Liver Adenoma	M	10.0%	4.0%	–	–	40.8%	–	–	33.3%	–	32.0%
	F	4.0%	2.0%	–	–	4.2%	–	–	6.1%	–	44.0%
Liver Carcinoma	M	0%	4.0%	–	–	8.2%	–	–	14.6%	–	66.0%
	F	2%	0%	–	–	0%	–	–	0%	–	50.0%
Rats											
Liver Adenoma	M	1.6%	–	0%	2.1%	–	21.3%	n.d.	27.7%	n.d.	–
	F	3.4%	–	0%	6.12%	–	n.d.	8.3%	n.d.	31.9%	–
Liver Carcinoma	M	0%	–	0%	2.1%	–	12.8%	n.d.	25.5%	n.d.	–
	F	0%	–	0%	2.0%	–	n.d.	10.4%	n.d.	14.9%	–

nd: no data as dose level not used in this sex (higher dose levels for female rats as males were more sensitive to propaquizafop).
-: dose level not given in the species.
This table includes multiple studies in mice and rats that were cited in the DAR for propaquizafop in 2006.