Induction of Transporter Expression and Modulation of Hepatobiliary Disposition using Sandwich Cultures of Human Hepatocytes

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Introduction / Abstract

Transporter-related drug interactions are emerging as an important consideration in the clearance of drugs. In particular, hepatic clearance is important due to the liver's role in xenobiotic defense. However, few in vitro assays are available to quantify hepatobiliary transport, and even fewer studies have described the effect of altered transporter expression on hepatic transport. Historically, most studies investigating transporter modulation have focused on mRNA content providing little data on transporter activity following drug treatment. The current study examines the use of a sandwich cultured hepatocyte model (B-CLEAR®, Qualyst, NC) to quantify the effects of various transporter expression modulators on hepatobiliary transport. Multiple models have been used to assess hepatic transporter activity (e.g. transiently and stably transfected cell lines); however, cell lines traditionally have been shown to lack many of the hepatic regulatory pathways (e.g. nuclear receptors) necessary to model hepatic induction. The sandwich-culture hepatocyte model maintains many of these critical cell-signaling pathways and also forms functional bile canalicular networks with liver-like polarization of hepatic uptake and efflux transporters (1). This provides an integrated and physiologically relevant model to quantify changes in transporter activity as a result of alterations in expression. Primary human hepatocytes in sandwich-culture were treated for 3 days with DMSO (0.1%), PB (1 mM), RIF (10 µM), CDCA (100 µM), Colchicine (0.5 µM), Mancozeb (40 µM), or Bensulfuron-methyl (5 µM). Messenger RNA content of efflux (ABCB1, ABCG2, ABCB11, ABCC1, ABCC2, and ABCC3) and uptake transporters (SLCO1B1) was measured by TaqMan® Assays. Hepatobiliary transport activity was quantified by using selective transporter probe substrates (e.g. digoxin, taurocholate, and estradiol). Following treatment for 3 days with xenobiotics, preliminary data showed a 2.2-fold increase in the biliary excretion index of digoxin (2.2-fold) in PB treated hepatocytes. Similarly, changes in transporter mRNA expression were detected. This approach has the potential to become an effective tool in the prediction of transporter-related drug-drug interactions resulting from altered hepatobiliary transport.

Figure 1 – Induction of CYP mRNA Content

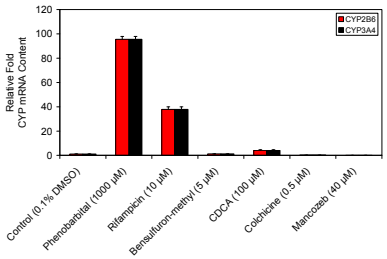


Figure 1-CYP2B6 and CYP3A4 mRNA content was evaluated 48 hours after xenobiotic treatment using TaqMan® methodology. Primary human hepatocytes were lysed and total RNA was isolated using the ABI6100 Nucleic Acid Prepstation with ABI chemistry following manufacturer's procedure (Life Technologies, Carlsbad, CA). Total RNA (200 ng) was converted to cDNA using the High Capacity cDNA Archival Kit from ABI following manufacturer's instructions.

Hepatic CYP and transporter cDNA from primary hepatocytes was analyzed 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 7900HT Real-Time PCR System in relative quantification mode for 40 amplification cycles. Relative-fold mRNA content was determined for each treatment group relative to the 0.1% DMSO vehicle control.

Table 1 – Induction of CYP Enzyme Activity

Treatment	Mean CYP2B6 Activity Bupropion Hydroxylation (pmol/min/million cells)	Standard Error	Fold Over Control
Control (No DMSO)	4.41	0.28	
Control (0.1% DMSO)	7.00	0.19	1.0
PB (1000 µM)	151	6	21.6
RIF (10 µM)	77.8	4.3	11.1
Bensulfuron-Methyl (5µM)	6.66	0.55	1.0
CDCA (100 µM)	11.7	0.8	1.7
Colchicine (0.5 µM)	3.72	0.11	0.5
Mancozeb (40 µM)	0.374	0.024	0.1
Treatment	**Mean CYP3A4 Activity Testosterone 6ß-Hydroxylation (pmol/min/million cells)**	**Standard Error**	**Fold Over Control**
Control (No DMSO)	99.9	7.1	
Control (0.1% DMSO)	128	12	1.0
PB (1000 µM)	1070	20	8.3
RIF (10 µM)	1380	30	10.8
Bensulfuron-Methyl (5µM)	159	11	1.2
CDCA (100 µM)	125	20	1.0
Colchicine (0.5 µM)	61.1	6.0	0.5
Mancozeb (40 µM)	4.76	0.51	0.0

Table 1-CYP enzyme activity was evaluated 72 hours after xenobiotic treatment in primary hepatocyte cultures. Incubations were performed *in situ* with HBSS containing 500 µM bupropion or 200 µM testosterone. HBSS containing bupropion or testosterone was added directly to the hepatocyte monolayers and incubated at 37°C for 20 or 14 minutes, respectively. All samples were extracted using a solution of isoamyl alcohol/ethyl acetate (1:100). The organic layer was transferred to new deep-well plates, evaporated to dryness under a stream of nitrogen gas, and reconstituted in 75 µL of mobile phase.

The marker metabolites OHBP and 6ßT were quantitated using a Micromass Quattro LC-MS/MS equipped with a turbo ion spray interface operated in positive ion mode. OHBP & 6ßT and internal standards (OHBP-$_{d6}$ & 6ßT-$_{d3}$) were monitored by MRM for the transition of precursor-to-product ions at the following m/z: OHBP 257→239/ OHBP-d6 263→245 & 6ßT 305→269/6ßT-d3 308→272.

Figure 2 – B-CLEAR® Methodology

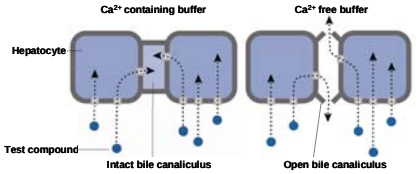


Figure 2-Hepatobiliary disposition (hepatocyte uptake and biliary clearance) was characterized in confluent sandwich cultured hepatocytes with functional bile canalicular networks. Freshly isolated human hepatocytes were incubated in sandwich culture for 4-7 days prior to xenobiotic treatment. This preparatory phase optimizes cellular morphology, allowing for insertion of transporters into the cell membranes and formation of bile canaliculi (1). In vitro biliary clearance was assessed as the difference between accumulation of transporter probe substrate in hepatocyte monolayers incubated in a physiological buffer and in a buffer lacking divalent cations (Ca2+ and Mg2+). In the physiological buffer, transporter probe substrates (taurocholate, digoxin, or estradiol) accumulate in the cells and canalicular networks. In the depleted buffer, the substrates only accumulate in cells owing to the opening of the tight junctions. The difference between substrate concentrations in the monolayers incubated with these two buffers is used to calculate the biliary excretion index and biliary clearance (2, 3).

Figure 3 – Drug Induced Alterations in Hepatic Transporter mRNA

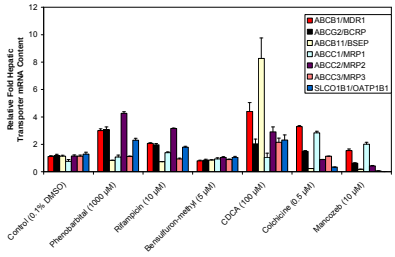
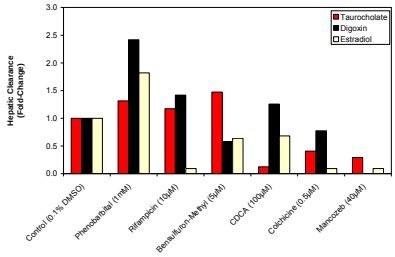


Figure 3-Hepatic transporter mRNA content was evaluated 48 hours after xenobiotic treatment using TaqMan® methodology.

Figure 4-Hepatic clearance was evaluated 72 hours after xenobiotic treatment using B-Clear methodology. Twenty-four hours after the last dosing, hepatocyte cultures were conditioned with Ca+ or with Ca+ Free buffer for 10 minutes at 37ºC. After conditioning, media was removed and all hepatocytes were submerged in Ca+ buffer. The transporter probe substrates, taurocholate-H^3, digoxin-H^3, or estradiol-H^3 were then added directly to the hepatocyte sandwich cultures and incubated for 10 minutes at 37ºC. After incubation, substrate solutions were removed for analysis and hepatocyte monolayers were frozen.

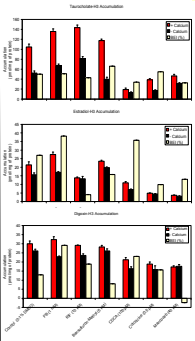
Figure 4 – Drug Induced Alterations in Hepatic Clearance



Hepatocytes were thawed and lysed with Triton X. Protein determination was performed on the cell lysates using Pierce® BCA Kit following manufacturer's instructions. Following protein determinations, a small aliquot of cell lysate from each sample was added to scintillation cocktail in a 96-well plate. Similarly, a small aliquot of substrate solution from each sample was added to scintillation cocktail in a 96-well plate. All samples were analyzed using a Chameleon V plate scintillation counter (Bioscan, Washington, DC). Once DPM's were determined for each sample the following equation was used to calculate accumulation of substrate. The accumulation values determined from the following equation are used to estimate %BEI and biliary clearance.

$$\text{Accumulation}_{\text{sample}}\ [\text{pmol/mg protein}] = \frac{\left(\frac{\text{DPM}_{\text{sample}}}{2.22 \times 10^{6}\ (\text{DPM/µCi})}\right) \times \left(\frac{\text{Concentration}_{\text{radioactive}} + \text{Concentration}_{\text{non-radioactive}}}{\text{Concentration}_{\text{radioactive}}}\right)}{\text{Specific Activity [µCi/pmol]}} \Big/ \text{Protein Concentration [mg/mL]} \times \text{Volume}_{\text{sample}}\ [\text{mL}]$$

Figure 5 – Drug Induced Alterations in Hepatobiliary Disposition



Taurocholate							
Treatment	Accumulation (pmol/mg of protein) + Calcium	Std. Error	- Calcium	Std. Error	BEI (%)	Std. Error (%)	Cl_{int} (mL/min/Kg)
Control (0.1% DMSO)	104.85	5.68	52.17	4.20	50.24	1.40	34.80
PB (1 mM)	139.60	4.12	66.65	3.61	90.85	1.44	45.70
RIF (10 µM)	143.62	4.90	81.77	3.57	43.07	1.26	49.80
Bensulfuron-Methyl (5 µM)	117.45	3.04	59.65	4.32	68.24	1.26	51.30
CDCA (100 µM)	19.13	3.08	12.60	1.82	34.15	1.16	4.50
Colchicine (0.5 µM)	38.84	2.38	17.44	1.17	55.11	1.42	14.10
Mancozeb (40 µM)	46.41	3.35	31.17	1.86	32.65	1.22	10.10

Estradiol							
Treatment	Accumulation (pmol/mg of protein) + Calcium	Std. Error	- Calcium	Std. Error	BEI (%)	Std. Error (%)	Cl_{int} (mL/min/Kg)
Control (0.1% DMSO)	21.45	1.71	14.63	1.19	26.72	2.82	4.40
PB (1 mM)	27.51	1.28	17.01	0.50	38.16	3.69	8.00
RIF (10 µM)	13.86	0.40	13.30	1.39	4.05	4.41	0.40
Bensulfuron-Methyl (5 µM)	23.54	0.59	19.83	0.33	15.75	2.12	2.80
CDCA (100 µM)	10.97	0.70	7.05	0.24	35.73	0.81	3.00
Colchicine (0.5 µM)	4.87	0.16	4.39	0.33	9.95	0.64	0.40
Mancozeb (40 µM)	3.64	0.22	3.17	0.23	12.95	1.19	0.40

Digoxin							
Treatment	Accumulation (pmol/mg of protein) + Calcium	Std. Error	- Calcium	Std. Error	BEI (%)	Std. Error (%)	Cl_{int} (mL/min/Kg)
Control (0.1% DMSO)	29.66	1.57	25.10	0.89	12.66	2.26	3.10
PB (1 mM)	32.29	1.54	22.87	0.38	29.18	3.92	7.50
RIF (10 µM)	29.06	0.25	23.92	0.84	18.80	1.34	4.40
Bensulfuron-Methyl (5 µM)	28.27	0.83	26.02	1.20	7.99	0.96	1.80
CDCA (100 µM)	21.19	1.30	16.31	1.01	23.03	2.60	3.90
Colchicine (0.5 µM)	18.80	1.22	15.85	2.00	15.70	0.85	2.40
Mancozeb (40 µM)	17.24	0.66	17.64	0.91	-2.54	1.25	-0.93

Figure 5-Accumulation of transporter probe substrates, percent biliary excretion index (BEI), and hepatic clearance was determined from primary hepatocyte cultures treated with various xenobiotics for 72 hours.

Results and Conclusions

- CYP2B6 & CYP3A4 mRNA and enzyme activity was significantly induced by prototypical inducers PB and RIF indicating that culture conditions were appropriate to maintain critical cell-signaling pathways such as CAR and PXR.
- PB was shown to induce mRNA content of the hepatic transporters MDR1, BCRP, and MRP2 ≥ 3-fold above vehicle control. PB was also shown to increase the hepatic clearance of digoxin, a known substrate of PgP and BCRP, by ~2.4-fold. Hepatic clearance data and mRNA data are consistent suggesting that PB has potential to increase transporter mRNA content and activity resulting in increased hepatic clearance of co-administered compounds.
- Colchicine and mancozeb were shown to suppress CYP2B6 & CYP3A4 mRNA content and enzyme activity. Similarly, these compounds were shown to suppress BSEP, MRP2, and OATP1B1 mRNA content. Consistent with transporter mRNA data, colchicine & mancozeb were shown to decrease the hepatic clearance of estradiol, a known OATP1B1 substrate, by ≥ 10-fold. Overall, these data suggest that these compounds have the potential to decrease transporter mRNA content and activity resulting in decreased hepatic clearance of co-administered compounds.
- PB, colchicine, and mancozeb data support the use of such models (B-CLEAR®) to estimate a compound's potential to alter hepatobiliary disposition as a result of altered gene expression.

References

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