Bile Acid Basolateral Efflux: A Compensatory Mechanism that Prevents Cholestatic DILI

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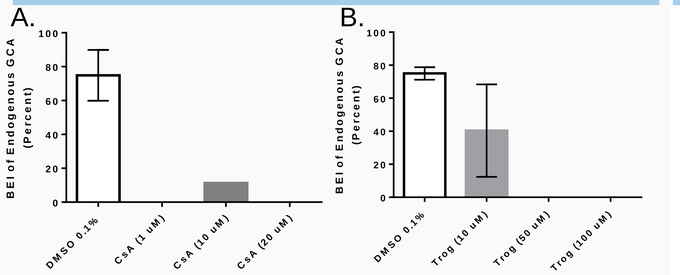
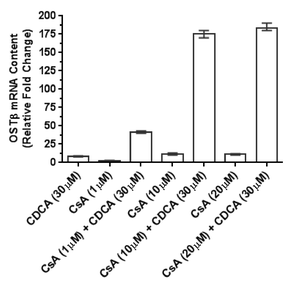
Abstract:(3560/P535)

Figure 1. Effects of CsA and Trog on Excretion of GCA into Bile Pockets of SCHH.



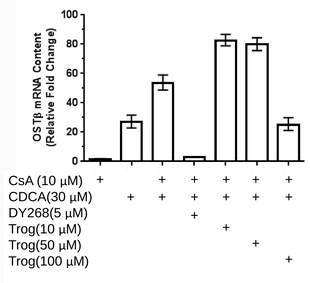
Following exposure to well-known BSEP inhibitors, the biliary excretion of endogenous GCA was evaluated in SCHH utilizing B-CLEAR® technology. BEI is the fraction of molecules taken up into the hepatocyte and then excreted into the bile pocket. Biliary efflux of GCA was reduced in SCHH following 72 hours of treatment with A) CsA (1, 10, 20 μM) and B) Trog (50 and 100 μM). Mean and SD of triplicate wells are reported .

Figure 2. The Basolateral Efflux Transporter OST is Upregulated by BSEP Inhibition in SCHH.



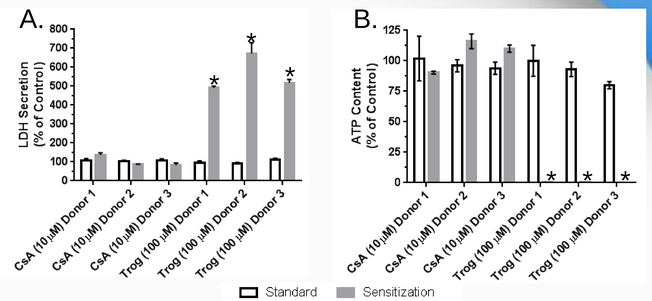
The expression of the basolateral efflux transporter monomer OSTβ was evaluated in SCHH following 72 hr treatment. The BSEP inhibitor CsA (10 and 20 μM) increased OSTβ mRNA content ≥ 11-fold. The FXR agonist CDCA (30 μM) increased OSTβ mRNA ≥ 8-fold. A synergistic increase in OSTβ mRNA content was observed in SCHH co-treated with CsA (1, 10, 20 μM) and CDCA (30 μM). Values represent the RQ mean and error bars represent 95% confidence intervals.

Figure 4. FXR Antagonism by Trog Reduces Basolateral Efflux Compensatory Mechanism in SCHH.



Disruption of FXR activation reduces the BA efflux compensatory response, OSTβ. SCHH were treated for 24 hr. A synergistic increase in OSTβ mRNA content was observed in SCHH co-treated with CsA and CDCA. DY268, a specific FXR antagonist, prevented the synergistic increase in OSTβ mRNA content. The synergistic response of OSTβ gene expression was removed in the presence of Trog (100 μM). Values represent the RQ mean and error bars represent 95% confidence intervals. .

Figure 5. Evaluation of Cholestatic Liver Toxicity Screen Across Multiple Donors



Utilizing the molecular mechanism described herein, we developed a cholestatic liver toxicity screening assay. The C-DILI™ assay (patent pending) was evaluated across 3 different donors to screen for Trog cholestatic liver toxicity. SCHH were cultured and treated using either standard or sensitization medium, a propriety media. SCHH must be conditioned to identify toxicity due to disruption of the BA homeostasis adaptive response. Cytotoxicity was measured by leakage of LDH and by changes in ATP content. CsA, a BSEP inhibitor, exposure had no effect of hepatocyte health across all three donors in either media. In comparison, Trog (100 μM) demonstrated to be both a BSEP inhibitor and FXR antagonist significantly increased A) LDH secretion and B) depleted ATP content across all three donors in the sensitization medium. LDH and ATP values represent the mean and SD of triplicate wells. A two-way ANOVA followed by a Tukey's multiple comparison test was performed on LDH and ATP data, * denotes P-value of 0.05 when compared to respective solvent control.

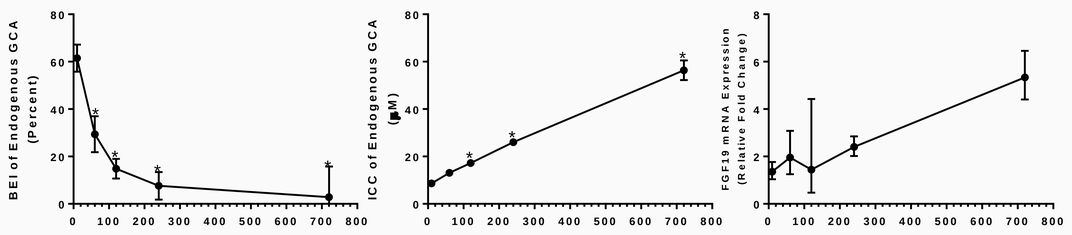
Introduction

Bile acid (BA) homeostasis is essential for liver health and disruption leads to hepatocellular damage. The ability to evaluate a new drug candidate's potential to cause cholestatic drug induced liver injury (DILI) is an important component of drug discovery research. Currently, most cholestatic DILI screening efforts in early discovery research focus on a drug candidate's potential to inhibit canalicular bile salt export pump (Kis et al. 2012, Morgan et al., 2013). Screening new drug candidates for BSEP inhibition using vesicle technology has become the accepted norm for evaluating cholestatic liver toxicity risk.

Many potent BSEP inhibitors (based on vesicle data) including cyclosporine A (CsA; BSEP IC_{50} = 0.5 μM; Morgan et al., 2013) rarely result in cholestatic liver injury. In large clinical trials, initiation of CsA therapy was associated with only mild elevations in serum bilirubin levels, often without significant increases in serum ALT or alkaline phosphatase (LiverTox Database). Therefore, looking only at BSEP inhibition in an isolated model, such as transporter vesicles overlooks the role that BA homeostatic mechanisms (e.g. adaptive response) play in the prevention of BA hepatotoxicity. Not accounting for this adaptive response reduces both sensitivity and specificity when attempting to identify drug candidates with true potential to induce cholestatic liver toxicity.

To investigate this apparent lack of correlation between *in vitro* BSEP inhibition potency and *in vivo* cholestatic DILI severity, we investigated the response of the BA homeostasis feedback mechanism to well-known BSEP inhibitors, CsA and troglitazone (Trog), in sandwich-cultured Transporter Certified™ human primary hepatocytes. The primary aim of this study was to evaluate our hypothesis that inhibition of BSEP triggers a transient increase in endogenous BA (e.g. glycocholic acid (GCA)) resulting in the activation of FXR initiating the previously described basolateral BA efflux compensatory mechanism (Jackson et al., 2016). In addition, we explored interference of this adaptive mechanism by Trog, a well-established inducer of cholestatic DILI.

Figure 3. Effects of CsA on GCA Biliary Excretion, GCA Intracellular Concentration, and Induction of the FXR target gene FGF19.



We evaluated the kinetics of the hepatic bile acid homeostasis mechanism in SCHH following 10, 60, 120, 240, and 720 minutes of exposure to CsA (10 μM). A) Biliary excretion of GCA was reduced in a time-dependent manner reaching statistical significance by 60 minutes of exposure to CsA. B) Concomitant time-dependent increases in the intracellular concentration (ICC) of GCA were observed reaching statistical significance by 120 minutes of exposure to CsA. C) Concomitant time-dependent increases in FGF19, an FXR target gene, mRNA content were also observed. BEI and ICC values represent the mean and SD of triplicate wells. mRNA content values represent the RQ mean and error bars represent 95% confidence intervals. A one-way ANOVA followed by a Tukey's multiple comparison test was performed on BEI and ICC data, * denotes P-value of 0.05 when compared to 10 minute time point.

Figure 6. BA Basolateral Efflux Prevents Cholestatic DILI

Basolateral efflux mechanism (OST) prevents cholestatic DILI. A) Normal BA flow. B) Inhibition of canalicular BA efflux (BSEP) results in transient increase in the ICC of endogenous BA activating FXR. C) Basolateral efflux (OST) becomes active via FXR activation lowering the ICC of endogenous BA to prevent BA toxicity. Once ICC of BA return to normal and BSEP inhibition is removed, compensatory mechanism dissipates allowing BA flow to return to native state. D) **Cholestatic DILI**: Hepatic ICC of BA rise to toxic levels as a result of BSEP inhibition plus FXR antagonism (e.g. Trog), or basolateral efflux inhibition (OST/MRP3/4), or all of the above.

Conclusions

- **CsA and Trog exposure inhibited canalicular BA efflux (BSEP) in SCHH**
- **Inhibition of canalicular BA efflux (BSEP) by CsA resulted in increased ICC of endogenous BA and activation of BA homeostasis adaptive response in SCHH**
  - **Inhibition of canalicular BA efflux (BSEP) by CsA resulted in FXR activation**
  - **FXR activation initiated basolateral efflux compensatory mechanism in SCHH**
- **Trog exposure disrupts FXR activation**
- **Cholestatic DILI requires inhibition of BSEP plus**
  - **FXR antagonism (e.g. Trog)**
  - **Or Basolateral efflux inhibition**
  - **Or all the above**

C-DILI™ Assay

- **Mechanism based assay**
- **Evaluated cholestasis hepatotoxicity potential**
- **Correctly assigned CsA as low C-DILI potential in 3 out of 3 donors**
  - **No clinical evidence of liver injury in CsA therapy (LiverTox Database)**
- **Correctly assigned Trog as high C-DILI potential in 3 out of 3 donors**
  - **Clinical incidence of Trog induced liver injury 1:1000 (LiverTox Database)**

Materials & Methods

Preparation of sandwich-culture hepatocytes:

Sandwich cultured human hepatocytes (SCHH) were established with Transporter Certified™ cryopreserved cells. Once thawed the cells were suspended in QualGro™ Seeding Medium, a QTS proprietary medium, at a density of 0.8 million viable cells/mL and seeded onto BioCoat® 24-well cell culture plates purchased from Corning. Following the initial seeding, cells were allowed to attach for 2-4 hours, then rinsed and fed with 500 μL/well warm (37C) QualGro™ Seeding Medium. After a culture time of 18-24 hours, the seeding medium was removed and the cells were fed and overlaid with QualGro™ Culture Induction Medium, a QTS proprietary medium, supplemented with 0.35 mg/mL Matrigel® purchased from Corning. Cells were maintained in QualGro™ Culture Induction Medium until consumed. All hepatocytes were consumed on day 5 of culture.

Quantitation of GCA Biliary Efflux:

To investigate the effects of CsA and Trog on efflux of BA into SCHH bile pockets, SCHH were prepared and treated as follows. Stock solutions of CsA and Trog were prepared in DMSO and then diluted with QualGro™ Induction Medium daily to yield the desired final concentrations. SCHH were established and treated as described above. Treatments were refreshed daily with fresh treatment solution. At the completion of the exposure period, the hepatobiliary disposition of the endogenous bile acids, GCA, was determined using QTS B-CLEAR® technology as previously described (Jackson et al., 2016). Briefly, hepatocytes in parallel cultures, were washed twice with Plus (+) buffer (buffer containing Ca++) or Minus (-) buffer (buffer without Ca++). The wash solutions were removed and replaced with fresh Plus (+) or Minus (-) buffer and incubated for 10 minutes at 37°C to modulate tight junctions (Jackson et al., 2016). Following the incubation, the wash solution was removed and the hepatocytes were then washed three times with ice-cold Plus (+) Buffer. The plates were frozen at -80°C until processed for bioanalysis to determine protein content and disposition of endogenous BA. Protein content was determined using Pierce™ BCA protein assay kit (Thermo Fisher Scientific) following manufacturer's instructions.

Bioanalysis of Bile Acid Disposition Assessment:

Endogenous BA (taurocholic acid (TCA), GCA, cholic acid (CA), taurochenodeoxycholic (TCDCA) acid, glycochenodeoxycholic acid (GCDCA), and chenodeoxycholic acid (CDCA)) were extracted from cell lysates derived from the 24-well cell culture plates saved from the BA biliary efflux evaluations. LC/MS/MS was performed using a Thermo Electron TSQ® Quantum Discovery MAX™ (ThermoFisher Scientific) with an Ion Max ESI source operated in negative electrospray ionization mode. Chromatographic separation for the endogenous bile acids and their deuterated counterparts (for use as analytical standards and dosing material for d5-CDCA) was achieved using a Shimadzu binary HPLC system with LC-10ADvp pumps equipped with a Thermo Scientific Hypersil Gold™ 100 x 1.0 mm, 3 μm with matching guard and pre-column filter, using a linear gradient of 45-100%B, at a flow rate of 0.05 mL/min, with mobile phase A as 20% methanol with 0.25 mM ammonium acetate and mobile phase B as 80% methanol with 0.25 mM ammonium acetate, both at native pH.

Total RNA Isolation and qRT-PCR:

To evaluate treatment effects on FXR activation in SCHH, mRNA content of FXR target genes, Ostβ or FGF19, was assessed. Following exposure period with specified treatment, total mRNA was extracted using a Qiagen RNeasy® kit, according to the manufacturer's instructions. Prior to extraction the cells were lysed by adding a 0.3 mL aliquot of Qiagen RLT lysis buffer (supplemented with β-mercaptoethanol) to each plate. The plates were then frozen at -80ºC to allow cells to lyse and then thawed to continue the RNA isolation procedure. Total RNA was isolated from each treatment group. The RNA isolated from three individual wells within a group were pooled. The isolated RNA was quantified using the Quanti-iT RiboGreen® RNA assay kit (ThermoFisher Scientific) according to the manufacturer's instructions. Pooled total RNA samples were used to prepare cDNA using 500 ng in the High Capacity cDNA Archive Kit (ThermoFisher Scientific) according to the manufacturer's instructions. Changes in gene expression was measured by analyzing the cDNA prepared from each treatment group with using gene-specific TaqMan® assays (ThermoFisher Scientific). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous house-keeping gene to normalize each sample. Polymerase chain reaction (PCR) was performed on a ViiA 7 system Real-Time PCR System (Thermo Fisher Scientific) in relative quantification mode for 45 amplification cycles. Standard conditions for TaqMan-based assays were used. The determination of threshold cycles (Ct) was determined by the Vii7 system software for both target and endogenous control (GAPDH) genes. Relative-fold mRNA content was determined for each treatment group relative to the endogenous control gene expression and the calibrator, 0.1% DMSO vehicle control using the ViiA™ 7 system software. Confidence intervals of 95% were calculated for each target gene relative quantitation (RQ) mean by the ViiA™ 7 system software.

C-DILI™ Assay:

SCHH were established by thawing Transporter Certified™ cryopreserved cells according the manufacturer's instructions. Once thawed the cells were suspended in QualGro™ Seeding Medium at a density of 0.8 million viable cells/mL and seeded onto BioCoat® 96-well cell culture plates purchased from Corning. Following the initial seeding, cells were allowed to attach for 2-4 hours, then rinsed and fed with 200 μL/well warm (37C) QualGro™ Seeding Medium. After a culture time of 18-24 hours, the seeding medium was removed and the cells were fed and overlaid with QualGro™ Culture Medium supplemented with 0.35 mg/mL Matrigel®. Cells were maintained in QualGro™ Culture Medium until day four of culture. On day four of culture, SCHH were utilized in the C-DILI™ assay, a QTS propriety assay (patent pending). Briefly, SCHH hepatocytes were exposed to CsA (10 μM) or Troglitazone (100 μM) for 24 hours. Compounds were diluted directly into standard or QualGro™ sensitization medium, a QTS proprietary medium (contains a physiological mixture of bile acids and lipids). Following the exposure period cellular ATP was determined using CellTiter-Glo™ luminescent cell viability assay from Promega (Madison, WI). LDH leakage was determined using CytoTox-ONE™ homogeneous membrane integrity fluorescence assay from Promega. Both assays were performed according to the manufacturers instructions. Luminescence and fluorescence were measured with a BioTek Synergy 4 Plate Reader. Each treatment group was conducted in triplicate wells across 3 Transporter Certified™ cryopreserved hepatocyte lots.

References

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