

# Prochloraz: Further *In vitro* data comparing responses in male wild-type mouse and human cultured hepatocytes completes the human relevance framework

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## INTRODUCTION

The fungicide prochloraz has induced hepatocellular tumors after chronic administration to CD-1 mice, but not to Sprague Dawley or Fisher 344 rats. Genotoxicity can be confidently excluded. Previous *in vivo* studies using wild-type mice, constitutive androstane receptor /pregnane X receptor (CAR/PXR) knock-out (KO) mice and humanised CAR/PXR mice have demonstrated that prochloraz induces liver tumours *via* a CAR/PXR-dependent Mode of Action that is considered not to be of relevance to humans (Melching-Kollmuss *et al.*, 2015).

To confirm non-human relevance, the responses of cultured mouse and human hepatocytes to prochloraz were compared.

## METHODS

Hepatocytes, isolated from male CD-1 mice and cryopreserved male human hepatocytes were cultured in the presence of prochloraz for 96 hr (Mitchell *et al.*, 1985).

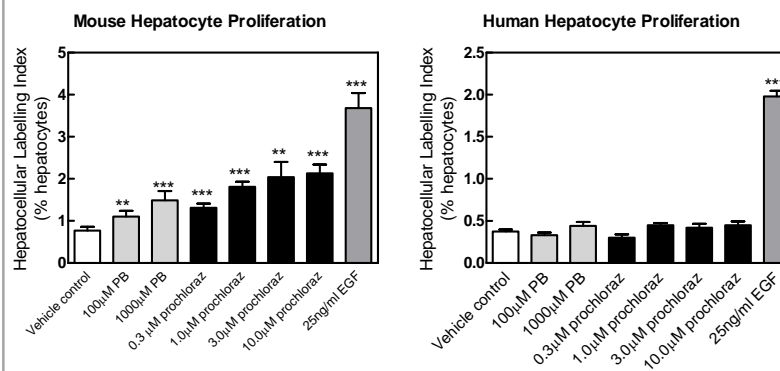
Cytotoxicity was assessed by measurement of cellular ATP concentrations and cell proliferation (S-phase) was evaluated using BrdU incorporation. CAR and PXR activation were assessed indirectly by evaluating the expression of CYP2B and CYP3A mRNA by Taqman. Phenobarbital (PB) and EGF were used as positive controls.

Values are Mean ± SD (n = 6 for Taqman and n = 5 for S-phase. A Student's t-test (2-sided) was performed on the results; \*statistically different from control p<0.05; \*\*p<0.01, \*\*\*p<0.001.

## RESULTS

- Cell proliferation and enzyme induction were assessed at 0.3-10µM, as a dose of 10µM Prochloraz and above caused cytotoxicity (ATP↓) in human cells.
- Prochloraz induced Cyp2b10 and Cyp3a11 in mice (2- and 8-fold, respectively), and CYP2B6 and CYP3A4 expression in humans (2- and 3-fold, respectively).
- Prochloraz exhibited a dose-response for stimulation of hepatocyte proliferation (labelling index) in mouse hepatocytes, while no increase in proliferation was seen in human hepatocytes. The positive controls PB (1000µM) and EGF produced the expected increases in cell proliferation and confirmed sensitivity of the test system (Fig. 1).

**Figure 1. Comparison of Mouse and Human Hepatocytes: Cell Proliferation.**



## SUMMARY

Key events for proposed mode of action and their occurrence in mice

Dose (ppm in diet) & mouse strain	Key Event 1 CAR/PXR Receptor Activation Cyp2b10 Transcripts & Protein 7 and 14 days	Key Event 2 Hepatocellular Proliferation 7 days	Key Event 3 Increased Hepatocellular Tumours 18 Months
78 Wt			-
325 Wt	++	+	+
325 CAR/PXR KO	-	-	
325 hCAR/hPXR	(+)	-	
1300 Wt	++	++	++

Wt, wild type; KO, knockout; h, humanized

- Temporality (sequence of events leading to adverse outcome) is shown towards the end of life span
- Dose-response of effects is illustrated
- Alternative modes of action can be excluded (not genotoxic based on robust study package; no AHR- or PPAR-activation as no induction specific receptor-regulated enzymes; no chronic inflammation/cytotoxicity in histopathology of the (sub)chronic studies).

The collective (*in vitro* and *in vivo*) data presented can be summarised as follows:

Key Event	Qualitative Concordance		Quantitative Species Concordance	Quantitative Dose Response	Confidence/Uncertainty
	Evidence in Mice	Evidence in Humans			
CAR/PXR Receptor Activation Cyp2b10 Transcripts & Protein	Yes	Yes	No major differences between PB-mediated CYP induction in mouse and human hepatocytes	Clear dose response in wild type mice with prochloraz, generally weaker response in humanized, no response in KO at tumorigenic dose	Medium to high confidence/ indirect proof of receptor binding, but very robust transcript and protein response
Hepatocellular Proliferation	Yes	No	Not applicable	Clear dose response in mice with prochloraz, no proliferation in human	High confidence
Increased Hepatocellular Tumours	Yes	No		Clear dose response in mice with prochloraz, no evidence in epidemiology of PB carcinogenicity in humans	Medium to high confidence. Epidemiology on PB is limited

## CONCLUSIONS

- Prochloraz activates CAR and possibly PXR in both mouse and human hepatocytes. While stimulation of cell proliferation was shown in mouse hepatocytes, increased cell proliferation was not demonstrated in human hepatocytes exposed to prochloraz.
- Temporality and dose-response of key events are shown. Alternative modes of action were investigated and could be excluded. Biological plausibility exists.
- As cell proliferation is the key event in CAR-mediated induction of rodent liver tumours (Elcombe *et al.*, 2014); the absence of cell proliferation in prochloraz-exposed human hepatocytes strongly suggests that the increase in liver tumour incidence caused by prochloraz in mice is not relevant to humans.

## REFERENCES

- Elcombe *et al.*, (2014). *Crit. Rev. Toxicol.* **44**: 64-82.  
 Melching-Kollmuss *et al.*, (2015). *The Toxicologist*, 1634.  
 Mitchell *et al.*, (1984). *Arch. Toxicol.* **55**, 239-246.