AN INTEGRATED IN VITRO SCREEN USING SANDWICH-CULTURED HUMAN HEPATOCYTES FOR PREDICTION OF CHOLESTATIC HEPATOTOXICITY

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ABSTRACT

Cholestatic DILI in humans has been associated with bile salt export pump (BSEP) inhibition; however, in vitro BSEP IC50 concentrations do not correlate with in vivo cholestatic DILI severity. Sandwich-cultured human hepatocytes (SCHH) when treated with BSEP inhibitors respond to the resulting increased intracellular concentration (ICC) of bile acids (BA), by activation of FXR (adaptive response). This results in decreased synthesis of BA and increased expression of basolateral and canalicular efflux transporters for BA via OST alpha/beta, and BSEP which prevents cholestatic hepatotoxicity. We evaluated the time course of this adaptive response, changes in the ICC of BA, the effects of FXR antagonists, the in vivo relevance, and whether integration of FXR regulatory effects would improve the prediction of cholestatic DILI. Cryopreserved, TRANSPORTER CERTIFIED™ human hepatocytes in a sandwich configuration were cultured using QUALGRO™ Media for 5 days. On Day 5 of culture, the time course of the adaptive response was determined by determining the effect of cyclosporine A on the biliary excretion, and ICC of endogenous bile acids (LCMS analysis), in parallel with FXR activation (gene expression - TaqMan® primer/probe sets). Mechanistic modeling was used to determine the functional effects of mRNA based changes in FXR activation. The effect on the ER stress biomarker, CHOP, following 12 hours of exposure to CsA (10 µM), Trog (100 µM), or DY268 (5 µM) under sensitization conditions (250 µM BA pool + 1 mM free fatty acids (FFA)) was also evaluated. In a separate study, 49 compounds with varying degrees of BSEP inhibition and DILI (NIH LiverTox database) were evaluated (24 hr exposure, sensitization conditions) for their potential to affect the adaptive response. Cyclosporine A decreased the biliary excretion of endogenous bile acids in a time dependent manner, with a parallel increase in the ICC of BA, followed by activation of FXR. FXR activation resulted in a 2X increase in the biliary efflux clearance, and a 6X increase in the basolateral efflux clearance (adaptive response). Co-administration of FXR antagonists reduced the FXR mediated response to 50 and 5% of control for troglitazone and DY268, respectively. Following 12 hours of exposure, CHOP mRNA content was induced ≤ 2.0-fold above solvent control in SCHH treated with CsA (10 µM) or DY268 (5 µM) in the presence of a BA pool + FFA. CHOP mRNA content was increased to 7.1-fold above solvent control in SCHH treated with Trog (100 µM) in the presence of BA pool + FFA. Integration of the effect on the adaptive response in addition to the effect on BSEP inhibition improved the accuracy for prediction of cholestatic DILI from 22% (BSEP inhibition alone) to 93%. In addition to BSEP inhibition, integration of inhibition of basolateral efflux and/or interference with the adaptive response (FXR antagonism) allows for more accurate prediction of cholestatic DILI.

OBJECTIVES

Demonstrate:

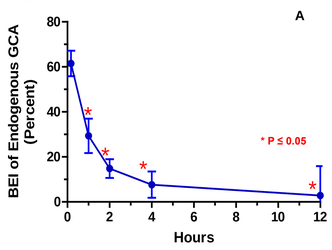
- BSEP inhibition "triggers" FXR activation initiating BA compensatory mechanism to prevent BA-induced hepatotoxicity
- Basolateral efflux is an important BA efflux pathway following FXR activation
- Interference with BA compensatory mechanism (e.g. FXR) key factor in BA-induced (e.g. cholestatic) DILI
- C-DILI™ Assay accurately predicts DILI potential

METHODS

Human Hepatocytes Cryopreserved, TRANSPORTER CERTIFIED™ human hepatocytes in a sandwich configuration were cultured using QualGro ™ Media for 5 days. **Treatments** On Day 4 of culture, hepatocyte cultures were exposed to test compounds (Table 1) at 20X (or limit of solubility) of their systemic C_{max} in QUALGRO™ Sensitization Media (physiologically relevant concentrations of lipids and bile acids) for 24 hours. Approximately 49 compounds with a range of BSEP IC_{50}s and associated with clinical DILI were tested. Treatment with vehicle control and standard QualGro™ Media was used as a control. **Gene Expression** mRNA content of various transporters, synthetic enzymes, and regulatory factors from SCHH was determined from each RT reaction using gene-specific TaqMan® primer/probe sets. All reactions were normalized to the endogenous control GAPDH. Amplifications were performed on an ABI ViiA7 Real-Time PCR System in relative quantification mode. Relative-fold mRNA content was determined for each treatment group relative to the vehicle control. **Endogenous Bile Acids** LC-MS/MS which employed reversed-phase HPLC and electrospray ionization was used to quantitate endogenously generated cholic acid (CA), CDCA, and their taurine (TCA, TCDCA) and glycine (GCA, GCDCA) conjugates in cells, bile and cell culture media.

RESULTS AND DISCUSSION

Figure 1



Exposure to Cyclosporine A (10 µM), a potent BSEP inhibitor leads to a **rapid, time dependent decrease** in biliary excretion of endogenous bile acids.

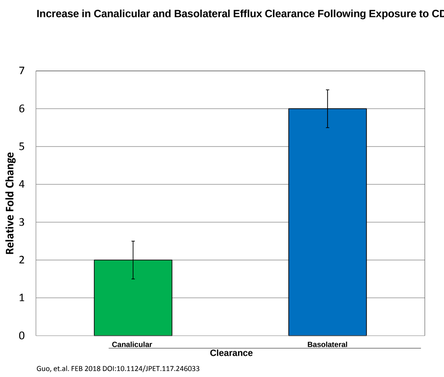
Inhibition of biliary excretion leads to an increase in the intracellular concentration of endogenous bile acids.

Increased intracellular concentrations of bile acids activate FXR (increased FGF19).

Figure 2

Chronic exposure to CDCA increases mRNA expression of BSEP (canalicular efflux transporter) and OSTα/β (basolateral efflux transporters) by approximately 8X and 80X, respectively[2]

Molecular modeling was utilized to estimate changes in the canalicular and basolateral efflux clearances following 72 hours of exposure to CDCA (100 µM). Consistent with the changes in mRNA, the canalicular clearance increased by approximately 2X while the basolateral clearance increased by 6X.
In the induced state basolateral efflux clearance mediated by OSTα/β represents an important elimination pathway.



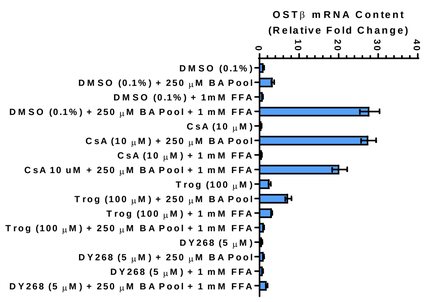
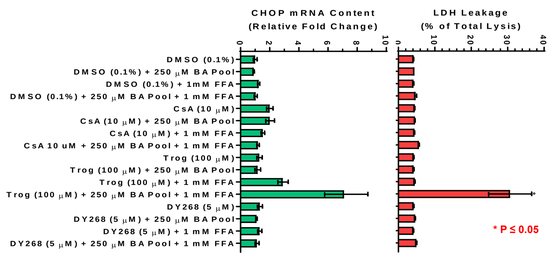


Figure 3

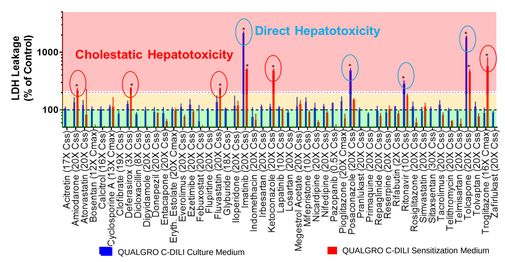
Following 12 hours of exposure, synergistic increases of OSTβ (27.8-fold) mRNA content were observed in SCHH treated under sensitization conditions (BA Pool + FFA) in the absence or presence of Cyclosporine A (CsA). Trog or DY268 (potent FXR antagonist) exposure markedly reduced OSTβ mRNA content ≥ 74% and ≥91%, respectively. These results suggested troglitazone antagonizes FXR reducing the effectiveness of the BA efflux compensatory mechanism (OSTα/β)

Figure 4



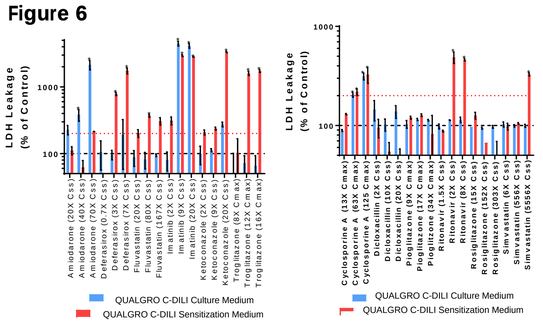
CCAAT/enhancer-binding protein homologous protein (CHOP) is a key component in the ER stress-mediated cell death pathways. CHOP mRNA expression was increased > 3-fold in SCHH treated with troglitazone under sensitization conditions (e.g. BA pool + FFA) for 12 hours. Concomitant increase in LDH leakage > 7.3-fold above solvent control was also observed. These results suggested troglitazone treatment under sensitization conditions induced ER stress resulting in BA-induced (e.g. cholestatic) hepatocyte injury.

Figure 5



49 compounds with varying degrees of BSEP inhibition and clinical DILI (NIH LiverTox database) were evaluated in the C-DILI™ Assay. Test concentrations were based on reported plasma C_{max} or steady state (C_{ss}) values. Portal vein concentrations of orally administered medications can be order of magnitudes higher than systemic exposure; therefore, 20X C_{max} or C_{ss} test concentrations were targeted unless limited by solubility. * p-value ≤ 0.05

Figure 6



A selection of compounds were re-evaluated across a range of concentrations in a separate lot of hepatocytes to evaluate dose-dependency and to confirm previous results. * p-value ≤ 0.05

Figure 7

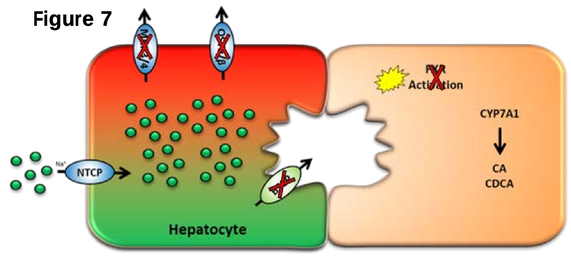


Table 1

49 compounds with varying degrees of BSEP inhibition and clinical DILI (NIH LiverTox database) were evaluated in the C-DILI™ Assay. The C-DILI™ Assay reduces the number of false positives predicted by hBSEP IC_{50} cutoff of 25 µM and identifies mode of toxicity. NA-not applicable.

Table 1 Compounds Evaluated in C-DILI™ Assay

Drug	hBSEP Vesicle IC_{50} (µM)[3]	C-DILI™ Assay Hepatotoxicity Potential	C-DILI™ Assay Hepatotoxicity Mechanism
Acitretin	38	Low Potential	NA
Amiodarone	43	High Potential	General
Atorvastatin	13	Low Potential	NA
Bosentan	23	Low Potential	NA
Calcitrol	40	Low Potential	NA
CsA	0.5	Low Potential	NA
Clofibrate	71	Low Potential	NA
Deferasirox	58	High Potential	Cholestatic
Dicloxacillin	56	Low Potential	NA
Dipyridamole	3.8	Low Potential	NA
Donepezil	78	Low Potential	NA
Entacapone	56	Low Potential	NA
Eryth. Estolate	13	Low Potential	NA
Everolimus	2.0	Low Potential	NA
Ezetimibe	56	Low Potential	NA
Febuxostat	43	Low Potential	NA
Flupirtine	36	Low Potential	NA
Fluvastatin	36	High Potential	Cholestatic
Glyburide	5.0	Low Potential	NA
Iloperidone	23	Low Potential	NA
Imatinib	25	High Potential	General
Indomethacin	42	Low Potential	NA
Irbesartan	7.3	Low Potential	NA
Ketoconazole	3.4	High Potential	Cholestatic
Lapatinib	6.5	Low Potential	NA
Losartan	8.5	Low Potential	NA
Megestrol Acetate	18	Low Potential	NA
Mifepristone	2.0	Low Potential	NA
Nicardipine	7.9	Low Potential	NA
Nifedipine	64	Low Potential	NA
Pazopanib	10	Low Potential	NA
Pioglitazone	0.3	Low Potential	NA
Posaconazole	8.1	High Potential	General
Pranlukast	2.9	Low Potential	NA
Primaquine	33	Low Potential	NA
Repaglinide	22	Low Potential	NA
Reserpine	8.4	Low Potential	NA
Rifabutin	27	Low Potential	NA
Ritonavir	1.6	High Potential	Cholestatic
Rosiglitazone	2.8	Low Potential	NA
Simvastatin	25	Low Potential	NA
Sitaxsentan	13	Low Potential	NA
Tacrolimus	7.2	Low Potential	NA
Telithromycin	5.0	Low Potential	NA
Telmisartan	16	Low Potential	NA
Tolcapone	37	High Potential	General
Tolvaptan	9.9	Low Potential	NA
Troglitazone	2.7	High Potential	Cholestatic
Zafirlukast	11	Low Potential	NA

Inhibition of BSEP along with antagonism of FXR activation in response to increased intracellular concentrations of bile acids, and/or inhibition of bile acid basolateral efflux transporters can lead to increased cholestatic hepatotoxicity.

Drugs that impact multiple pathways or single pathways when given in combination with other drugs that affect alternate pathways could also result in hepatotoxicity. E.g. drug interaction with a BSEP inhibitor and FXR antagonist or basolateral efflux transporter inhibitor.

C-DILI™ Assay

The cultures were measured for ATP content (CellTiter-Glo® Promega) and LDH secretion (CytoTox-ONE™ Promega) following 24 hours of treatment. The LiverTox data base was used to identify compounds that were consistent with hepatocellular injury. LDH readout was used as a surrogate marker of cholestatic bile acid toxicity.

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CONCLUSIONS

The C-DILI™ Assay correctly predicted compounds with significant clinical hepatocellular cholestatic toxicity:

- Integrates effects on BSEP, OSTα/β, MRP3/4, and FXR to delineate hepatotoxicity resulting from a build up of intracellular bile acids.
- BSEP inhibition alone does not result in BA-induced (e.g. cholestatic) liver injury
- Contingency Analysis: 95% Accuracy; 81% Sensitivity Score (ability to predict toxicity); 100% Specificity Score (ability to predict no toxicity)
- C-DILI™ Assay results consistent with clinical evidence of liver injury

Key assay features:

- TRANSPORTER CERTIFIED™ human hepatocytes in a model that maintains transporter expression, localization and function
- Convenient 24 hour incubation in 96-well format in optimized media
- Assess effects of parent and metabolites simultaneously
- Integrated effects of transporter inhibition and hepatocyte adaptive response in a single toxicity readout
- Evaluate cholestatic vs. direct toxicity
- Available as a service or kit

[1] Dawson et.al., Drug Metab Dispos 40,130, 2012, [2] Jackson et.al., App In Vitro Tox 2, 4, 2016, [3] Morgan et.al. Tox Sci 136,1, 2013, [4] https://livertox.nih.gov/ [5] Malinen et.al. Am J Physio – GI & Liver Physio. Feb 2018