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Safety and Health Research

TOXICOLOGY SERVICES

- General toxicology in all species
- Special toxicology
  - infusion
  - Inhalation
  - Dermal
  - Ocular
- Immunotoxicology
- Regenerative medicine
- Reproductive toxicology including minipigs and NHPs
- Carcinogenicity studies also in rasH2 and p53+/- mice
- Genetic toxicology: ICH compliant package
- *In vitro* toxicology: BCOP, h-CLAT, KeratinoSens™, DPRA, Photo 3T3-NRU, Episkin™, chicken eye test
- Agrochemical / chemical / REACH
- QSAR
- Physico-chemical testing
- Ecotoxicology: wide range of test species

SAFETY PHARMACOLOGY

- Integrated safety pharmacology in toxicology studies
  - CV (JET), BP
  - Respiratory (JET), plethysmography
  - CNS (FOB) and JET-EEG
- Safety pharmacology core battery
- *In vitro* assays
  - GLP compliant ion channel testing panel (hERG +5)
  - CNS *ex vivo* models for seizure liability screening
- Screening and follow-up models
  - Rodent and non-rodent LVP telemetry
  - Anesthetized models
  - Polysomnography
  - Gastrointestinal motility

DMPK, BIOANALYSIS, BIOMARKERS

- ^{14}C and ^{3}H ADME studies in all species
- *In vitro* metabolic clearance, metabolite ID and profiling, DDI package (metabolism and transporters)
- Bioanalysis: LC-MS/MS, GC-MS/MS, LC-ICP/MS, LC-Radiodetection, ELISA, RIA
- Toxicogenomics, miRNA: Affymetrix™ accredited service provider, next generation sequencing (Illumina®)
- Immunology: 10-color flow cytometer, Luminex, Meso Scale

MEDICAL DEVICE

- Biocompatibility testing
- Cardiovascular stents, electrophysiology and structural heart studies
- Long-bone defects and craniomaxillofacial/dental models
- Spinal fusion models
- Joint and cartilage repair models
- Regenerative medicine (growth factors, biomaterials, cell and gene therapy)

SPECIALIZED EXPERTISE

- Juvenile studies including minipigs
- Ototoxicity in rats
- Fertility studies in rodents and NHPs
- Radiation safety and efficacy studies
- Drug transporter studies and Drug-Drug Interactions
- Tissue Cross Reactivity (TCR): human and animal tissue banks
- Gene therapy vector biodistribution via qPCR
- ES cell testing: devTOX™ and cardioTOX™ (with Stemina)
- Lead optimization and predictive toxicology services: Leadscreen™

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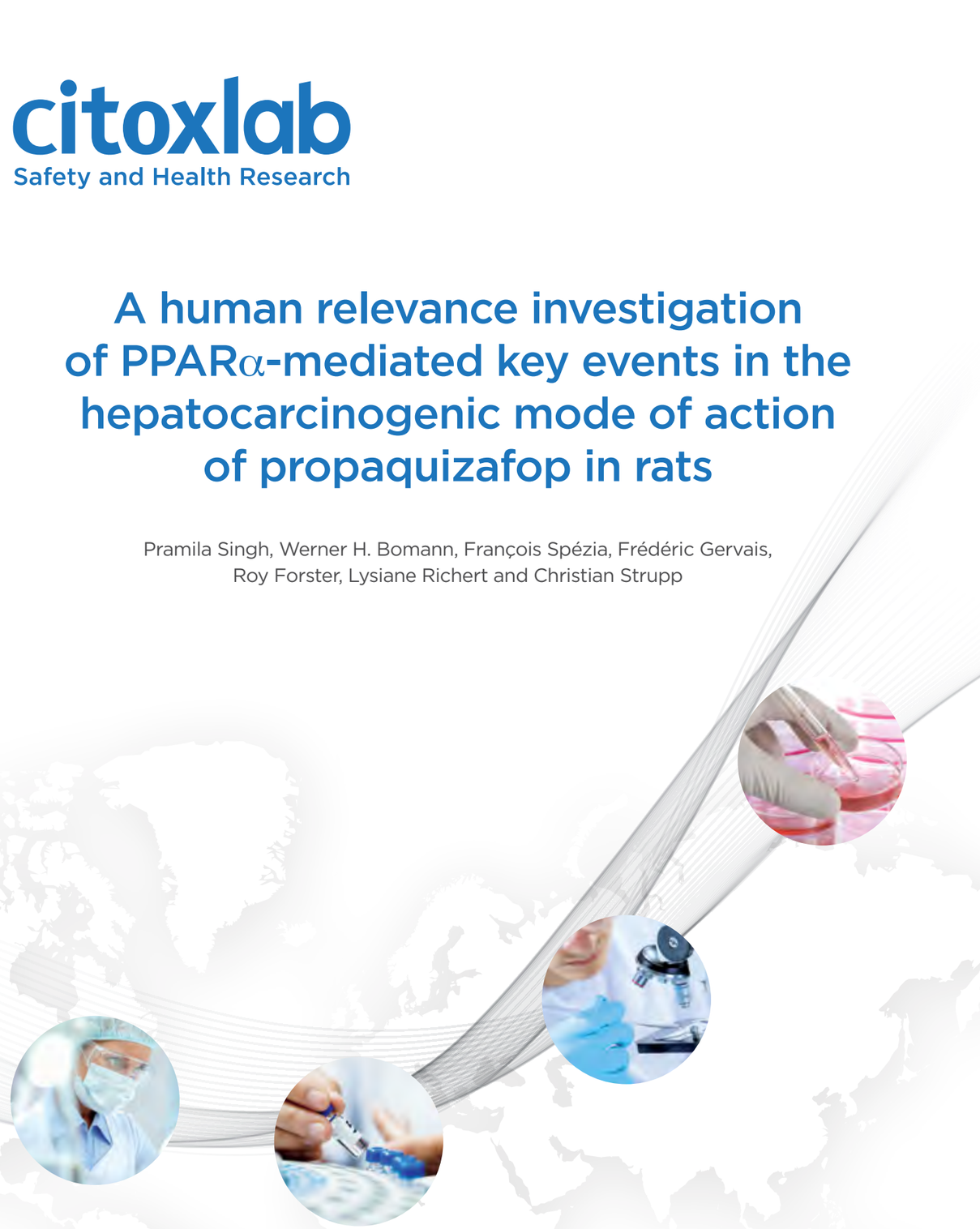
A human relevance investigation of PPARα-mediated key events in the hepatocarcinogenic mode of action of propaquizafop in rats

Pramila Singh, Werner H. Bomann, François Spézia, Frédéric Gervais, Roy Forster, Lysiane Richert and Christian Strupp



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

Pramila Singh [1], Werner H. Bomann [2], François Spézia [1], Frédéric Gervais [1], Roy Forster [1], Lysiane Richert [3,4] and Christian Strupp [5]

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INTRODUCTION

Propaquizafop is an herbicide with a long history of use. In regulatory toxicology testing, chronic dietary exposures in rodents led to liver tumors without genotoxicity; subchronic studies demonstrated dose-dependent increases in liver weights with hepatocellular hypertrophy; and two weeks administration in rats induced liver CYP4A and peroxisomal enzymes. The objective of this study was to investigate the rodent toxicological mode of action (MOA) of propaquizafop in order to use the information together with existing data to evaluate the human relevance of rodent liver tumors. A postulated rodent-specific MOA via activation of the peroxisome proliferator-activated receptor α (PPARα), followed by increased liver peroxisomal activity, hypertrophy and enlargement, subsequent cell proliferation and adenoma formation has been based on the consistency of these findings with an established hepatocarcinogenic MOA of fibrate drugs in rodents. Experience with PPARα-inducing pharmaceuticals indicates a lack of human relevance. The present study evaluated propaquizafop-induced liver tumors in rodents with a 2-week MOA investigation in male, Sprague-Dawley wildtype (WT) and PPARα-knockout (KO) rats to confirm the PPARα-activation dependency of key events in the hepatocarcinogenic MOA of propaquizafop in rats. A known PPARα activator, WY-14643, was used as a positive control, and alternative pathways such as oxidative stress, apoptosis, aromatase (CYP19), Ah-R and CAR-mediated pathways were evaluated and largely excluded in a preliminary study.

RESULTS

In WT rats, propaquizafop and the positive control, WY-14643, induced marked increases in relative liver weights (+84% and 89%) that correlated with liver enlargement and hepatocellular hypertrophy, along with increased peroxisomal lauric acid hydroxylase (CYP4A) activity (2.4 and 1.5 fold) and acyl-CoA oxidase activity (10.0-23.3 fold) *versus* control, while in KO rats only increased relative weight (24%) was observed without any of the other effects, thus confirming the PPARα-dependency of the changes. BrdU labeling resulted in higher numbers and density of positive hepatocytes on day 15 compared to the untreated control group, indicating increased mitotic activity and cell proliferation.

Table 1: Preliminary Study: Confirmation of PPARα Activation and Evaluation of Alternative Pathways in Control and Propaquizafop-Treated Male Sprague-Dawley Wildtype Rats (mean ± SD)

Dose	Total protein content		Total CYP content pmol/mg proteins	CYP1A1/2 Phenacetin O-deethylase activity pmol/min/mg proteins	CYP2B Pentoxy-resorufin O-dealkylase activity pmol/min/mg proteins	CYP3A1/2 16β Testosterone hydroxylase activity pmol/min/mg proteins	CYP4A Lauric acid hydroxylase activity pmol/min/mg proteins	CYP19 Aromatase synthase content µg/mg proteins
	Microsomal mg/g liver	Homogenate mg/g liver						
Control (n=5)	8.99 ± 1.20	194 ± 34	497 ± 211	442 ± 73	11.3 ± 3.5	44.6 ± 8.7	909 ± 184	5.65 ± 2.51
1500 ppm (n=5)	12.9 ± 2.53 (1.4)	200 ± 7 (1.0)	474 ± 212 (0.95)	501 ± 158 (1.1)	11.3 ± 1.9 (1.0)	97.7 ± 32.1 (2.2)	1684 ± 114 (1.8)	5.29 ± 1.62 (0.9)

( ): in brackets, fold difference vs. control; n: number of animals

Table 2: Preliminary Study: Liver Peroxisomal Acyl CoA Oxidase and Oxidative Stress Activities in Control and Propaquizafop-Treated Wildtype Rats (mean ± SD)

Dose	AcylCoA oxidase nmol/min/mg proteins	Glutathione peroxidase nmol/min/mg proteins	Reduced glutathione nmol/mg proteins	Oxidized glutathione nmol/mg proteins	Total glutathione nmol/mg proteins	Superoxide dismutase		Catalase nmol/min/mg proteins
						Cytosolic activity U/mg proteins	Mitochondrial activity U/mg proteins	
Control (n=5)	0.480 ± 0.295	589 ± 98	51.8 ± 2.7	69.8 ± 11.5	138 ± 16	34.6 ± 1.7	24.4 ± 6.5	1598 ± 98
1500 ppm (n=5)	11.8 ± 7.1 (24.6)	677 ± 114 (1.2)	111 ± 8 (2.1)	46.4 ± 6.1 (0.7)	167 ± 22 (1.2)	33.7 ± 2.5 (1.0)	19.8 ± 3.5 (0.8)	1843 ± 25 (1.2)

( ): in brackets, fold difference vs. control; n: number of animals

Figure 1: BrdU-positive hepatocellular nuclei in rats:
A: WT control - B: WT propaquizafop-treated - C: KO control - D: KO propaquizafop-treated
Marked increase of positive nuclei in the propaquizafop-treated wildtype rats only (arrows show BrdU -positive hepatocellular nuclei)

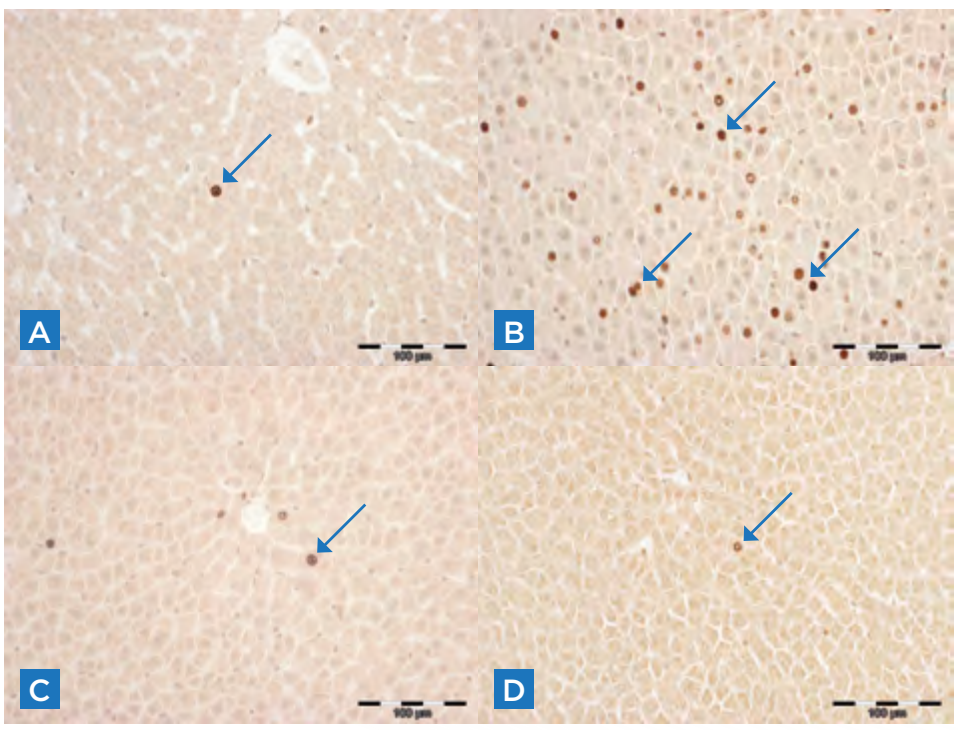

Table 3: Hepatic changes

Dose level	Units	Wildtype rats (WT)				PPAR-α knockout rats (KO)			
		Propaquizafop (ppm)			WY-14643 (mg/kg/day)	Propaquizafop (ppm)			WY-14643 (mg/kg/day)
		75	500	1000	50	75	500	1000	50
Animals examined	number	16	18	18	8	18	18	17	8
Final body weight	% vs control	-2	-2	-11**	-7*	+1	-1	-2	+1
Relative liver weight	% vs control	+9	+69**	+84**	+89**	+3	+10	+25**	+14
Liver enlargement	number	1	14	13	4	0	0	2	2
Hepatocellular hypertrophy	number	0	17	18	8	0	0	8	1
Grade 1								8	1
Grade 2			17	8	2				
Grade 3				10	6				
BrdU-positive hepatocytes	number	17	18	18	8	18	18	17	8
Grade 1		17	5	3	1	18	18	17	8
Grade 2			11	9	3				
Grade 3			2	5	4				
Grade 4				1					

Statistical significance vs. control: *: p < 0.05, **: p < 0.01. BrdU scoring [positive cells per high power field (x400)]: Grade 1: less than 10, grade 2: 10-20, grade 3: 20-40, grade 4: more than 40

Table 4: Peroxisomal acyl CoA oxidase and lauric acid hydrolase (CYP4A) liver enzyme activity

Activity		AcylCoA oxidase		Lauric acid hydroxylase (CYP4A)	
Compound	Dose	Fold vs. control		Fold vs. control	
		WT	PPARα-KO	WT	PPARα-KO
Propaquizafop	Control	(1.31)[a]	(1.57)[a]	(823)[a]	(655)[a]
	75 ppm	1.3	1.2	2.6	0.9
	500 ppm	15.2	1.0	2.1	1.1
	1000 ppm	10.0	0.7	2.4	1.1
WY-14643	50 mg/kg/day	23.3	1.0	1.5	0.9

a: control values in nmol/min/mg proteins

Table 5: Postulated Hepatocarcinogenic MOA of Propaquizafop in Rats vs Humans

		Propaquizafop results		
Event	Relationship	In WT Rats	In KO Rats	in Humans
PPARα activation	Causal; molecular initiating event	Indirectly demonstrated as observed in mRNA and protein activity profiles	Absent: ACO and CYP4A mRNA expression or enzyme activity were unaffected	Possible
Expression of peroxisomal genes	Associative	Demonstrated increased CYP4A activity	Absent: ACO and CYP4A mRNA expression or enzyme activity were unaffected	Possible; different transcriptional network (Corton et al., 2014)
Peroxisome proliferation	Associative	Demonstrated by electron microscopy and indirectly increased lauric acid hydroxylase/ ACO activity	Absent: ACO and CYP4A mRNA expression or enzyme activity were unaffected	Not observed: No increase in palmitoyl CoA oxidation or lauric acid hydroxylation in human, marmoset and guinea pig hepatocytes in vitro (while strong in rat and mouse) (DAR 2006; Corton et al., 2014)
Cell proliferation	Causal	Demonstrated	Not observed	PPARα activation
Hepatocyte oxidative stress	Associative	Changes in oxidative stress pathways in the liver were observed in the preliminary study using UV fluorescence	Not evaluated	Highly unlikely in absence of the peroxisome proliferative response (Klaunig *et al.*, 2003)
Apoptosis	Associative	Observed in the preliminary study: some evidence in H&E staining of liver in some animals, but not confirmed by TUNEL. In the main study, there were no differences across the groups	No difference across groups	
Occurrence of liver tumors	Adverse outcome	Demonstrated in carcinogenicity study		

Table 6: Human Relevance Framework Evaluation of Propaquizafop MOA

Concordance of dose-response relationship	- In subchronic studies, dose-dependent increases in liver weight and hepatocellular hypertrophy - In the carcinogenicity studies, liver tumors occurred dose-dependently
Temporal association	All phases of tumor development are in good agreement with the temporal succession of the MOA steps: - Early start of liver cell proliferation as demonstrated by BrdU results after 14-day treatment; hepatomegaly in 2- and 13- week studies; hypertrophy in 13 week studies; liver tumors after longer treatment duration, in 104- and 80-week studies in rats and mice, respectively.
Strength, consistency and specificity of association of tumor response with key events	- importance of peroxisome proliferation as key event for liver tumor induction clearly demonstrated in PPARα-KO rats, in which no liver cell proliferation occurred, whereas wildtype animals showed liver cell proliferation - Comparative in vitro studies in mouse, rat, marmoset and guinea pig hepatocytes with propaquizafop showed that rat and mouse hepatocytes reacted to treatment by increased activity of peroxisome-associated enzymes, whereas hepatocytes from guinea pigs and marmosets did not react - In vitro studies in human hepatocytes with propaquizafop did not show a peroxisome proliferating potential - In dog studies, no evidence of a peroxisome proliferating potential was seen
Biological plausibility and coherence	- Succession of key events and liver tumor development in rodents in agreement with knowledge about biological processes in the liver - In agreement with broadly accepted knowledge that cell proliferation is the main process leading to the development of tumors by non-genotoxic compounds - Support by another peroxisome-proliferating compound, WY-14,643, which causes liver tumors via a similar MOA - Similarity downstream to phenobarbital-type MOA which leads, via increased induction of certain liver enzymes, to increased liver cell proliferation and finally to liver tumors, giving plausibility to the rodent-specific liver tumor MOA
Other possible MOAs	- The other main possible MOA, i.e. by genotoxicity, can be excluded since propaquizafop was fully investigated and did not indicate a genotoxic potential - Effects on apoptosis or CAR/PXR activation were not evident and thus are excluded as alternative MOA possibilities - Other nuclear-receptor-mediated pathways, such as AhR-mediated or CAR-mediated can be excluded
Uncertainties, inconsistencies and data gaps	- No inconsistencies due to clear concordance between dose and time relationships of MOA key events and rodent liver tumor increases - Hypertrophy and hyperplasia were noted with only a low incidence in the carcinogenicity studies (probably a consequence of the late termination) - Hyperplasia was not observed in the subchronic studies, probably due to short treatment duration - Oxidative stress was observed, but is not considered as a primary MOA
Assessment of postulated mode of action	- High reliability of MOA as strong concordance between dose, temporality, and the expected sequence of events for tumorigenicity in the liver and agreement with broad published data base in this area - Further support due to congruence with the MOA of positive control, WY-14643 - Agreement with the wide literature basis for this MOA via increased peroxisome proliferation, leading to increased liver cell proliferation and eventually to tumors - This MOA is not considered to be relevant to humans, based on published scientific consensus, on the long history of use in human medicine and on epidemiological evidence from extensive human use of clofibrate, which has the same MOA.

BIBLIOGRAPHY

IARC Technical Report No.24, Lyon, 1995 Peroxisome Proliferation and its Role in Carcinogenesis. Views and expert opinions of an IARC Working Group. Lyon, 7-11 December 1994. https://monographs.iarc.fr/ENG/Publications/techrep24/IARCrep24.pdf. Corton C. *et al.* (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): 1-49. doi:10.3109/10408444.2013.835784. Draft Assessment Report (DAR), Initial risk assessment provided by the rapporteur Member State Italy for the existing active substance, propaquizafop, of the third stage (part A) of the review Programme referred to in Article 8(2) of Council Directive 91/414/EEC; Vol 3, Annex B, B6, part 3. June 2006. Klaunig J.E., Babich M.A., Baetcke K.P., Cook J.C., Corton J.C., David R.M., DeLuca J.G., Lai D.Y., McKee R.H., Peters J.M., Roberts R.A., Fenner-Crisp P.A. (2003). PPARalpha agonist-induced rodent tumors: modes of action and human relevance. Crit. Rev. Toxicol. 33(6): 655-780. DOI:10.1080/713608372.

CONCLUSION

We evaluated the data according to the MOA-Human Relevance Framework and our findings support the conclusion that liver tumors observed in rodents after dietary propaquizafop administration do not pose a relevant health risk to humans.