# C-DILI™ Assay: Integrating BSEP Inhibition and FXR Antagonism to Improve Prediction of Cholestatic Drug Induced Liver Injury

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## INTRODUCTION

Cholestatic Drug Induced Liver Injury (DILI) in humans has been associated with bile salt export pump (BSEP) inhibition ($IC_{50}$s < 25 µM)[1]; however, *in vitro* BSEP $IC_{50}$ 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 concentrations of bile acids (BA), via activation of FXR (adaptive response). This results in decreased synthesis of BA and increased expression of basolateral and canalicular efflux of BA via OSTα/β, and BSEP which prevents cholestatic hepatotoxicity. We propose that BSEP inhibition alone may not be sufficient to induce toxicity due to this adaptive response. In addition to BSEP inhibition, inhibition of basolateral efflux and/or interference with the adaptive response (FXR antagonism) may lead to increases in drug-induced cholestatic bile acid hepatotoxicity. Such mechanisms must be incorporated to accurately predict *in-vivo* cholestatic drug induced liver injury (DILI).

We have determined the time dependence of this adaptive response, linking inhibition of BSEP, increases in the intracellular concentration of BA, activation of FXR, and evaluated the effects of FXR antagonists. A predictive model for cholestatic hepatotoxicity was developed (C-DILI™ Assay) which integrates the effects of BSEP inhibition, basolateral efflux inhibition, and FXR antagonism using a human hepatocyte system.

## 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 Qual-Gro™ 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.

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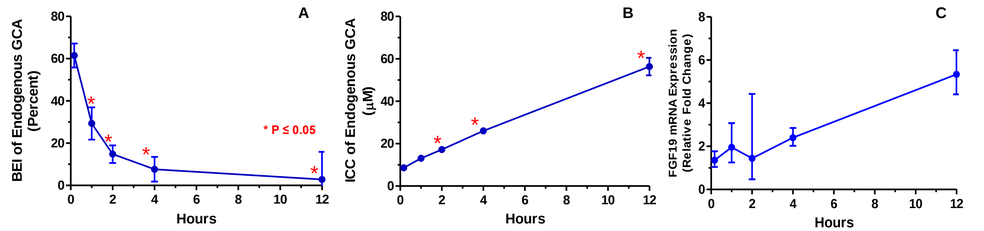
## RESULTS AND DISCUSSION

**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.

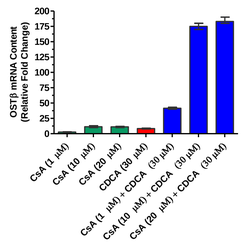
Inhibition of biliary excretion leads to an increase in the intracellular concentration of endogenous bile acids.

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

**Figure 2** Increased OSTβ expression through activation of FXR (adaptive response) observed:

- in the presence of a BSEP inhibitor (CsA)
- Increased intracellular concentration of bile acids by addition of CDCA

Synergistic effect observed in the presence of CsA **and** CDCA due to greatly increased intracellular concentrations of bile acids



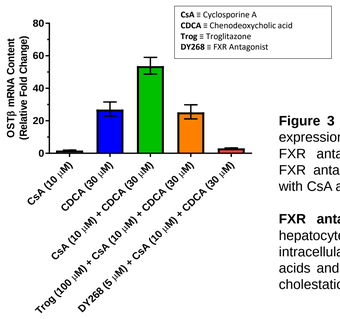
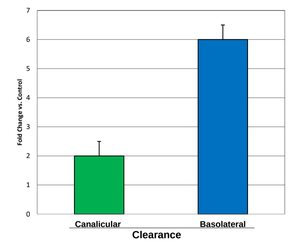




**Figure 3** Decrease in OSTβ mRNA expression when troglitazone (weak FXR antagonist) or DY268 (strong FXR antagonist) are co-administered with CsA and CDCA

**FXR antagonism** can prevent the hepatocyte from responding to high intracellular concentrations of bile acids and increasing the potential for cholestatic hepatotoxicity

**Increase in Canalicular and Basolateral Efflux Clearance Following Exposure to CDCA**




Guo, et.al. ITC Workshop, 13 March 2017

**Figure 4** 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.

**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.

**Table 1** Compounds were from selected publications[1,3] based on BSEP $IC_{50}$ values, and hepatotoxicity information from the NIH LiverTox[4] database.

| Drug | DILI Category | C-DILI™ Assay Potential | Correct Call | Clinical DILI |
|---|---|---|---|---|
| Acitretin | Idiosyncratic | Low | True negative | Yes |
| Amiodarone | Steatotic | High | False positive | Yes |
| Atorvastatin | Idiosyncratic | Low | True negative | Yes |
| Bosentan | Idiosyncratic | Low | True negative | Yes |
| Calcitriol | NID | Low | --- | NID[3] |
| CsA | Lack of causal evidence | Low | True negative | No |
| Clofibrate | Cholelithiasis | Low | True negative | Yes |
| Deferasirox | DILI | High | True positive | Yes |
| Dicloxacillin | Idiosyncratic | Low | True negative | Yes |
| Dipyridamole | Lack of causal evidence | Low | True negative | No |
| Donepezil | Lack of causal evidence | Low | True negative | No |
| Entacapone | Lack of causal evidence | Low | True negative | No |
| Eryth. Estolate | Idiosyncratic | Low | True negative | Yes |
| Everolimus | Lack of causal evidence | Low | True negative | No |
| Ezetimibe | Lack of causal evidence | Low | True negative | No |
| Febuxostat | Lack of causal evidence | Low | True negative | No |
| Flupirtine | NID | Low | --- | NID[3] |
| Fluvastatin | DILI | High | True positive | Yes |
| Glyburide | Lack of causal evidence | Low | True negative | No |
| Iloperidone | Lack of causal evidence | Low | True negative | No |
| Imatinib | DILI | High | True positive | Yes |
| Indomethacin | Idiosyncratic | Low | True negative | Yes |
| Irbesartan | Idiosyncratic | Low | True negative | Yes |
| Ketoconazole | DILI | High | True positive | Yes |
| Lapatinib | DILI | Low | False negative | Yes |
| Losartan | Idiosyncratic | Low | True negative | Yes |
| Megestrol Acetate | NID | Low | --- | NID[3] |
| Mifepristone | NID | Low | --- | NID[3] |
| Nicardipine | Lack of causal evidence | Low | True negative | No |
| Nifedipine | Idiosyncratic | Low | True negative | Yes |
| Pazopanib | DILI | Low | False negative | Yes |
| Pioglitazone | Lack of causal evidence | Low | True negative | No |
| Posaconazole | DILI | High | True positive | Yes |
| Pranlukast | NID | Low | --- | NID[3] |
| Primaquine | No clinical evidence | Low | True negative | No |
| Repaglinide | Idiosyncratic | Low | True negative | Yes |
| Reserpine | Lack of causal evidence | Low | True negative | No |
| Rifabutin | Lack of causal evidence | Low | True negative | No |
| Ritonavir | Difficult to separated from viral infection | High | True positive | Yes |
| Rosiglitazone | Lack of causal evidence | Low | True negative | No |
| Simvastatin | Idiosyncratic | Low | True negative | Yes |
| Sitaxsentan | Idiosyncratic | Low | True negative | Yes |
| Tacrolimus | Lack of causal evidence | Low | True negative | No |
| Telithromycin | Idiosyncratic | Low | True negative | Yes |
| Telmisartan | Lack of causal evidence | Low | True negative | No |
| Tolcapone | DILI | High | True positive | Yes |
| Tolvaptan | Observed in cirrhosis and ADPKD patients | Low | True negative | Yes |
| Troglitazone | DILI | High | True positive | Yes |
| Zafirlukast | Idiosyncratic | Low | True negative | Yes |

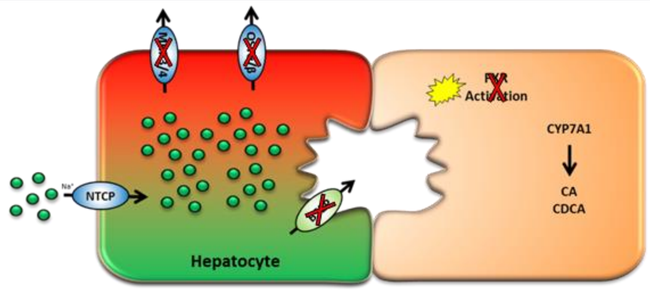



**Figure 5** Inhibition of BSEP along with antagonism of FXR activation in response to increased intracellular concentrations of bile acids, and/or inhibition of bile acid basolateral efflux transporters can lead to increased cholestatic hepatotoxicity.

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.

**Table 2** The C-DILI™ Assay correctly predicted hBSEP False Positives (Pioglitazone, Rosiglitazone) as having low potential for hepatocellular cholestasis, and hBSEP False Negative (Deferasirox) as having a high potential. Troglitazone and Ketoconazole, both True Positives in the hBSEP analysis were also correctly predicted in the C-DILI™ Assay. Cyclosporine A is a potent BSEP inhibitor, however the hepatocyte can adapt through FXR activation of basolateral efflux transporters, and is used as a negative control in the C-DILI Assay. Troglitazone is a potent BSEP inhibitor (sulfate metabolite) and a weak FXR antagonist, and recently the sulfate metabolite has been shown to inhibit OSTα/β[5]. Troglitazone is used as a positive control in the C-DILI assay.

Compared to use of hBSEP data alone, analysis with C-DILI™ increased the accuracy from 22% to 96%.

| Compound | hBSEP $IC_{50}$ (µM) | hBSEP Prediction | Correct Prediction[4] | Reports of Liver Injury | C-DILI™ Prediction |
|---|---|---|---|---|---|
| Cyclosporine A | 0.5 | Positive | Negative | None or rare | Low Potential |
| Pioglitazone | 0.5 | Positive | Negative | None or rare | Low Potential |
| Rosiglitazone | 3 | Positive | Negative | None or rare | Low Potential |
| Troglitazone | 3 | Positive | Positive | Hepatocellular | High Potential |
| Ketoconazole | 3 | Positive | Positive | Hepatocellular | High Potential |
| Simvastatin | 25 | Positive | Negative | None or rare | Low Potential |
| Imatinib | 25 | Positive | Negative | Hepatocellular | Direct Toxicity |
| Fluvastatin | 36 | Negative | Positive | Mixed or Rare | Moderate |
| Deferasirox | 58 | Negative | Positive | Mixed | High Potential |

## Applications:

**Discovery Stage**
- No information on clinical concentrations
- Screen at high concentrations (50 – 100 µM), and then follow up hits with a dose ranging study at lower concentrations

**Pre-Clinical Stage**
- Projected clinical concentrations
- Screen at concentrations that cover clinical $C_{max}$ or $C_{ss}$ and up to 20X to 50X to account for higher portal vein concentrations

**Clinical Stage**
- Known clinical concentrations
- Screen for potential drug interactions at 20X clinical $C_{max}$ or $C_{ss}$ for test compound and anticipated concentration range for co-administered compound

## 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.

**Key Assay Features:**

- Transporter Certified™ human hepatocytes in a model that maintains transporter expression, localization and function
- Optimized culture media
- Convenient 24 hour incubation in 96-well format
- Assess effects of parent and metabolites simultaneously
- Integrated effects of transporter inhibition (acute effects) and regulation (chronic effects) 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