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An Integrated Approach to More Accurately Assess Cholestatic Liability of Drugs

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ABSTRACT

Drug-induced interference with normal bile flow can result in relatively mild and transient cholestasis, or it can result in much more severe hepatocellular injury, leading to drug-induced liver injury (DILI). Drug hepatotoxicity is a common reason for the failure of pharmaceuticals during drug development, and DILI is the most common reason for removing approved medications from the market. Identifying drug candidates with the potential to cause DILI as early as possible in the development pathway is of paramount importance, and having tools available to conduct SAR studies to retain beneficial pharmaceutical properties while minimizing the DILI risk of those candidates could prove invaluable.

We have developed an integrated approach for assessing the DILI potential of drugs by combining two assay systems: a recombinant cell based system expressing key transporters involved in drug uptake and bile flow, and a holistic primary hepatocyte co-culture model which has been shown to more accurately predict intrinsic clearance, metabolite generation, and hepatotoxicity potential of drugs.

Using the first system, a panel of drugs known to have hepatic liabilities was tested against MDCK-II cells co-transfected with OATP1B1, NTCP, and BSEP transporters. Several of these drugs associated with cholestasis, but not necessarily hepatotoxic, were shown to inhibit bile salt uptake transporters (NTCP, OATP1B1), which would result in decreased bile flow. Drugs like rifampicin, which are known to be hepatotoxic, inhibited the bile salt efflux transporter (BSEP) but not the uptake transporter (NTCP), resulting in high cellular retention of bile acids, which could lead to cell death due to their detergent-like properties. Drugs that inhibit both uptake and efflux transporters may lead to cholestasis, but are less likely to lead to hepatocellular injury as they will not cause high intracellular concentrations of bile salts.

Using the second system, a selection of drugs were tested in the Hurel hepatocyte model to assess toxicity in the absence and in the presence of a mixture of bile acids. Drugs such as ritonavir and cyclosporine were found to increase hepatotoxicity in the presence of bile acids. Drugs which were found to not increase cellular retention of bile acids in the first system, like erythromycin, showed no increase in hepatotoxicity in the presence of bile acids in the second system. Unlike transfected cell lines, which do not have significant metabolic capability, the primary hepatocytes are capable of addressing potential toxicity due to drug metabolites and their longer term effects on bile acid accumulation. If bile acid enhanced toxicity arises due to a metabolite, the mechanism of that toxicity can then be further defined by using the first system.

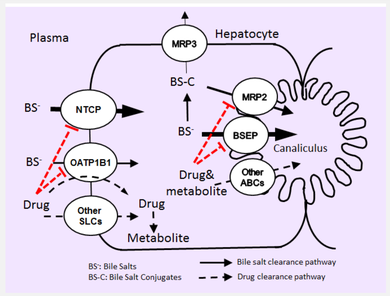
Combining the two assay systems results in a novel and powerful approach to assess the DILI potential of drugs and their metabolites.

BACKGROUND

Drug toxicity is not only a primary reason for the failure of pharmaceuticals during drug development, but drug-induced liver injury (DILI) is also the single most frequent reason for removing approved medications from the market [1]. With more than 1000 drugs and supplements reported to cause liver injury and more than 50% of fulminant hepatic failure cases due to drug toxicity [2,3], identifying drugs with the potential of causing DILI as early in the development pathway as possible is of paramount importance.

Transporters are responsible for the movement of bile salts in hepatocytes. Uptake across the basolateral (sinusoidal) membrane occurs primarily in a sodium-dependent manner and is mediated by the sodium-taurocholate cotransporting polypeptide, NTCP (SLC10A1), as well as by the sodium-independent organic anion-transporting polypeptides, or OATPs (SLCO family members), which are also mediators of drug and xenobiotic uptake. Export across the apical (canalicular) membrane occurs against a steep gradient and is mediated by the Bile Salt Export Pump, BSEP (ABCB11), which is the rate limiting step in the overall transport from the portal blood into the bile. BSEP is essential for keeping the intracellular level of bile salts low in hepatocytes, as these compounds can be cytotoxic due to their detergent properties, which can lead to mitochondrial stress and eventually to cell death. Since bile formation is an iso-osmotic process, bile salts are a major driving force for the generation of canalicular bile flow. When bile flow is reduced, a pathophysiological condition results called cholestasis. Under these conditions, bile salts are metabolized to sulphated and glucuronidated forms, which can be excreted into the bile by multidrug resistance proteins MRP2 (ABCC2), or back into the blood by MRP3 (ABCC3) and MRP4 (ABCC4). These salvage systems can help to reduce potentially cytotoxic levels of bile salts inside the hepatocytes.

Therapeutics that interfere with these transporters, especially BSEP, are often associated with cholestasis and eventually liver damage. A recent study of more than 200 compounds demonstrated a strong correlation between the degree of BSEP interference and the severity of liver injury [4]. However, inhibition of BSEP alone is not always a perfect predictor of potential liver injury, as some compounds with low IC_{50} values are not known to have effects on liver, while others with higher IC_{50} values are associated with liver injury – perhaps because of 1) the involvement of multiple other bile salt transporters *in vivo*, 2) the effects from active drug metabolites, and 3) the difference between plasma and hepatic concentrations of drugs and their metabolites (Fig. 1A).



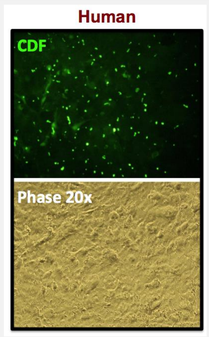


Fig 1. A) Simple schema of hepatic clearance pathways of bile salts (BS) and drugs, and sites of drug induced inhibition of major BS transporters B) Phase image of human co-culture hepatocytes and CDF staining of the bile canaliculi.

MATERIALS AND METHODS

Hurel's Co-culture Based Cholestasis Assay	
Culture System	Hurel Hepatic Co-Cultures
Lot/Species	Human (Hurel H1004)
Test Compounds	Cyclosporin A (0-50 μM), Ritonavir (0-200 μM), Bosentan (0-199 μM)
Bile Acids Mix (40x)	52.8 μM glycochenodeoxycholic acid 15.6 μM chenodeoxycholic acid 15.2 μM glycodeoxycholic acid 16 μM deoxycholic acid 14 μM glycocholic acid
Incubation Time	1 hr, 24hr, 48hr
Toxicity Assays	Albumin Assay (Liver-Specific Function)
Replica	n = 2

Mechanistic transporter models: MDCK-II cells were maintained in DMEM with low glucose and 10% FBS. Cells were seeded in Millipore 24/96-well insert plates (PCF-0.4 μm) and then transfected using a novel *in situ* transfection technology, Opti-Expression™, which allows consistent and effective transfection of polarized cell monolayers. Cells were either transfected with one or more plasmids encoding the appropriate transporter gene(s) or GFP as a mock control. Assays were conducted by placing test compounds in basal chamber, measuring transmonolayer bile salt transport into apical chamber and its cellular accumulation.

RESULTS

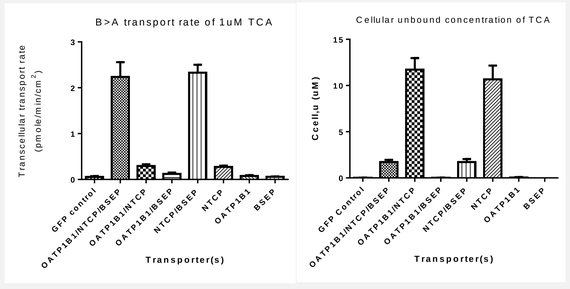


Fig 2. The use of mechanistic recombinant models to determine contribution of individual transporters to Taurocholate transport. (A) Basal to apical transmonolayer transport, and (B) Intracellular accumulation of taurocholate in MDCK cells expressing various combinations of transporters.

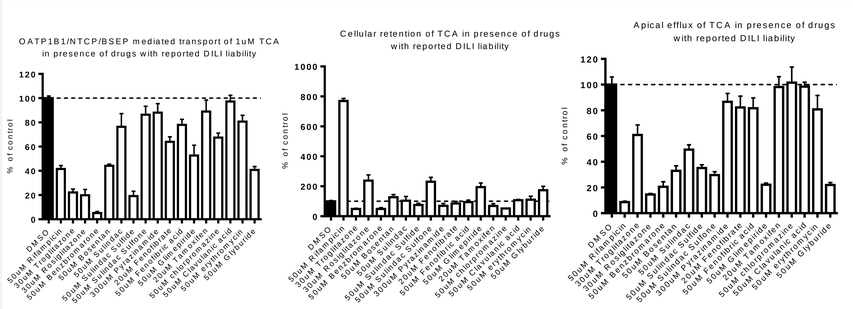


Fig 3. Drugs with reported DILI were tested with OATP1B1+NTCP+BSEP MDCK model for their net effects on taurocholate transport and transporter inhibition mechanism (A) Transmonolayer transport (B) Intracellular accumulation (both are dependent on NTCP and BSEP), and (C) Apical efflux (primarily mediated by BSEP)

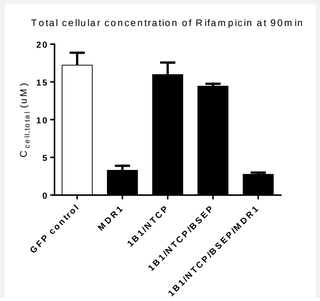


Fig 4. Mechanistic study using clearly defined MDCK models expressing various transporters revealed that Rifampicin is a substrate of P-gp, but is not transported by NTCP nor BSEP, and its intracellular concentration heavily depends on P-gp.

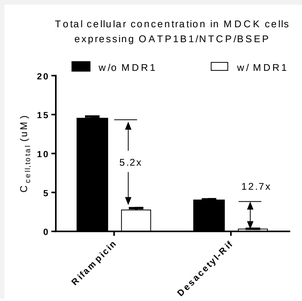


Fig 5. P-gp Dramatically reduces the intracellular concentrations of Rif and Des-Rif in cells expressing P-gp. Thus, P-gp may be a key determinant of the hepatic concentration and liver toxicity of Rif and Des-Rif.

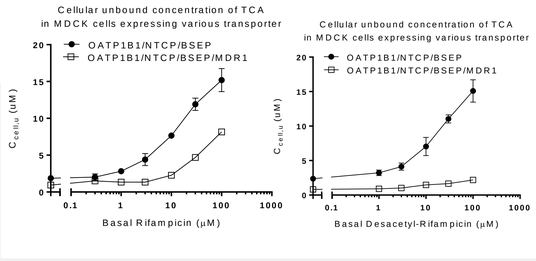


Fig 6. By decreasing their intracellular concentrations, P-gp significantly reduces the effects of Rifampicin (Left) and Des-Rifampicin (Right) on inhibiting BSEP intracellularly, therefore, resulting in less elevation of intracellular taurocholate in MDCK cells. The results suggest that drug-induced P-gp inhibition may potentiate hepatocyte BSEP inhibition by Rif and Des-Rif.

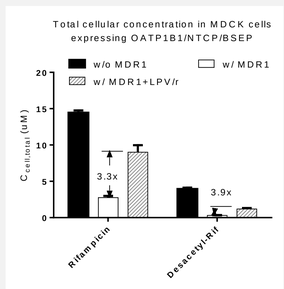


Fig 7. Protease Inhibitors Ritonavir and Lopinavir increase intracellular concentrations of Rif and Des-Rif in MDCK models through P-gp Inhibition, which suggests that when co-administrated with Rif, they may aggravate Rif hepatotoxicity.

RESULTS (CONT'D)

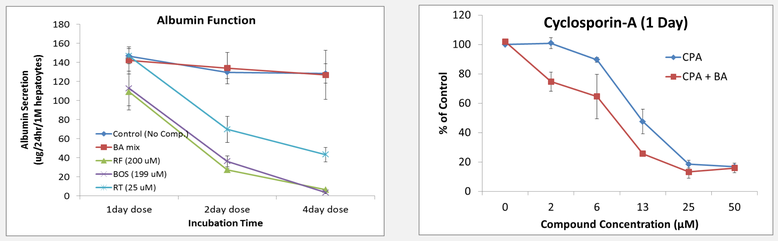


Fig 8. Albumin Assay for Liver-Specific Function. Albumin secretion in Hurel Hepatic Co-Cultures was measured in the presence of bile acids or various hepatotoxic drugs. A) Albumin is a good and stable marker for hepatotoxicity. B) The effects of bile acid addition can be seen after 24 hours for compounds like Cyclosporine A.

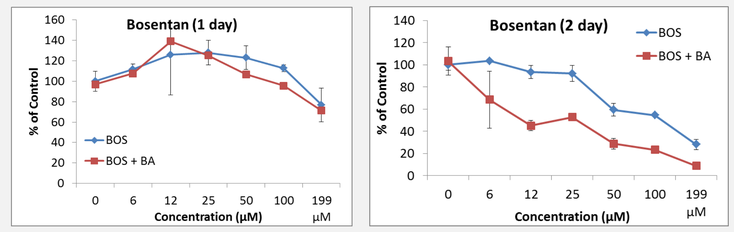


Fig 9. Hepatotoxicity of Bosentan in the absence or presence of a mixture of bile salts using Hurel Hepatic Co-Cultures. For compounds like bosentan, the toxicity can take up to 48 hours to present and get enhanced by bile salt addition.

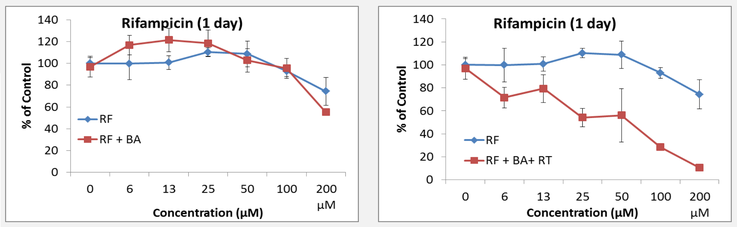


Fig 10. Hepatotoxicity of Rifampicin in the absence (A) or presence (B) of ritonavir using Hurel Hepatic Co-Cultures. Hepatotoxic response of rifampicin is greatly enhanced in the presence of 25 μM ritonavir, a potent P-gp inhibitor.

CONCLUSIONS

The clearance of bile salts is heavily dependent on transporters. Drug induced inhibition of bile salt uptake and/or efflux can cause blockage of bile salt hepatic clearance (resulting in cholestasis), and potentially result in increased hepatic bile salt accumulation (resulting in hepatocellular damage).

A variety of drugs with reported DILI liability were shown to inhibit transporters involved in bile flow. Some drugs were shown to increase cellular retention of bile salts because of higher inhibition potency toward BSEP. Here we showed that albumin is a valid marker for measuring hepatotoxicity of primary hepatocytes in co-culture. The albumin assay in hepatic co-cultures showed no hepatotoxicity in the presence of bile salts alone, but hepatotoxicity was seen in the presence of a variety of drugs with known DILI liability. For cyclosporine, rifampicin and bosentan, their hepatotoxicity was potentiated in the presence of bile salts, suggesting that blocking bile salt clearance via inhibiting bile salt transporters likely attributes to their liver toxicity.

Using mechanistic transporter models, rifampicin and its metabolite desacetyl-rifampicin were shown to be potent BSEP inhibitors that do not block NTCP, and their intracellular concentrations are mediated by P-gp. Therefore, P-gp inhibition by a co-medication such as ritonavir, can increase rifampicin and desacetyl-rifampicin intracellular concentrations. Such DDI may aggravate the hepatotoxicity of Rifampicin through sensitizing its inhibition on BSEP. The hepatocyte co-culture model substantiates these results, demonstrating that ritonavir potentiates the hepatotoxicity of rifampicin in primary hepatocytes in a bile salt dependent manner. These results may provide a mechanistic explanation on the high incidence of unexpected liver injuries in HIV-infected TB patients treated with Rifampicin and several protease inhibitors that inhibit P-gp [5,6].

Well-defined transporter models are useful tools for mechanistic studies of bile salt transport and its inhibition by drugs, while the resultant hepatotoxicity can be confirmed with the hepatocyte co-culture model. Using MDCK cells transfected with a variety of transporters in conjunction with primary hepatocyte-co-cultures allows for the ability to run detailed mechanistic transporter studies that can identify potential transporter interactions as well as primary hepatocyte based studies that can confirm hepatotoxicity and identify parent vs. metabolite toxicity profiles.

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