In vitro toxicity assessment of the nucleoside analog fialuridine using micropatterned primary hepatocyte cocultures and discovery of a non-toxic isomer

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INTRODUCTION

Fialuridine, a nucleoside analog designed for the treatment of hepatitis B virus infection, failed in clinical trials due to fatal hepatotoxicity, occuring after 13 weeks of 0.25 mg/kg qd dosing in HBV infected patients. Both *in vitro* assays as well as preclinical *in vivo* tests in mice, rats, dogs, and primates could not predict its hepatotoxic potential. Fialuridine toxicity is thought to be mediated by human-specific impaired mitochondrial function after long-term exposure. We used the micropatterned primary hepatocyte coculture model, HepatoPac™, to assess fialuridine-mediated toxicity *in vitro*. To confirm the human specific toxicity, also rat, dog and monkey HepatoPac™ were exposed to fialuridine.

Another nucleoside analog, sofosbuvir, which is on the market for the treatment of hepatitis C virus infection and shows no signs of hepatotoxicity in the clinic, was used to study whether this approach would be able to distinguish a nucleoside analog with clinical chronic DILI finding from a nucleoside analog without DILI findings.

METHODS

In the HepatoPac™ model, primary parenchymal liver cells are seeded onto collagen spots and subsequently surrounded by mouse 3T3-J2 fibroblasts as supporting cell line. The cultures were exposed to increasing concentrations of fialuridine up to 9 days in serum-free medium, which was refreshed every two days. Toxicity was evaluated by the nondestructive endpoints albumin and urea secretion, and the lysate-based endpoint ATP-content. In addition, viability was measured using a resazurin-based assay (PrestoBlue).

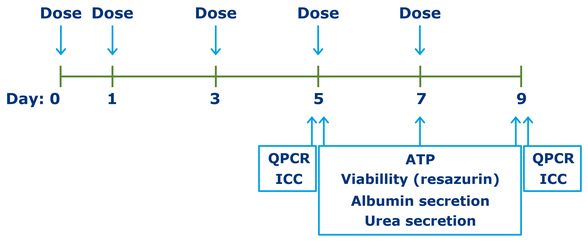

Fig. 1: Schematic overview of the experimental setup

Fialuridine:	0.015	0.045	0.137	0.412	1.23	3.71	11.1	33.3	100	300
Sofosbuvir:	0.412	3.71	33.3							

Table 1: Incubated concentrations in µM

	Dose:	Cmax:	Fup:	Free plasma Cmax:
Fialuridine:	17.5 mg qd	1.14	0.18	0.205
Sofosbuvir:	400 mg qd	0.64	0.36	0.230

Table 2: Clinical total and free Cmax values of fialuridine and sofosbuvir in µM

RESULTS

Discovery of a non-toxic fialuridine isomer

Initial studies indicated notable differences in the hepatotoxic potential of different fialuridine batches acquired from different commercial vendors. Further analysis by ^{1}H-NMR spectroscopy revealed that some of these batches did not contain fialuridine (FIAU) but its 2' epimer (FIRU), *i.e.* with respect to the base, the fluorine atom is oriented at the opposite face of the sugar ring.

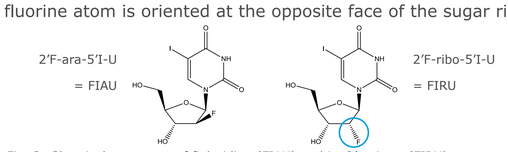

Fig. 2: Chemical structures of fialuridine (FIAU) and its 2' epimer (FIRU)

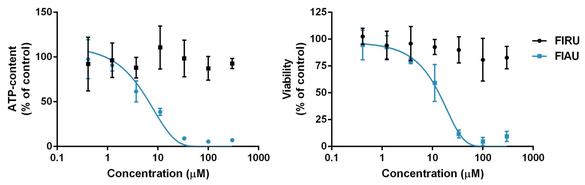

Fig. 3: Effects of FIAU and FIRU on ATP content and viability of human HepatoPac™ cultures after 9 days of exposure. Data represent mean ± SEM of triplicate incubations

A clear concentration-dependent inhibition of ATP content and viability was found for fialuridine, whereas FIRU does not appear to be toxic to the human hepatocyte cocultures.

Fialuridine-mediated toxicity in human HepatoPac™

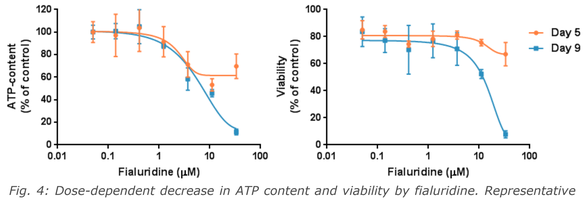

Fig. 4: Dose-dependent decrease in ATP content and viability by fialuridine. Representative data from one out of three independent experiments is depicted (mean ± SEM of three incubations).

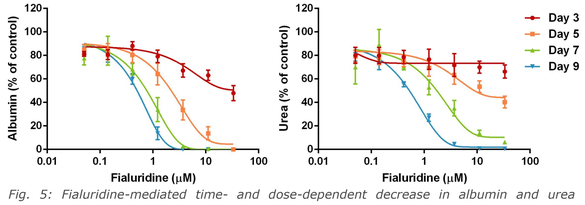

Fig. 5: Fialuridine-mediated time- and dose-dependent decrease in albumin and urea secretion. Representative data from one out of three independent experiments is depicted (mean ± SEM of six incubations).

All parameters tested were dose-dependently affected by fialuridine, although the severity of the effects were highly dependent on the endpoint.

Mitochondria-related hepatotoxicity of fialuridine

To further unravel the mechanism of fialuridine-mediated hepatotoxicity, secreted lactate levels were determined as a surrogate measure for the rate of glycolysis and the expression of the mitochondrially encoded cytochrome C oxidase subunit 1 (MTCO1) was assessed.

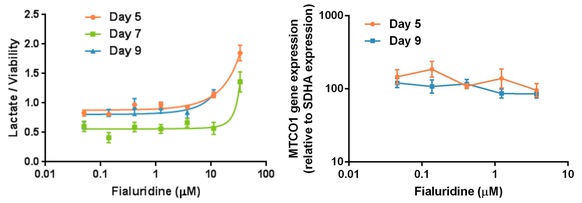

Fig. 6: Lactate levels in medium after fialuridine exposure, normalized to viability. Data represent mean ± SEM of triplicate incubations. Absolute lactate levels increased at concentrations > 3.7 µM for day 3, 5 and 7 but not for day 9 (data not shown).

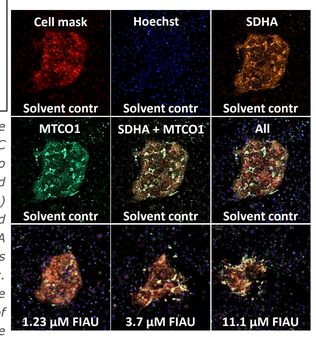

Fig. 7: mRNA and protein expression of the mitochondrially encoded cytochrome C oxidase subunit 1 (MTCO1) relative to expression of the nuclear encoded succinate dehydrogenase subunit A (SDHA) obtained by QPCR (above) and immunocytochemistry (bottom). mRNA samples of high fialuridine concentrations could not be measured due to toxicity. Data represent mean ± SEM of triplicate incubations. Representative stainings of fialuridine-treated cultures at day 5 were shown. Protein expression levels were determined by image analysis (not shown).

Fialuridine-mediated increase in relative lactate levels indicate an increase in glycolysis, which might be related to impaired mitochondrial function as a compensation mechanism to prevent energy depletion. A proposed hypothesis that fialuridine would interfere with mRNA synthesis of mitochondrially encoded genes was not confirmed by QPCR and immunocytochemistry analysis.

Effects of sofosbuvir on human HepatoPac™

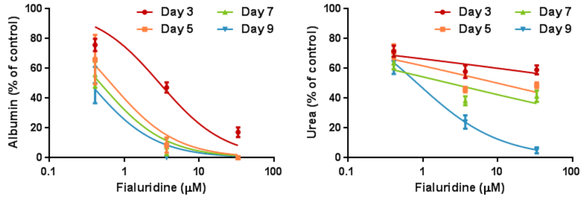

Fig. 8: Effect of sofosbuvir albumin and urea secretion. Data represent mean ± SEM of triplicate incubations

Sofosbuvir did not affect viability of the HepatoPac cultures and only minimally affected ATP content at day 9 (data not shown). In contrast, albumin and urea secretion was clearly attenuated.

Effect of fialuridine in preclinical species

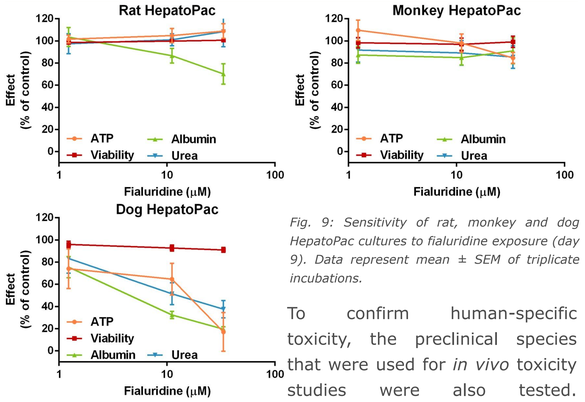

Fig. 9: Sensitivity of rat, monkey and dog HepatoPac cultures to fialuridine exposure (day 9). Data represent mean ± SEM of triplicate incubations.

To confirm human-specific toxicity, the preclinical species that were used for *in vivo* toxicity studies were also tested. No significant toxicity was found in rat and cynomolgus monkey HepatoPac cultures, whereas dog HepatoPac cultures showed sensitivity to fialuridine at ≥ 11.1 µM. The largest effects were seen on day 9. Earlier time points have been evaluated but not depicted.

Overview of the results

		Fialuridine				Sofosbuvir*			
		Day 3	Day 5	Day 7	Day 9	Day 3	Day 5	Day 7	Day 9
Human:	ATP	ND	>33	ND	12	X	>33	X	>33
	Viability	ND	>33	ND	7	X	>33	X	>33
	Albumin	>33	2	0.6	0.4	0.4-3.7	0.4-3.7	0.4-3.7	0.4-3.7
	Urea	>33	15	1	0.4	>33	>33	3.7-33	0.4-3.7
Dog:	ATP	ND	>33	ND	11-33				
	Viability	ND	>33	ND	>33				
	Albumin	>33	15	10	5				
	Urea	>33	ND	25	14				

*Table 2: IC50 values in µM. *For sofosbuvir, no IC50 values could be determined because only three concentrations were used.*

IC50 values of hepatocyte-specific functions (albumin and urea secretion) were much lower than IC50 values of general cytotoxicity endpoints (ATP content and viability). Possible explanations include that, in this donor, albumin and urea are most sensitive as hepatotoxicity markersm, or that fialuridine provokes hepatocyte dedifferentiation.

CONCLUSIONS

- Our results indicate that HepatoPac is a useful model to identify non-acute hepatotoxicants and study their mechanism of toxicity
- We show for the first time a multiple-species *in vitro* approach to pick up human-specific fialuridine-mediated hepatotoxicity
- *In vitro* observations from this study were not in line with the reported differences in clinical outcome between fialuridine and sofosbuvir
- The precise mechanism of toxicity needs further investigation, as well as the potential mechanistic differences between fialuridine- and sofosbuvir-mediated toxicity

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