Isoproturon

Investigative Studies on the Mode of Action of and Human Relevance of Liver Tumors in Rats

Expert Statement

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1. Introduction

In a chronic toxicity and carcinogenicity study in rats with dietary administration of concentrations of 0, 80, 400 and 2000 ppm Isoproturon technical (IPU) over 2 years, increased incidences of hepatocellular adenomas and carcinomas were seen. The increases were more pronounced in males than in females (Table 1). No increase in these tumors was observed in mice (Table 2).

Table 1: Incidence of liver adenomas and carcinomas in the rat carcinogenicity study with Isoproturon [redacted] al., 1981)

	Incidence (%)				HCD (%)*
	Rat study [redacted] al, 1981; TOX9551907)				
Dose level (ppm)	0	80	400	2000	
	males				
Hepatocellular adenoma	1.2	2.5	2.5	8.7	1.3-18.2
Hepatocellular carcinoma	6.2	0	11.2	78.7	1.1-9.1
	females				
Hepatocellular adenoma	1.2	0	0	11.2	1.0-5.5
Hepatocellular carcinoma	0	1.2	1.2	16.2	1.0-4.0

* Historical control data (Charles River, 1992, April 1984 - Feb. 1989)

Table 2: Incidence of liver adenomas and carcinomas in the mouse carcinogenicity study with Isoproturon [redacted] al., 1982)

	Incidence			
	Mouse study (Hunter et al, 1982, TOX9551906)			
Dose level (ppm)	0	50	200	800/1600*
	males			
Hepatocellular adenoma	7.7	17.3	13.5	23.1
Hepatocellular carcinoma	23.1	32.7	25	32.7
	females			
Hepatocellular adenoma	7.7	0	1.9	9.6
Hepatocellular carcinoma	0	1.9	1.9	1.9

* Dose level was increased after 8 weeks of treatment

IPU has been intensively investigated and was found to have no genotoxic potential so that this mechanism can be confidently excluded.

Table 3: Genotoxicity data on Isoproturon

Bacterial *in vitro* tests (reserve mutation)	
Salmonella typhimurium (2)	Negative
Salmonella typhimurium (2)	Positive
Salmonella typhimurium, Escherichia coli	Positive
Salmonella typhimurium, Escherichia coli	Negative
Salmonella typhimurium, Escherichia coli	Negative
Escherichia coli	Negative
Fungal *in vitro* tests	
Mitotic recombination (crossing-over, gene conversion), forward mutation	
Saccharomyces cerevisiae MP-1	Negative
Saccharomyces cerevisiae D7 (2)	Negative
Mitotic recombination (gene conversion)	
Saccharomyces cerevisiae D7	Negative
Forward mutation	
Schizosaccharomyces pombe PL	Negative
Mammalian cell *in vitro* tests	
Gene mutation	
L5178Y mouse lymphoma cells	Negative
Chromosomal aberration	
Human lymphocytes	Negative
DNA demage/repair	
UDS; rat primary hepatocytes (3)	Negative
UDS; human fibroblasts	Negative
Cell transformation	
BHK21/C13 cells	Negative
Host-mediated assays	
Mouse/Salmonella typhimurium TA 100 (2)	Positive
Mouse/Salmonella typhimurium TA 100	Negative
Mouse/L5178Y mouse lymphoma cells	Negative
Mammalian *in vivo* tests	
Somatic cells	
Micronucleus; hamster bone marrow (2)	Negative
Chromosomal aberration; mouse bone marrow (2)	Negative
Chromosomal aberration; hamster bone marrow	Negative
Covalent binding to DNA; rat liver	Negative
UDS; rat liver	Negative
Germ cells	
Dominant lethal mutation; mouse (3)	Negative
Chromosomal aberration; mouse spermatogonia	Negative
Chromosomal aberration; mouse spermatocytes	Negative

(number of tests if more than one)

Based on the observed tumor profile and histopathology, together with information on liver toxicity from other apical studies, a receptor-mediated rodent-specific mode of action of tumorigenicity is suggested.

Table 4: Findings in apical studies that indicate the mode of action (Subacute, subchronic rat studies and chronic/carcinogenicity rat study)

Study	Parameter	Low dose	Mid dose	High dose
14 d study in rats	mg/kg bw	80	200	500
	Liver enzyme induction	Induction of phase I and II liver enzymes	Induction of phase I and II liver enzymes	Induction of phase I and II liver enzymes
	Liver weights	-	Rel. Liver weights significantly increased	Rel. Liver weights significantly increased, prolif. of smooth ER*
90 d dietary study in rats	ppm	80	400	10000/20000
	Liver weights	-	-	Relative weights ↑ (+88% (m)/+78% (f))
	Histopathology of the Liver	Not conducted	Not conducted	Not conducted
90 d dietary study in rats	ppm	400	1500	5000
	Liver weights	-	-	Increased
	Histopathology of the Liver	-	-	-
90 d dietary study in rats	ppm	400	1200	2400
	Liver weights	-	-	Increased (m, f)
	Histopathology of the Liver	-	-	-
90 d dietary study in rats	ppm	400	800	8000
	Liver weights	-	-	Relative weights increased (m, f)
	Histopathology of the Liver	-	Pigment deposition, minimal basophilic foci	Pigment deposition, minimal basophilic foci
90 d gavage study in rats	mg/kg bw	85	250	750
	Liver weights	Not given	Not given	Not given
	Histopathology of the Liver	-	Few animals with slight degenerative changes	Animals with slight to moderate degenerative changes
Chronic study in rats	ppm	80	400	2000
	Liver weights	-	-	Increased (m)
	Histopathology of the Liver	-	-	Degeneration, necrosis, fibrosis, areas of vacuolated hepatocytes (m)

- = no effect

* Endoplasmatic reticulum

The postulated mode of action is well described in the scientific literature and involves sustained liver activation as a consequence of binding to the liver nuclear receptors CAR and / or PXR. The Constitutive Androstane Receptor (CAR) and Pregnane X Receptor (PXR) are important nuclear receptors involved in the regulation of cellular responses from exposure to many xenobiotics and various physiological processes. Phenobarbital is a non-genotoxic CAR activator which induces cytochrome P450 and other metabolizing enzymes and produces liver tumors in rats and mice by changed gene expression, increased cell proliferation, formation of altered hepatic foci and finally development of liver tumors. This mechanism is rodent-specific and not relevant to humans since this CAR and PXR activation only stimulates hepatocellular

S phase DNA synthesis in rodent hepatocytes but not in human hepatocytes. This mechanism does not lead to tumor formation in humans which is proven for phenobarbital after its long use in human medicine epidemiologically. In animals with human CAR and PXR receptors or in CAR- and PXR-knockout rats no hyperplastic responses are seen (Ross, J. et al., 2010; Elcombe, C. et al., 2014; Yamamoto, Y. et al., 2004).
Therefore, this mode of action was investigated in specific investigative studies on Isoproturon in order to demonstrate that the liver tumors after Isoproturon are not relevant to humans.

2. Investigative studies on Isoproturon

In vitro studies evaluated the ability of Isoproturon technical to induce typical CAR- and PXR-dependent cytochrome P450 activity (CYP2B/3A isoforms) and cell proliferation in isolated primary male wild type (WT) Sprague Dawley rat hepatocyte cultures and male human hepatocyte cultures.

The results are briefly summarized in the following table.

Table 5: Summary of results of the *in vitro* studies

IPU

Parameters	Rat hepatocytes		Human hepatocytes
	Wild-type rat	CAR knockout rat	
CYP2B/3A activities	↑	↑	(↑)
Cell proliferation	↑	-	-

As an overall conclusion of these in vitro tests it can be stated that IPU caused a more or less distinct induction of CYP2B and CYP3A activities and cell proliferation in wild-type rats, whereas in CAR-knockout rat hepatocytes or human hepatocytes no cell proliferation was seen after IPU-exposure, despite some variability of the inductions of CYP2B and CYP3A activities. This is regarded as evidence that the observed effects of IPU and its metabolites in rats are not relevant to humans.

3. Mode of Action Human Relevance Framework analysis according to IPCS

Based on the results of all relevant studies, especially the available results of the mechanistic in vitro studies, a receptor-mediated rodent-specific mode of action is proposed with liver activation as a consequence of binding of IPU to the liver nuclear receptors CAR and/or PXR. A Mode of Action Human Relevance Framework analysis according to IPCS is shown in the following table.

Table 6: Mode of Action Human Relevance Framework analysis according to IPCS

Key events	Liver activation as a consequence of binding to the liver nuclear receptors CAR and PXR, leading to hyperplasia and finally liver tumors in rodents.
Concordance of dose-response relationship	In a subchronic rat study, relative liver weight was increased as indicator of liver enzyme induction. In a 14-day rat study, dose-related liver enzyme induction, liver weight increases and a proliferation of the smooth endoplasmic reticulum of the liver cells was demonstrated.
Temporal association	In a 14-day rat study, dose-related liver enzyme induction occurred, furthermore, in the subchronic and chronic studies liver weight increases were seen which are indicative of live renzyme induction.
Strength, consistency and specificity of association of tumor response with key events	Evidence of liver enzyme induction was seen in the rat. Furthermore, the special in vitro studies demonstrated an activation of the liver nuclear receptors CAR and PXR in the rat which support the proposed rodent-specific mechanism of CAR and PXR activation which finally leads to tumors in rats. It could be demonstrated in vitro that despite CAR and PXR activation in the KO rats no proliferation occurred in the measurements. This mechanism is not relevant to humans. This is supported by the in vitro studies in human hepatocytes in which no proliferation was seen.
Biological plausibility and coherence	The plausibility of this mechanistic explanation was demonstrated for many compounds, e.g. phenobarbital, perfluooctanoate, sulfoxaflor and others, for which this rodent-specific mechanism was clearly demonstrated (Elcombe (2010, 2014)).
Other modes of action	IPU is not genotoxic so that a genotoxic mechanism can be excluded. Investigations of a possible tumor-initiating potential were not conclusive and since the special studies show evidence of a mechanism via CAR and PXR receptors, this is more plausibel and supported by the literature.
Assessment of postulated mode of action	The available experimental data were already supportive of a liver tumor-inducing mechanism via activation of CAR and PXR receptors in the liver. The conducted special in vitro studies demonstrated an activation of the liver nuclear receptors CAR and PXR in the rat which support the proposed rodent-specific mechanism of CAR and PXR activation which finally leads to tumors in rats. It could be demonstrated that despite CAR and PXR activation in the KO rats no proliferation occurred. This mechanism is not relevant to humans. This is supported by the in vitro studies in which no proliferation in human hepatocytes was seen. Aspects, like dose response, temporal association and consistency are clearly fulfilled.
Uncertainties, inconsistencies and data gaps	In the long-term studies in rats the step from hypertrophy via hyperplasia leading to tumors is not well-described but can be assumed. In the subchronic studies hyperplasia in the liver was not seen, but also not expected due to the short exposure time. Results from the mechanistic in vitro studies were able to demonstrate the lack of proliferation in the KO rats and, thus demonstrating also the lack of proliferation. This would support that under suppression of rat CAR and PXR no tumor induction will occur.

5. Conclusion

In a chronic toxicity and carcinogenicity study in rats with dietary administration of Isoproturon technical (IPU) over 2 years increased incidences of hepatocellular adenomas and carcinomas were seen. Since IPU has an overall negative genotoxic potential such mechanism can be excluded and a threshold effect be assumed. Based on the existing data, a receptor-mediated rodent-specific mode of action was suggested with sustained liver activation as a consequence of binding to the liver nuclear receptors CAR and/or PXR, leading to hyperplasia and finally tumors. This mechanism is regarded as rodent-specific mechanism which is not relevant to humans.
The results of a special study support this mechanism since the findings from BrdU determinations demonstrated an increase of hepatocellular proliferation in wild type rat hepatocytes, whereas in contrast, hepatocytes from transgenic (CAR-KO) rats and humans did not reveal indicators for proliferation. This mechanism, therefore, would not justify a classification with R40.

5. Literature

Elcombe, C. (2010): Hepatocellular hypertrophy and cell proliferation in Sprague–Dawley rats following dietary exposure to ammonium perfluorooctanoate occurs through increased activation of the xenosensor nuclear receptors PPARα and CAR/PXR. Archives of Toxicology October 2010, Volume 84, Issue 10, pp 787-798.

Elcombe, C. (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. Critical Reviews in Toxicology, January 2014, Vol. 44, No. 1 , Pages 64-82 (doi:10.3109/10408444.2013.835786).

Lemaire, G. et al. (2006): Identification of New Human Pregnane X Receptor Ligands among Pesticides Using a Stable Reporter Cell System. Toxicol. Sci. 91 (2); 501-509.

Ross, J. et al. (2010): Human Constitutive Androstane Receptor (CAR) and Pregnane X Receptor (PXR) support the hypertrophic but not the hyperplastic Response to the Murine Nongenotoxic Hepatocarcinogens Phenobarbital and Chlordane in vivo. Toxicological Sciences 116(2), 452-466.

Yamamoto, Y. et al. (2004): The orphan nuclear receptor constitutive active/androstane receptor is essential for liver tumor promotion by phenobarbital in mice. Cancer Res. 64, 7197–7200.