Hepatotoxic Mechanisms by Drug-Induced Cholestasis and Reactive Metabolites in Human Hepatocyte Co-Culture System

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Abstract

Background and Purpose: Hepatotoxicity is a critical concern in drug development because the liver is the primary organ responsible for drug metabolism and detoxification. Drug-induced cholestasis (DIC) represents a major subtype of drug-induced liver injury (DILI) that arises when compounds disrupt bile acid synthesis, uptake, or efflux in hepatocytes. This leads to bile acid accumulation and liver injury. Early detection of cholestatic potential is essential, as DIC is often species-specific and not reliably predicted by conventional animal models. Reactive drug metabolites are unstable intermediates formed during xenobiotic biotransformation, primarily mediated by hepatic cytochrome P450 (CYP) enzymes. These metabolites can covalently bind to cellular macromolecules such as proteins, lipids, and DNA, causing oxidative stress, mitochondrial dysfunction, and immune-mediated hepatocellular injury. Traditional liver toxicity assessments relying on animal models and isolated liver tissues have limitations including high cost, low throughput, interspecies variability, ethical concerns, and limited predictivity for human outcomes. We previously developed an advanced hepatocyte co-culture system that maintains long-term viability (>4 weeks) and stable metabolic function. These models (human, dog, and rat) were validated with 29 reference compounds, demonstrating dose-dependent hepatotoxicity, species-specific responses, and improved sensitivity (83-92%) compared to hepatocyte monocultures (50%). In the present study, we utilized the human hepatocyte co-culture system to evaluate compounds associated with DIC and determine the effect of reactive metabolites on hepatotoxicity.

Methods: The human hepatocyte system was generated by co-culturing cryopreserved primary human hepatocytes with stromal fibroblast cells in collagen-coated 96-well plates for one week prior to test compound exposure. For the DIC study, cholestatic compounds (cyclosporin A and bosentan) were tested at seven concentrations in the presence and absence of a bile acid (BA) mixture to mimic physiological cholestatic conditions. For the reactive metabolite study, compounds known to produce bioactivated intermediates (cyclophosphamide and aflatoxin B_1) were tested at seven concentrations with and without a broad pan-CYP inhibitor, aminobenzotriazole (1 mM). Hepatotoxicity was assessed using an ATP cell viability assay after 7-day exposure to the test compounds. TC_{50} values were calculated from non-linear regression curves fitted with GraphPad software.

Results: The results demonstrated dose-dependent hepatotoxic effects for the test compounds. In the DIC study, TC_{50} values for both cyclosporin A and bosentan were significantly lower with BA supplementation, indicating bile acid associated hepatotoxicity. In the reactive metabolite study, co-treatment with the CYP inhibitor significantly reduced the cytotoxicity of both cyclophosphamide and aflatoxin B_1. TC_{50} values for these compounds were significantly higher with the CYP inhibitor, confirming that their hepatotoxicity is mediated by reactive metabolite formation.

Conclusions: The human hepatocyte co-culture model provides a robust and predictive *in vitro* platform for elucidating hepatotoxic mechanisms associated with cholestasis and reactive metabolites. These models enable identification of cholestatic risk, mechanistic evaluation of bioactivation and detoxification pathways, and detection of delayed or cumulative hepatotoxicity from repeated exposure. Overall, these models reduce reliance on animal testing and improve human-relevant risk assessment, supporting the design and development of safer drug candidates.

Methods

Hepatocyte Co-cultures: Cryopreserved hepatocytes and non-parenchymal stromal cells were cultured with HUREL maintenance media. Cells were seeded into collagen coated 96-well plates. After culturing for 7 days to stabilize the cultures, cells were treated with drug compounds.

Compound Dosing: Toxicity effects were tested at seven concentrations. Cell cultures were re-dosed every 48 hours. Fresh media and compound were added at each re-dosing. For the DIC study, cholestatic compounds (cyclosporin A and bosentan) were tested in the presence and absence of a bile acid (BA) mixture to mimic physiological cholestatic conditions. The final concentrations of bile acids mix were 52.8 µM glycochenodeoxycholic acid, 15.6 µM chenodeoxycholic acid, 15.2 µM glycodeoxycholic acid, 16 µM deoxycholic acid, and 14 µM glycocholic acid. For the reactive metabolite study, compounds known to produce bioactivated intermediates (cyclophosphamide and aflatoxin B_1) were tested with and without a broad pan-CYP inhibitor, aminobenzotriazole (1 mM).

Morphology and Immuno-Staining: Cellular morphology was visualized using phase-contrast microscopy. Bile canaliculi were stained with Carboxy-DCFDA and imaged via fluorescence microscopy.

Cell Viability Assay and Data Analysis: Cellular toxicity was measured with the ATP cell viability assay using the CellTiter-Glo ®(Promega). Luminescence signals were measured by the microplate reader. The ATP assay data was used to determine TC_{50} values. GraphPad software was used for fitting curves to data using non-linear regression analysis. TC_{50} values were determined from the curve fitted data.

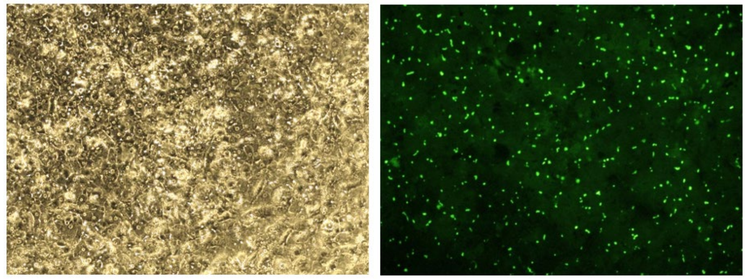

Figure 1: Morphology and bile canalicular formation in H*µ*REL*humanPool*™ hepatocyte co-culture model (day 14). 10x magnification.

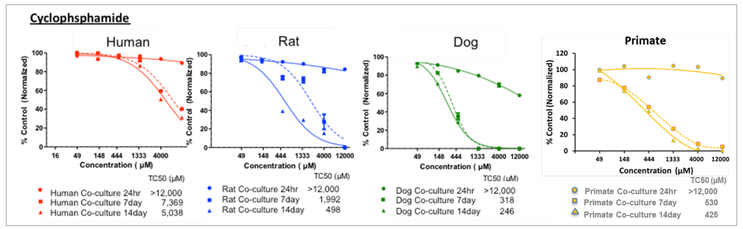

Figure 2: Dose-dependent cytotoxicity after single and repeated treatments in HUREL primary hepatic co-cultures. Non-linear regression curves estimating dose-dependent cytotoxicity after single and repeated treatments of the HUREL human, monkey, dog, and rat hepatic co-cultures with cyclophosphamide, respectively. In each instance, the greatest increase in cytotoxicity was measured between 24 h and Day 7, with some additional increase measured between Day 7 and Day 14. *Data from Novik et al, Toxicology and Applied Pharmacology [1].*

Table 1: Sensitivity and specificity of cytotoxic response to compounds of Sample Set I. *Data from Novik et al, Toxicology and Applied Pharmacology [1].*

			A	B			C	D			E		
Compound	Clinical DILI	Human C_{max} (µM)	Human mono-culture TC50/C_{max}	Human co-culture TC50/C_{max}			Rat (fresh) mono-culture TC50/C_{max}	Rat (cryo) co-culture TC50/C_{max}			Dog co-culture TC50/C_{max}		
			24 hr	24 hr	7 day	14 day	24 hr	24hr	7 day	14 day	24 hr	7 day	14 day
Troglitazone	Positive	6.39	25	15	9	7	14	13	13	17	13	12	14
Nefazodone		0.92	41	74	68	59	75	76	86	53	83	69	70
Benzbromarone		4.30	>104	44	8	9	12	29	12	7	37	9	9
Amiodarone		0.81	41	>215	15	11	38	>215	28	11	>215	30	22
Flutamide		6.00	25	>167	73	70	14	>167	75	69	132	77	73
Bosentan		2.00	382	807	104	41	407	469	158	59	721	102	63
Bicalutamide		1.97	>187	>187	>187	123	71	>187	>187	>187	>187	141	70
Diclofenac		4.20	212	250	109	75	75	213	74	56	184	59	59
Tacrine		0.06	2,016	>4,048	2,349	1,381	2,683	>4,048	1,937	635	>4,048	1,778	1,762
Chlorpromazine		0.84	25	83	36	33	37	83	30	31	88	81	86
Cylophosphamide		143.00	>84	>84	52	35	16	>84	14	4	>84	2	2
Acetaminophen		130.00	45	>308	60	43	105	>308	87	75	>308	52	37
	True Positive		6	4	8	10	9	4	9	10	4	9	11
	Total Positive		12	12	12	12	12	12	12	12	12	12	12
	Sensitivity (%)		50%	33%	67%	83%	75%	33%	75%	83%	33%	75%	92%
Propranolol	Negative	0.20	525	1,350	465	310	695	1,760	610	355	1,420	665	495
Rosiglitazone		1.04	432	678	408	327	399	620	517	351	723	570	150
Diphenhydramine		0.30	510	>1,667	580	547	690	>1,667	800	383	>1,667	>1,667	>1,667
Isoproterenol		0.006	>83,333	>250,000	>250,000	>250,000	>83,333	>250,000	>250,000	>250,000	>250,000	>250,000	>250,000
Kanamycin		72.20	>554	>554	245	169	285	>554	401	193	>554	>554	250
Macitentan		0.30	2,159	1,706	316	347	575	2,038	359	334	3,669	263	353
Primidone		4.67	>298	>298	>298	>298	>298	>298	>298	>298	>298	>298	>298
	True Negative		7	7	7	7	7	7	7	7	7	7	7
	Total Negative		7	7	7	7	7	7	7	7	7	7	7
	Specificity (%)		100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

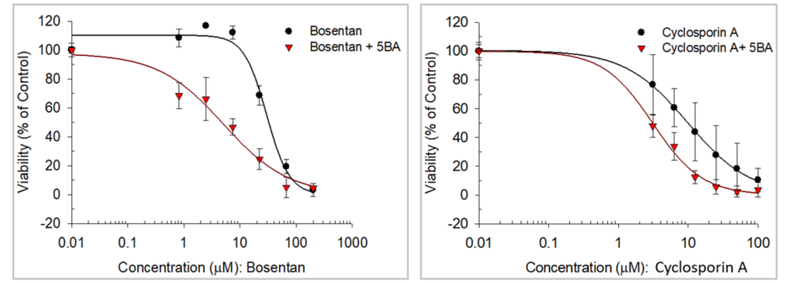

Figure 3: Drug-induced cholestatic injury (DICI) in H*µ*REL*humanPool*™ hepatocyte co-culture model (day 7). Compounds with known cholestatic liability and bile acids (Bosentan and Cyclosporin A) were tested in the presence and the absence of 5 bile acid mixture (5BA).

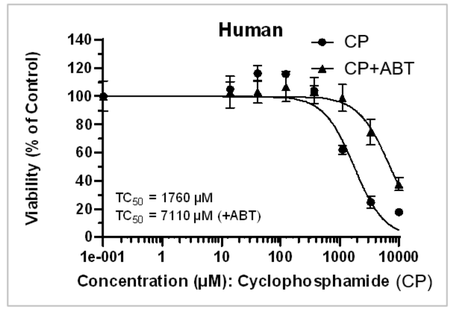

Figure 4: Effect of hepatotoxicity by reactive metabolites in H*µ*REL*humanPool*™ hepatocyte co-culture model (day 7). Compounds known to produce bioactivated intermediates (cyclophosphamide and aflatoxin B_1 (data not shown)) were tested at seven concentrations with and without the broad pan-CYP inhibitor, aminobenzotriazole (ABT, 1 mM).

Conclusion

- Time- and dose-dependent hepatotoxicity was observed in all species cultures treated with positive control compounds. With the increase of compound concentration and incubation time, the cell viability decreased.
- For the DIC study, TC_{50} values for both cyclosporin A and bosentan were significantly lower with bile acid supplementation, indicating hepatotoxicity caused by cholestasis.
- In the reactive metabolite study, co-treatment with the CYP inhibitor significantly reduced the cytotoxicity of cyclophosphamide, confirming that their hepatotoxicity is mediated by reactive metabolite formation.
- The HUREL human hepatocyte co-culture model provides a robust and predictive *in vitro* platform for elucidating hepatotoxic mechanisms associated with cholestasis and reactive metabolites.
- Overall, these models reduce reliance on animal testing and improve human-relevant risk assessment, supporting the design and development of safer drug candidates.

References

Novik E, Dwyer J, Morelli J, Parekh A, Cho C, Pludwinski E, Shrirao A, Freedman R, MacDonald J and Jayyosi Z. “Long-enduring primary hepatocyte-based co-cultures improve prediction of hepatotoxicity”. Toxicology and Applied Pharmacology, 336 (2017) 20-30