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EVALUATION OF CLINICALLY RELEVANT INHIBITORS OF BSEP USING B-CLEAR® HUMAN SANDWICH-CULTURED HEPATOCYTES TO BETTER PREDICT INHIBITION AND CHOLESTASIS

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Introduction

Drug-induced liver injury (DILI) is characterized as liver injury due to intake of medications or xenobiotics, which leads to liver abnormalities or dysfunction.[1] There are a large number of drugs that have been withdrawn from the market due to acute DILI; including, troglitazone (antidiabetic and anti-inflammatory), benzbromarone (gout), and sitaxsentan (pulmonary arterial hypertension).[2] The mechanism behind DILI is multifaceted and can be exacerbated by unpredictable metabolism and bodily response, as well as the complex relationship between an individual's genetic makeup and environmental risk factors.[3] Furthermore, several factors may contribute to hepatocellular injury and cholestasis; including, acute hepatocyte necrosis, the production of reactive metabolites (and oxidative stress) during cytochrome P450 metabolism of the parent drug, activation of stress signaling, and mitochondrial dysfunction.[4]

Bile acids are water soluble end products of cholesterol metabolism which are highly regulated by metabolism, excretion, absorption, and feedback mechanisms, in order to limit their intracellular accumulation. It is hypothesized that dysfunction to the bile salt efflux pump (BSEP) may contribute to the mechanism of action behind DILI.[5] Current preclinical, *in vitro* models of BSEP inhibition may not accurately predict the potential for DILI. Therefore, an *in vitro* model which better reflects *in vivo* transporter expression and activity may better predict the potential for novel xenobiotics to cause DILI in the clinic. The purpose of this work was to evaluate the use of B-CLEAR® human sandwich-cultured hepatocytes to investigate the hepatobiliary disposition of taurocholic acid (TCA) in the presence of cholestatic agents.

Materials & Methods

Materials: Human hepatocytes were isolated and purified at XenoTech, LLC and characterized for use at Qualyst Transporter Solutions, LLC. Seeding, overlay, and culturing media, and Plus (+) and Minus (-) Assay Buffers were obtained from Qualyst Transporter Solutions. [^{3}H]-taurocholic acid (TCA) was obtained from Perkin Elmer Life and Analytical Sciences (Waltham, MA, USA). Bovine serum albumin, carbamazepine, cisapride monohydrate, cyclosporine A, fluphenazine, glyburide, nicardipine, omeprazole, sulpiride, and troglitazaone were obtained from Sigma (St. Louis, MO, USA). Ezetimibe and olmesartan medoxomil were obtained from Caymen Chemicals (Ann Arbor, MI, USA).

Cell Culture: Human hepatocytes were seeded at a density of 1.3x10^6 cells/mL in 24 well cell culture plates. Approximately 3 hours after seeding, matrigel overlay (0.25 mg/mL) was added to the wells and allowed to polymerize overnight. Cells were fed everyday for 5 days and kept at 37°C, 95% relative humidity, and 5% CO_2.

B-CLEAR® Assay: On day 6, cells were removed from the incubator and rinsed 3 times with Plus (+) Buffer. Cells were then pre-incubated in 300 µL Plus (+) or Minus (-) Buffer for 10 min at 37°C with one of the following inhibitors: carbamazepine, cyclosporine A (CspA), cisapride monohydrate, ezetimibe, fluphenazine hydrochloride, fluvastatin, glyburide, Nicardipine, olmesartan, omeprazole, sulpiride, troglitazone. Pre-incubation buffer was then removed and replaced with 300 µL Plus (+) buffer containing 2.5 µM taurocholic acid (TCA) and the inhibitors previously listed and incubated in the presence of 4% BSA for an additional 30 min at 37°C. At the end of the incubation, cells were washed three times in Plus (+) buffer, lysed with 0.1N NaOH and the lysate was analyzed using liquid scintillation counting. In follow up studies, a dose-response was generated with seven concentrations of selected compounds (nicardipine, ezetimibe, fluvastatin, cyclosporine A, and cisapride monohydrate) under the same conditions described above.

Results

Table 1. Summary of screening results for clinically relevant cholestatic agents

Inhibitor	Predicted Cholestasis Concern from BSEP IC50	Inhibitor Dose (µM)	ICC of TCA (µM)	BEI (%)	Kp	% Change of Control Kp
Solvent Control	**NA**	**NA**	**17.5**	**31.4**	**6.83**	**100%**
Carbamazepine	Unknown	100	17.9	42.9	7.18	105%
Cisapride Monohydrate	**Yes**	**20**	**24.3**	**14.6**	**9.72**	**142%**
Cyclosporine A	**Yes**	**1**	**9.91**	**11.8**	**3.96**	**42%**
Ezetimibe	**No**	**100**	**9.26**	**43.3**	**3.70**	**46%**
Fluphenazine	Yes	100	19.7	46.1	7.89	116%
Fluvastatin	**Maybe**	**100**	**14.1**	**43.4**	**5.65**	**83%**
Glyburide	Yes	1	19.4	31.5	7.78	114%
Nicardipine	**Yes**	**5**	**22.0**	**27.6**	**8.78**	**129%**
Olmesartan medoxomil	No	1	16.4	43.5	6.57	96%
Omeprazole	Yes	30	16.4	45.5	6.55	96%
Sulpiride	Yes	500	19.2	36.9	7.68	112%
Troglitazone	Yes	1	15.8	34.9	6.31	92%

Table 1 lists the results of screening a panel of clinically relevant cholestatic agents for their effect on intracellular concentration (ICC), biliary excretion index (BEI), and Kp (ratio of ICC to extracellular concentration). Results indicate that compared to solvent control (0.1% DMSO), there was a dramatic shift in the ICC of TCA in the hepatocytes for cisapride monohydrate, cyclosporine A, ezetimibe, fluvastatin, and nicardipine. Furthermore, BEI and Kp were also greatly impacted by exposure to these inhibitors, leading to changes in the hepatobiliary disposition. Follow up studies investigated a range of concentrations for the bolded inhibitors below.

References

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Results Continued

Figure 1. Cisapride monohydrate total accumulation (pmol/mg) and BEI (%) (*n=1)*

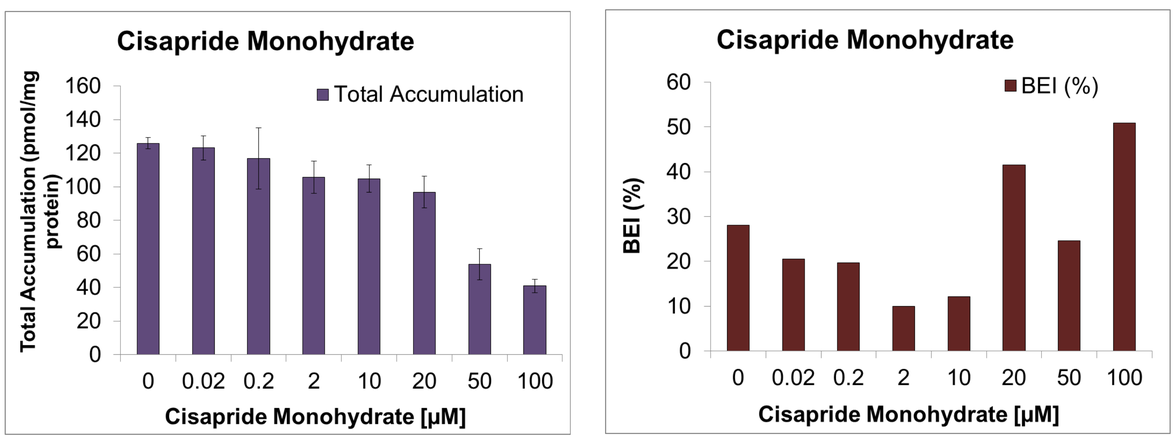


Figure 2. Cyclosporine A total accumulation (pmol/mg) and average BEI (%) (*n=2)*

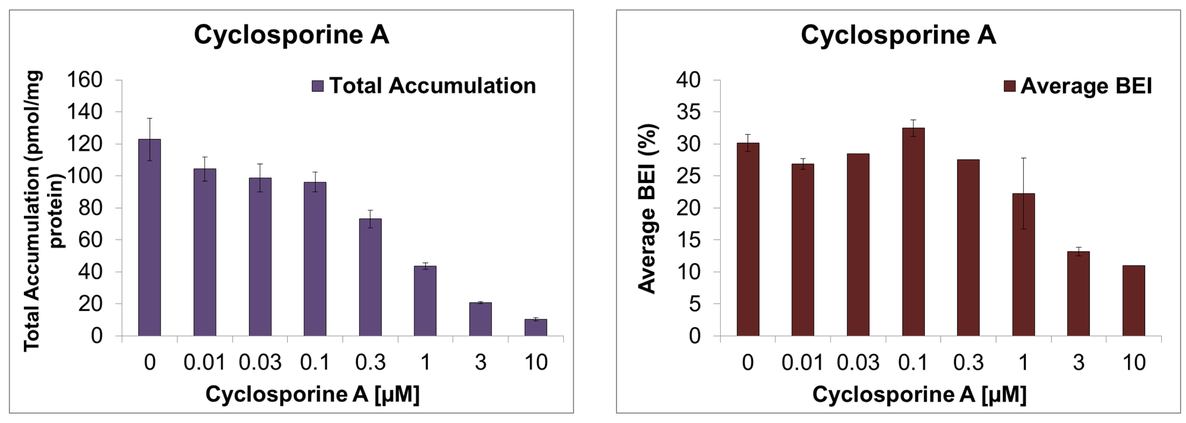


Figure 3. Ezetimibe total accumulation (pmol/mg) and average BEI (%) (*n=3)*

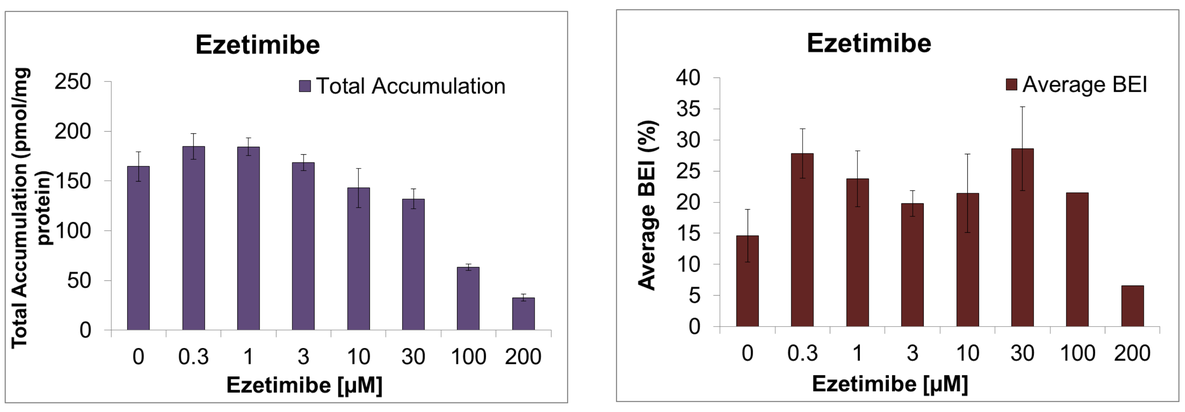


Figure 4. Fluvastatin total accumulation (pmol/mg) and average BEI (%) (*n=3)*

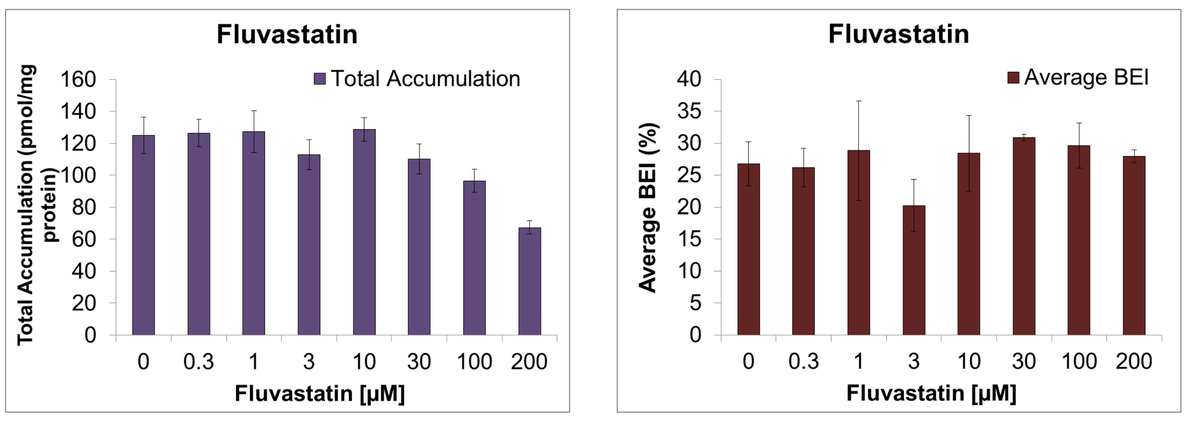
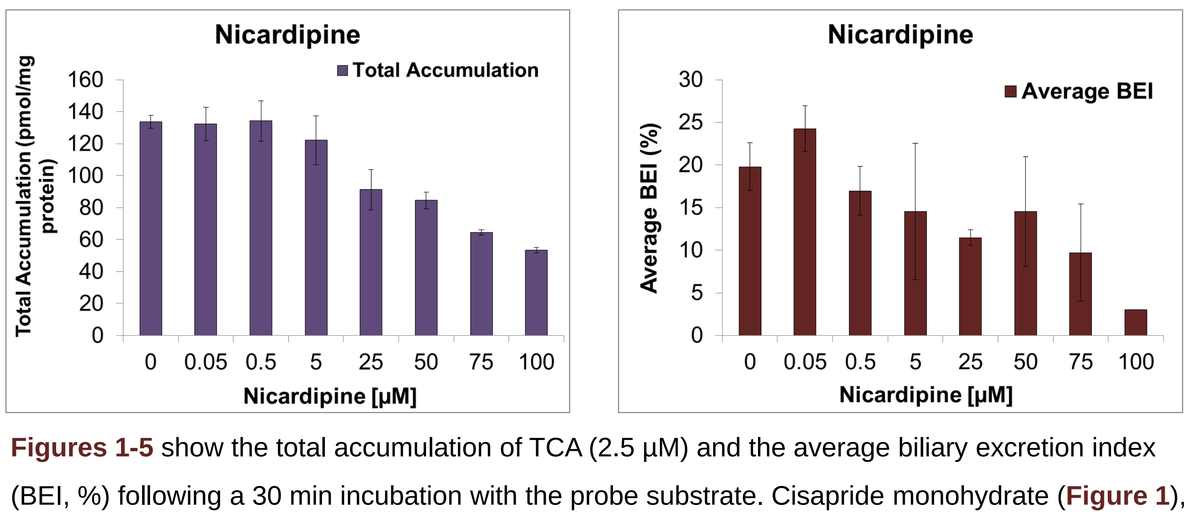


Figure 5. Nicardipine total accumulation (pmol/mg) and average BEI (%) (*n=3*)



Figures 1-5 show the total accumulation of TCA (2.5 µM) and the average biliary excretion index (BEI, %) following a 30 min incubation with the probe substrate. Cisapride monohydrate (**Figure 1**), demonstrated a concentration dependent decrease in total accumulation of TCA, and conversely, exhibited an increase in BEI as levels of cisapride increased. **Figure 2** displays the results for cyclosporine A, which exhibited a concentration-dependent decrease in total accumulation, with an initial increase in BEI, followed by a decrease as the level of compound in the system rose. Ezetimibe (**Figure 3**) demonstrated a decrease in total accumulation of TCA as the concentration of the inhibitor increased, but displayed an initial increase in BEI, followed by a subsequent decrease in the average BEI. While total accumulation of TCA in the presence of fluvastatin decreased in a concentration-dependent manner (**Figure 4**), the average BEI was not altered. Finally, nicardipine (**Figure 5**), demonstrated a concentration-dependent decrease in total accumulation of TCA at concentrations greater than 5 µM and a concentration dependent decrease in the BEI.

Conclusions

The B-CLEAR® human hepatocyte sandwich cultured assay demonstrates the ability to utilize the accumulation of a probe substrate to assess both inhibition of uptake as well as efflux. Many xenobiotics which inhibit efflux out of hepatocytes can lead to a risk of hepatotoxicity. It is important to note though, that a compound capable of inhibiting uptake into hepatocytes may lead to a risk of systemic toxicity. Therefore, the use of an *in vitro* system which more accurately models *in vivo* conditions can help to better predict the overall effect of xenobiotics on uptake, clearance and potential toxicity. These data presented here demonstrate that hepatobiliary uptake and clearance are complicated processes which are influenced by a number of factors. The Qualyst Transporter Solutions B-CLEAR® technology paired with XenoTech-produced sandwich-cultured hepatocytes are able to better assess inhibition of both uptake and efflux in hepatocytes. Future directions include further investigation of toxicity utilizing human sandwich-cultured hepatocytes in order to better understand the effect of acute and chronic exposure to these clinically relevant cholestatic agents.

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