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Inhibition of bile salt transport by drugs associated with liver injury in primary hepatocytes from human, monkey, dog, rat, and mouse

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Abstract

Interference of bile salt transport is one of the underlying mechanisms for drug-induced liver injury (DILI). We developed a novel bile salt transport activity assay involving in situ biosynthesis of bile salts from their precursors in primary human, monkey, dog, rat, and mouse hepatocytes in suspension as well as LC-MS/MS determination of extracellular bile salts transported out of hepatocytes. Glycine- and taurine-conjugated bile acids were rapidly formed in hepatocytes and effectively transported into the extracellular medium. The bile salt formation and transport activities were time- and bile-acid-concentration-dependent in primary human hepatocytes. The transport activity was inhibited by the bile salt export pump (BSEP) inhibitors ketoconazole, saquinavir, cyclosporine, and troglitazone. The assay was used to test 86 drugs for their potential to inhibit bile salt transport activity in human hepatocytes, which included 35 drugs associated with severe DILI (sDILI) and 51 with non-severe DILI (non-sDILI). Approximately 60% of the sDILI drugs showed potent inhibition (with IC₅₀ values <50 μM), but only about 20% of the non-sDILI drugs showed this strength of inhibition in primary human hepatocytes and these drugs are associated only with cholestatic and mixed hepatocellular cholestatic (mixed) injuries. The sDILI drugs, which did not show substantial inhibition of bile salt transport activity, are likely to be associated with immune-mediated liver injury. Twenty-four drugs were also tested in monkey, dog, rat and mouse hepatocytes. Species differences in potency were observed with mouse being less sensitive than other species to inhibition of bile salt transport. In summary, a novel assay has been

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developed using hepatocytes in suspension from human and animal species that can be used to assess the potential for drugs and/or drug-derived metabolites to inhibit bile salt transport and/or formation activity. Drugs causing sDILI, except those by immune-mediated mechanism, are highly associated with potent inhibition of bile salt transport.

Keywords

Bile salt export pump; BSEP; ABCB11; Drug-induced liver injury; DILI; Bile salts; Hepatocytes; Species difference

1. Introduction

Drug induced liver injury (DILI) accounts for more than 50% of the cases of clinical acute fulminant hepatic failure [1]. It is a common cause for failures in drug development, boxed warnings on marketed drugs, and market withdrawals of approved drugs [2]. Cholestatic and mixed hepatocellular cholestatic (mixed) liver injuries are the two most common phenotypes of DILI. Drugs related to these injuries, such as troglitazone and ketoconazole, have been reported to cause severe DILI (sDILI), including acute liver failure [1]. Interference of liver transporters involved in bile formation, in particular the bile salt export pump (BSEP) and multidrug resistance protein 3 (MDR3), have been implicated in drug-induced cholestatic and mixed liver injury [1,3,4].

BSEP is responsible for biliary secretion of bile salts, which is the primary driving force for enterohepatic recirculation of bile salts and for maintaining bile flow. BSEP is a 1321 amino acid *p*-glycoprotein encoded by gene *ABCB11* and primarily expressed on the canalicular membrane of hepatocytes [5]. Genetic dysfunction of BSEP is associated with various liver diseases such as progressive familial intrahepatic cholestasis type 2, intrahepatic cholestasis of pregnancy, and DILI [6]. In recent years, BSEP inhibition has been widely recognized to be one of the underlying mechanisms for DILI [7-11]. Several BSEP activity assays have been developed to test drugs and new chemical entities for their potentials to inhibit BSEP function. These assays include: a BSEP-expressed Sf9 insect cell membrane vesicle system, a canalicular membrane vesicle system derived from rat and human whole liver, a sandwich-cultured hepatocyte system prepared using primary animal or human hepatocytes, and a doubly-transfected with BSEP and sodium taurocholate co-transporting polypeptide cell system [12-16]. Currently, the membrane vesicle assay is the most commonly used assay for drug screening due to its convenience of operation [3,7-10]. However, this membrane vesicle assay lacks the capacity to assess the role of in situ drug metabolism, and therefore could generate false negative results if a metabolite causes inhibition or false positive results if a drug is rapidly metabolized to inactive metabolites, when compared with clinical outcomes [3,8,9]. The doubly-transfected with BSEP and sodium taurocholate co-transporting polypeptide cell assay also lacks the ability to assess in situ drug metabolism [15]. The sandwich-cultured hepatocyte assay requires a multiple-day cell culture procedure, which limits the assay's throughput and reproducibility for drug screening [17,18]. Therefore, a need remains for a physiologically relevant, robust, and flexible BSEP assay that can be used in various drug screening paradigms.

Species differences in DILI are well-recognized [19,20]. The concordance between preclinical animal safety data and human clinical liver toxicity is less than 60%, based on a survey of 150 development compounds. The reasons for the poor correlation may relate to species differences in drug metabolism and disposition, immune-mediated reactions, and inter-individual genetic variations. Recently, several groups have reported differences in BSEP (Bsep in animals) expression and activity among species [17,21,22]. Moreover, loss-of-function mutations for BSEP in humans causes nearly complete disruption of biliary secretion of bile salts, which can eventually lead to progressive familial intrahepatic cholestasis type 2, a severe liver disease characterized by early-onset progressive degenerative injury and eventually liver failure [23]. However, in a Bsep knockout (KO) mouse model, animals developed only mild cholestasis with bile flow reduced to 25% of that in non-KO mice [24]. Therefore, it is important to investigate the species difference in inhibition of bile salt transport activity to understand the possible mechanisms for poor DILI correlation. To our knowledge, none of the previously available assay formats has the capacity to compare BSEP (Bsep) inhibition across the most commonly used animal species, namely monkey, dog, rat, mouse and human, for preclinical safety assessment. Here we report a novel bile salt transport activity assay involving in situ biosynthesis of bile salts from their corresponding bile acid precursors in metabolically competent primary hepatocytes in suspension and LC-MS/MS determination of the amount of bile salts transported into extracellular medium. The assay was characterized by examining the inhibition of bile salt transport activity in human hepatocyte suspensions by drugs associated with DILI. The assay was also applied to investigations of species difference in BSEP (Bsep) inhibition across human, monkey, dog, rat and mouse hepatocytes.

2. Materials and methods

2.1. Reagents

Cholic acid (CA), taurocholic acid (TCA), glycocholic acid (GCA), chenodeoxycholic acid (CDCA), taurochenodeoxycholic acid (TCDCA) and glycochenodeoxycholic acid (GCDCA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The tested drugs were purchased from Sigma-Aldrich, the US Pharmacopeia (<http://www.usp.org>), and Sequoia Research Products Ltd. (<http://www.seqchem.com/>). Some drugs that were not commercially available were synthesized at MonomerChem Inc. (Durham, NC). Pooled cryopreserved human, monkey, rat, and mouse hepatocytes, and InVitroGRO HT medium were obtained from Celsis IVT (Baltimore, MD). The human hepatocytes were pooled from 10 donors, including 5 males and 5 females at ages between 17 and 65. The 10 donors consisted of 8 Caucasians, 1 Black and 1 Hispanic. Williams Medium E was purchased from GIBCO-ThermoFisher Scientific (Waltham, MA). Other reagents were purchased from Sigma- Aldrich, unless stated otherwise in the text.

2.2. Compound selection

Eighty-six drugs were selected for use, based on the severity and clinical types of DILI. sDILI was defined according to the criteria proposed by Zhang, J. et al. in this issue [25], namely: (1) drugs listed in the World Health Organization “Consolidated List of Products Whose Consumption and/or Sale Have Been Banned, Withdrawn, Severely Restricted or Not

Approved by Governments,” or those received U.S. FDA boxed warning due to hepatotoxicity; (2) drugs associated with acute liver failure (ALF) reported in the United States and with adjudicated causality by either a standardized causality assessment tool and/or that were presented in the LiverTox website developed at the National Institute of Diabetes and Digestive and Kidney (NIDDK) diseases; and (3) drugs associated with ALF reported in the United States and/or associated with an increased reporting frequency of ALF incidents calculated as Empirical Bayes Geometric Mean (EBGM 2.0) in the WHO individual case safety report database (VigiBase™). The major DILI clinical types were classified according to two sources: (1) Zimmerman’s publication entitled “Hepatotoxicity: the adverse effects of drugs and other chemicals on the liver” [26] and (2) NIDDK’s LiverTox website [27]. The classifications were matched for most drugs with only two exceptions, fluconazole and itraconazole (Table 1).

2.3. Preparation of hepatocytes

Fifty mL InVitroGRO HT medium was pre-warmed in a 37 °C water bath. A vial of hepatocytes was removed from a liquid N₂ tank and quickly warmed up in a 37 °C water bath by holding in hand with slow rotation. As soon as the edge of the frozen cells was separated from the wall of the vial, the frozen cells were poured into a tube with pre-warmed InVitroGRO HT medium and the remaining cells in the vial were collected using pipette. The tube was centrifuged at 50 *g* at 25 °C for 5 minutes and the supernatant was removed. After the cell pellet was re-suspended in 8 mL of prewarmed Williams E buffer, cell numbers were counted in a hemacytometer. Cell viability was determined using the trypan blue method. Hepatocyte concentrations were adjusted with Williams E buffer to meet the objectives of various experiments.

2.4. Incubations with hepatocytes in suspension

Human hepatocytes at various concentrations were incubated in Williams E buffer with CA or CDCA at various concentrations at 37 °C under 5% CO₂ and saturated humidity for various times, depending on the purpose of the experiment.

For intrinsic clearance determination, human hepatocytes at 0.25×10^6 cells/mL were incubated with 1 μ M CA or CDCA for the time periods indicated in the text. The post-incubation hepatocyte suspensions were mixed with 2–3 $\times$ volume of acetonitrile followed by centrifugation at 3310 *g* for 15 min. The supernatants were analyzed by LC-MS/MS for the disappearance of CA or CDCA. The intrinsic clearance was estimated according to the method reported by Obach [28]. For metabolite identification, human hepatocytes at 0.25×10^6 cells/mL were incubated with 100 μ M CA or CDCA for 2 h. At the end of the incubation time, hepatocyte suspensions were mixed with 2–3 $\times$ volume of acetonitrile, followed by centrifugation at 3310 *g* for 15 min. The supernatants were analyzed by LC-MS/MS for metabolite formation from CA or CDCA.

For the effects of incubation time on the formation and transport of bile salts, human hepatocytes at 0.25×10^6 cells/mL were incubated with 100 μ M CA or CDCA for 0, 0.5, 1, 2, and 4 h, respectively. For the effects of hepatocyte concentration on the formation and transport of bile salts, human hepatocytes at concentrations ranging from $0-1 \times 10^6$ cells/mL

were incubated with 100 μM CA or CDCA for 1 h, respectively. For the effects of substrate concentration on the formation of bile salts formation, human hepatocytes at 0.25×10^6 cells/mL were incubated with CA or CDCA at concentrations ranging from 0 to 1000 μM for 1 h, respectively. For the inhibition experiments, human hepatocytes at 0.25×10^6 cells/mL were incubated with 10 μM CA in the presence of test drugs at various concentrations ranging from 0 to 625 μM for 1 h. Similar experiments with monkey, dog, rat, and mouse hepatocytes were performed using the same conditions used for human hepatocyte experiments. At the end of the incubation time, the extracellular medium was separated by centrifugation at 3310 g for 15 min. The resulting supernatant was mixed with $2\text{--}3 \times$ volume of acetonitrile containing the analytical internal standard (see below) for LC-MS/MS quantitation of bile salts. To determine the intracellular level of bile salts, the hepatocyte pellet after centrifugation was sonicated in the presence of 100 μL water for 5 min, followed by the addition of $2\text{--}3 \times$ volume of acetonitrile containing the analytical internal standard, mixing at 2000 rpm for 1 min, and centrifugation at 3310 g for 15 min. The supernatant was used for LC-MS/MS quantitation of bile salts.

2.5. LC-MS/MS determination of bile salts

Bile salts were determined by LC-MS/MS methods, using AB Sciex 4000 Q Trap LC-MS/MS system coupled with Shimadzu Prominence 20 HPLC. Each sample was loaded onto a Symmetry Shield RP8 (5 μm , 2.1 mm $\times$ 50 mm) reverse phase column from Waters (Milford, MA). The column chamber's temperature was ambient. The initial HPLC mobile phase conditions used for separation and elution of analytes comprised 2 mM ammonium acetate buffer in water containing 0.1% formic acid and 10% acetonitrile. The flow rate was 0.5 mL/min. The amount of acetonitrile in the mobile phase was ramped linearly up to 40% over a 2-min period followed by a rapid increase to 95% acetonitrile in 0.5 min. After holding at 95% acetonitrile for 1.2 min, the mobile phase was reset to the initial conditions in 0.1 min. The analytical column was equilibrated with the starting mobile phase for 1.2 min. The total run time for each sample analysis was approximately 5 min.

The HPLC eluate was injected into the LC/MS/MS system equipped with a turbo ionspray source set with a desolvation temperature of 450 $^{\circ}\text{C}$. Nitrogen was used as curtain gas, nebulizer gas, heater gas, and collision gas. Data for bile acids and salts was acquired in the positive ion mode using multiple reaction monitoring methods (MRM). The ion transitions of the MRM method for specific detection of GCA, TCA, GDCA, TDCA, GLCA, TLCA, GCDCA, and TCDCA were developed in standard fashion. Carbutamide was used as internal standard (IS), m/z 272/156. Ionspray voltage was set at 4000 V and the collision gas (CAD) set at 6. Declustering potential was set at 82, 46, and 49 for GCA, TAC, and internal standard (IS), respectively. Collision energy was set at 25, 33, and 23 eV for GCA, TAC, and IS. The lower limit of quantification for the bile salts was 1 nM and the linearity range was from 1 to 1000 nM.

2.6. Data analysis

Kinetic parameters were calculated using standard Michaelis- Menten kinetics. Mean and standard deviation were calculated using Excel functions. IC_{50} values were determined using

the median-effect equation [29]. Area under the dose-response curve (AUC) of BSEP activity was estimated using GraphPad Prism software.

The correlation between the AUC values of the dose-response curve for BSEP activity (Table 1) and the normalized AUC values of the reactive oxygen species (ROS)/ATP ratio, a composite biomarker reported in this issue [25], as well as ATP alone were further explored in this study. The 80 overlapped drugs were categorized into 5 groups based on the characteristics of liver injury: Group-1 is related to non-immune-mediated sDILI drugs, Group-2 to immune-mediated sDILI drugs, Group-3 to non-sDILI drugs with bland cholestasis, Group-4 to non-sDILI drugs with hypersensitivity which could lead to either VBDS or cholestatic hepatitis, and Group-5 to other non-sDILI drugs. Logarithmic values of all AUC values were used for the correlation analysis and logistic regression with receiver operating characteristic (ROC) were performed to identify the cut-off value that differentiate positive and negative drugs by optimal likelihood ratio which equals to sensitivity/(1-specificity).

3. Results

3.1. Metabolism of bile acids in human hepatocytes in suspension

As shown in Fig. 1, both CA and CDCA were rapidly metabolized in human hepatocytes, with projected hepatic extraction ratios of 0.62 and 0.90, respectively. The metabolites formed from CA and CDCA were profiled using LC-MS/MS methods (data not shown). GCA was the major metabolite formed from CA in human hepatocytes, with only minor amounts of TCA. GCDCA was the major metabolite formed from CDCA in human hepatocytes, with minor amounts of TCDCA. A trace amount of glucuronide conjugate of CDCA was also detected in human hepatocyte incubation. Monkey hepatocytes were similar to human hepatocytes, with GCA and GCDCA the major metabolites formed when incubated with CA and CDCA, respectively. In dog and mouse hepatocytes, TCA and TCDCA were the major metabolites formed from CA and CDCA, while in rat hepatocytes, GCA and TCA or both GCDCA and TCDCA were the major metabolites formed from CA or CDCA, respectively.

3.2. Formation and transport of bile salts in human hepatocytes

In human hepatocytes, the formation of GCA from CA and the formation of GCDCA from CDCA both increased with an increase in incubation time through 1 h (Fig. 2). After 1 h, there was no further increase in the formation of either GCA or GCDCA. Greater than 90% of the GCA and GCDCA produced by hepatocytes from CA and CDCA were transported into the extracellular medium after 1 h of incubation. In addition, the formation of GCA from CA or GCDCA from CDCA increased with hepatocyte concentrations up to 0.5×10^6 cells/mL (data not shown).

The formation rates in human hepatocytes of GCA from CA and GCDCA from CDCA both increased with increasing concentrations of CA and CDCA, with the formation of GCA and GCDCA both being saturated at CA and CDCA concentration of $>300 \mu\text{M}$ (Fig. 3). Apparent K_m values were estimated to be $>300 \mu\text{M}$ for both GCA and GCDCA. Similar

findings were observed for the transport of GCA and GCDCA in human hepatocytes (Fig. 3). The transport rates of GCA and GCDCA were plotted as a function of the concentrations of GCA and GCDCA formed in hepatocyte incubation, respectively (data not shown). The transport rates increased linearly with increasing concentrations of both GCA and GCDCA formed in situ in human hepatocyte incubation with CA and CDCA.

3.3. Inhibition of bile salt transport by known BSEP inhibitors

Ketoconazole, saquinavir, cyclosporine, and troglitazone displayed concentration-dependent inhibition of bile salt transport activity in human hepatocytes (Fig. 4). Their IC_{50} values were 3.0, 0.4, 0.1, and 0.5 μM , respectively. Incubations in human hepatocytes with the negative control furosemide showed no substantial inhibition of bile salt transport activity.

3.4. Effects of drugs associated with DILI on bile salt transport activity in human hepatocytes

Eighty six drugs, including 35 drugs associated with sDILI and 51 drugs with non-sDILI, were tested for their potential to inhibit bile salt transport activity in blinded experiments (Table 1). Among the 35 sDILI drugs, 21 drugs (approximately 60% of total), namely ritonavir, saquinavir, troglitazone, benzydaron, ciglitazone, ketoconazole, atorvastatin, indinavir, benzbromarone, cerivastatin, fipexide, fluoxetine, bosentan, cyclofenil, deferasirox, disulfiram, flutamide, bicalutamide, diclofenac, lumiracoxib and leflunomide, showed inhibition of bile salt transport activity with IC_{50} values of $<50 \mu M$. Among the 51 non-sDILI drugs, 10 drugs (approximately 20% of total), namely cyclosporine, rosiglitazone, pioglitazone, clotrimazole, haloperidol, lovastatin, chlorpromazine, glimepiride, buspirone, and trazadone, showed inhibition of bile salt transport activity with IC_{50} values of $<50 \mu M$.

The 14 sDILI drugs that did not display substantial inhibition of bile salt transport activity were bromfenac, imipramine, ibuprofen, isoniazid, itrazonazole, carbamazepine, abacavir, ciprofloxacin, clarithromycin, labetalol, amiodarone, fialuridine, azithromycin, and azathioprine. The majority of the non-sDILI drugs, approximately 80%, showed no substantial inhibition of bile salt transport activity (Table 1).

Biotin, furosemide, and penicillamine, which are not associated with DILI, were found not to inhibit bile salt transport activity. Ketoconazole (positive control) and furosemide (negative control) were assessed three times, and biotin (negative control) twice in blinded experiments. The IC_{50} values calculated from three blinded experiments for the positive control ketoconazole were 1.1 μM and 1.5 μM with testing concentrations ranging from 0.14 to 100 μM , and 3.0 μM with testing concentration ranging from 0.34 to 250 μM . All experiments showed the IC_{50} values for the negative controls furosemide and biotin were greater than the highest testing concentration at 100 μM or 250 μM .

3.5. Correlation between inhibition of bile salt transport activity and ROS/ATP ratio

The logarithmic AUC values of the BSEP dose-response curves were plotted as a function of that of either normalized ROS/ATP ratio or ATP depletion (Fig. 6). The plots were divided into four quadrants using cut-off values of 0.62 for ROS/ATP ratio, 1.73 for BSEP

inhibition, and 2.14 for ATP which were generated by likelihood ratio from receiving operating characteristics (Fig. 6). The four quadrants in the plot of BSEP vs. ROS/ATP are as follows: Quadrant-1 (higher ROS/ATP ratio and higher BSEP inhibition), Quadrant-2 (higher ROS/ATP ratio and lower BSEP inhibition), Quadrant-3 (lower ROS/ATP ratio and lower BSEP inhibition), and Quadrant-4 (lower ROS/ATP ratio and higher BSEP inhibition). Those in the plot of BSEP vs. ATP are as follows: Quadrant-1 (lower ATP level and higher BSEP inhibition), Quadrant-2 (lower ATP level and lower BSEP inhibition), Quadrant-3 (higher ATP level and lower BSEP inhibition), and Quadrant-4 (higher ATP level and higher BSEP inhibition).

As indicated in Fig. 6a and b, there was no apparent correlations between BSEP inhibition and ROS/ATP ratio ($r = -0.45$) as well as ATP depletion ($r = 0.54$). In the plot of BSEP vs. ROS/ATP ratio (Fig. 6a), most of the drugs in Quadrant-1 are associated with nonimmune mediated-sDILI (Group-1 drugs) including atorvastatin, benzbromarone, benzydaron, bicalutamide, cerivastatin, cyclofenil, deferasirox, disulfiram, fipexide, fluoxetine, flutamide, ketoconazole, ritonavir, saquinavir and troglitazone, and the others with bland cholestasis (Group-3), including chlorpromazine and rosiglitazone. In Quadrant-2 there are drugs associated with immunemediated sDILI (Group-2), including abacavir, azathioprine, azithromycin, bromfenac, diclofenac, imipramine, isoniazid, labetalol, leflunomide and lumiracoxib. In Quadrant-3 there are drugs associated with VBDS and cholestatic hepatitis (Group-5), including captopril, chlorpropamide, cimetidine, enalapril, erythromycin (free base), ethambutol, fenofibrate, griseofulvin, methimazole, norfloxacin, penicillamine, phenobarbital, piroxicam, sulindac, ticlopidine and tolbutamide, and other non-sDILI (Group-4, see Table 1). And in Quadrant-4 there are drugs associated with bland cholestasis (Group-3), including clotrimazole, cyclosporine, glimepiride, lovastatin, pioglitazone, trazadone.

3.6. Effects of selected DILI drugs on bile salt transport activity in human, monkey, dog, rat, and mouse hepatocytes

Troglitazone and glimepiride caused concentration-dependent inhibition of bile salt transport activity in human, monkey, dog, rat, and mouse hepatocytes (Fig. 5). Human hepatocytes appeared to be more sensitive (lower IC_{50} values) than monkey, dog, rat, and mouse hepatocytes for troglitazone-mediated inhibition of bile salt transport activity, while rat hepatocytes appeared to be more sensitive (lower IC_{50} value) than other species for glimepiride-mediated inhibition of bile salt transport activity.

We tested 24 drugs for their potential to inhibit bile salt transport activity in monkey, dog, rat, and mouse hepatocytes compared to human hepatocytes. The IC_{50} values are listed in Table 2. The IC_{50} values derived from mouse hepatocyte incubations for all tested drugs were much higher than those of other species. Saquinavir, cyclosporine, lovastatin, rosiglitazone, and troglitazone showed much lower IC_{50} values derived from human hepatocyte incubations than those of other species, while the IC_{50} values for atorvastatin and glimepiride derived from human hepatocytes was greater than those calculated for monkey, dog, rat, and mouse.

4. Discussion

Bile salts are synthesized from cholesterol via two primary pathways (classic and alternative) which involve multiple steps of oxidation and conjugation reactions in hepatocytes [30]. The classic pathway involves initial oxidation by cytochrome CYP7A1 (cholesterol 7 α -hydroxylase), while the alternative pathway starts with side chain hydroxylation by CYP27A1 (sterol 27-hydroxylase). Both pathways lead to the formation of the bile acids CA and CDCA. The bile acids are further amidated with glycine and taurine to form bile salts by the enzymes bile acid:CoA synthetase (BACS) and bile acid:amino acid transferase (BAT) [30]. In general, CYP7A1 is the rate-limiting enzyme in bile salt synthesis. Amidation of bile acids dramatically lowers their pKa values by 2.4–5 units [31]. Because of the differences in physicochemical properties between bile acids and bile salts, they have marked differences in cell permeability at physiological pH. At physiological pH, bile acids are readily permeable, while bile salts are impermeable. This difference in permeability requires transporters to allow bile salts to cross the hepatocyte plasma membrane. The primary transporter responsible for efflux transport of bile salts in human hepatocytes is BSEP [5,23]. The assay we report here involves incubation of hepatocytes with exogenous bile acids that allows for bypassing the de novo rate-limiting step of bile acid synthesis to form bile salts, followed by specific LC-MS/MS quantification of amounts of bile salts exported into an extracellular medium by BSEP.

In characterizing the hepatocyte in suspension assay, we initially investigated the metabolism of the bile acids CA and CDCA using human hepatocytes in suspension. CA and CDCA were both rapidly metabolized to form their corresponding glycine-amidated conjugates (Figs. 1 and 2). Furthermore, the formation rate of GCA and GCDCA in human hepatocytes in suspension was dependent on the concentrations of their corresponding exogenous bile acids (Fig. 3). Kinetic analysis indicated the involvement of low binding affinity/high capacity kinetics, suggesting that the amidation of exogenous bile acids was not readily saturated. Species differences were observed in the formation of glycine and taurine conjugates of CA and CDCA [32]. Human and monkey incubations with CA and CDCA predominantly formed the glycine conjugates GCA and GCDCA; in dog and mouse hepatocyte incubations, the corresponding taurine conjugates TCA and TCDCA were primarily produced. Rat hepatocytes differed from the other species in producing significant amounts of both the glycine and taurine conjugates with incubations of CA and CDCA. The reason for the species preference in the formation of glycine or taurine conjugate of bile acids in hepatocyte incubation is unclear.

Next we investigated the extracellular transport of bile salts formed from CA or CDCA by in situ metabolism in hepatocytes in suspension. Results demonstrated that the bile salts produced in situ were effectively and efficiently transported into the extracellular medium (Figs. 2 and 3). The formation and transport of bile salts was found to be linear through a 1-h incubation period and increased with increasing hepatocyte concentrations up to 0.5 million cells/mL. Moreover, the transport rate of bile salts displayed a positive linear relationship with the concentration of bile salts formed in situ (data not shown). Based on these results, incubation conditions for inhibition studies were optimized as indicated in the

Experimental Procedures section. In selection of the bile acid concentrations for inhibition studies, the physiological relevance was also taken into consideration [33].

To assess the ability of the assay using human hepatocytes to accurately measure inhibition of bile salt transport activity, experiments were conducted using both known BSEP inhibitors (positive controls) and non-BSEP inhibitors (negative controls). Results demonstrated that bile salt transport activities were inhibited by the known BSEP inhibitors ketoconazole, saquinavir, cyclosporine, and troglitazone, while the negative control furosemide did not show inhibition. The IC_{50} values were comparable to those reported [7,8]. These results demonstrate that isolated hepatocytes in suspension maintain not only the function of amidation of bile acids, but also the function of bile salt transport (BSEP) activity.

The loss of ATP-binding cassette drug efflux transporters during isolation and preparation of hepatocytes by a process termed internalization has been reported [34]. However, Lundquist et al. recently reported that the expression and activity of the ABC transporters P-glycoprotein, multidrug resistance-associated protein 2 (MRP2), MRP3, MRP4, and BSEP in commercially available human and rat cryopreserved hepatocytes exhibited expression levels similar to those found in fresh liver biopsies [35]. The ABC transporters in human cryopreserved cells were localized to the plasma membrane. Functional studies demonstrated P-glycoprotein activity in both human cryopreserved and fresh rat hepatocytes. The results presented here clearly demonstrate that bile salt transport activity is preserved in human, monkey, dog, rat, and mouse cryopreserved hepatocytes in suspension. Based on experimental results, we elected to use for further investigation the transport of GCA or TCA formed from CA in situ. Experiments with other bile acids are the subject of further investigations.

To assess the applicability and usefulness of the hepatocytes in suspension assay, we investigated 86 drugs in blinded experiments for their potential to inhibit bile salt transport activity in human hepatocytes (Table 1). Thirty five of these drugs were known to cause sDILI. Approximately 60% of the sDILI drugs (21 drugs) displayed potent inhibition of bile salt transport activity and included ritonavir, saquinavir, troglitazone, benziodarone, ciglitazone, ketoconazole, atorvastatin, indinavir, benzbromarone, cerivastatin, fipexide, fluoxetine, bosentan, cyclofenil, deferasirox, disulfiram, flutamide, bicalutamide, diclofenac, lumiracoxib and leflunomide. Many drugs in this group are associated with cholestatic and mixed liver injury, and several are associated with hepatocellular necrosis. Among the 51 non-sDILI drugs, approximately 20% (10 drugs) also showed potent inhibition of bile salt transport activity (with IC_{50} values of $<50 \mu M$), and included cyclosporine, rosiglitazone, pioglitazone, clotrimazole, haloperidol, lovastatin, chlorpromazine, glimepiride, buspirone, and trazadone. Several of these drugs are known to be associated with cholestatic or mixed liver injury. In general, for these non-sDILI drugs, C_{max} values are relatively low ($<1 \mu M$) except for trazadone (Table 1). A possible reason for the relatively less severe clinical outcomes for this group of drugs may be related to the relatively low systemic drug exposures. These results demonstrated a correlation between inhibition of bile salt transport activity and severity of liver injury, and indicated that systemic drug exposure should be taken into consideration.

The sDILI drugs that did not show substantial inhibition of bile salt transport activity are related to other mechanisms of liver injury. We reported in this issue a composite biomarker, ROS/ATP ratio, that could be used to identify sDILI [25]. The correlation between the inhibition of the bile salt transport activity and the ROS/ATP ratio was explored in the present study (Fig. 6). The analysis suggests that the inhibition of bile salt transport activity is not associated with immune-mediated sDILI. Most of non-immune mediated sDILI drugs with higher ROS/ATP ratio showed higher BSEP inhibition. The two non-sDILI drugs, amiloride and isocarboxazid, showed higher ROS/ATP ratio (false positive), but did not inhibit BSEP activity. These results suggest BSEP inhibition could complement the ROS/ATP ratio in screening compounds that may cause sDILI. Vanishing bile duct syndrome may involve interference of other molecular targets such as multidrug resistant protein 3 (MDR3) [4], which is responsible for the biliary secretion of phosphatidylcholines [36,37]. The phosphatidylcholines combine with bile salts in bile flow to form mixed micelles, which prevent the highly concentrated free bile salts from damaging biliary canaliculi epithelium cells. The interference in biliary secretion of phosphatidylcholines can result in the disruption of bile salts' phosphatidylcholines mixed micelles, which in turn can lead to deleterious effects of the highly concentrated bile salts on bile canaliculi epithelial cells. It is also important to point out that the genetic variants in BSEP, other transporters, and drug metabolism enzymes may predispose individual patients to DILI.

To investigate species differences, we tested 24 drugs for their potential to inhibit bile salt transport in primary hepatocytes from human, monkey, dog, rat, and mouse (Fig. 2, Table 2). In general, the ability of these drugs to inhibit bile salt transport activity is much lower in mouse hepatocytes compared to human and other species. This is consistent with the reported mild manifestations of liver injury in Bsep KO mice [24]. Some drugs showed a notable species difference in inhibition of bile salt transport activity. For example, troglitazone caused more inhibition of bile salt transport in human hepatocytes than other species. The species difference in inhibition of bile salt transport may be attributed to differences in drug metabolism and disposition, the contribution of transporters other than Bsep in excretion of bile salts, and/or in molecular interactions between the inhibitors and Bsep proteins among species, especially for mouse.

Several drugs such as flutamide and chlorpromazine did not inhibit BSEP activity in the membrane assay [7-10], yet they are potent inhibitors in our hepatocyte assay (Table 1). These drugs are known to undergo extensive metabolism to form several metabolites. Given that the primary hepatocytes used in the present study retain competent metabolic capacity, differences in BSEP inhibition between the membrane vesicle assay and the hepatocyte in suspension assay could be the result of in situ drug metabolism [35]. Primary hepatocytes are considered the gold standard and the most physiologically relevant model system for in vitro investigations of hepatic drug metabolism and disposition, and hepatotoxicity [35]. In terms of bile salt transport inhibition, potential inhibitors could be converted to metabolites with more or less potency, which could be a major contributing factor in the discrepancy between BSEP inhibition potency determined by using membrane vesicles with their in vivo hepatotoxicity profile. In addition, potential interference in amidation of bile acids in hepatocytes could also affect the extracellular levels of bile salts. BSEP inhibition and other mechanisms of drug-mediated toxicity such as mitochondria damage may act synergistically

to mediate the severity of hepatotoxicity, which could not be predicted by BSEP inhibition data generated with non-physiological assays [38]. The present method utilizes primary hepatocytes in suspension that are metabolically competent and maintain biological functions (mitochondria oxidation), and therefore, it has more physiologically relevance than membrane vesicle assays.

In summary, we report a novel assay that measures BSEP activity using primary hepatocytes from human, monkey, dog, rat, and mouse. Species differences are observed in BSEP inhibition. Drugs associated with cholestatic and mixed liver injury demonstrate potent inhibition of BSEP transport activity and show strong correlation with DILI severity.

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Abbreviations

BSEP	bile salt export pump
CA	cholic acid
CDCA	chenodeoxycholic acid
DILI	drug-induced liver injury
GCA	glycocholic acid
GCDCA	glycochenodeoxycholic acid
KO	knock-out
LC-MS/MS	high performance liquid chromatography tandem mass spectrometry
MDR3	multidrug resistance protein 3
NCTR	National Center for Toxicological Research
nsDILI	non-severe drug induced-liver injury
ROS	reactive oxygen species
sDILI	severe drug-induced liver injury
TCA	taurocholic acid
TCDCa	taurodeoxycholic acid

Fig. 1.
The disappearance of cholic acid (CA) and chenodeoxycholic acid (CDCA) in human hepatocyte incubations. Human hepatocytes (0.25 million cells/mL) were incubated with CA or CDCA (1 μ M) at 37°C for specified time points (N = 3).

Fig. 2.

Effects of incubation time on the formation and transport of glycocholic acid (GCA) (left panel) and chenodeoxycholic acid (GCDCA) (right panel) in human hepatocytes. Human hepatocytes (0.25 million cells/mL) were incubated with CA or CDCA (100 μ M) at 37 $^{\circ}$ C for various time points (N = 3). The amount of GCA or GCDCA in extracellular medium was used for the transport activity, and the sum of the amounts in both hepatocyte pellet and extracellular medium was used for the formation activity.

Fig. 3.

Effects of concentrations of cholic acid (CA) (right panel) and chenodeoxycholic acid (CDCA) (left panel) on the formation and transport of glycocholic acid (GCA) and chenodeoxycholic acid (GCDCA) in human hepatocytes. Human hepatocytes (0.25 million cells/mL) were incubated with CA or CDCA at various concentrations at 37 °C for 1 h (N = 3). The amount of GCA or GCDCA in extracellular medium was used for the transport activity, and the sum of the amounts in both hepatocyte pellet and extracellular medium was used for the formation activity.

Fig. 4. Effects of BSEP inhibitors on bile salt transport activity in human hepatocytes in suspension. Furosemide was used as negative control. Human hepatocytes (0.25 million cells/mL) were incubated with CA (10 μM) at 37°C for 1 h (N = 3). The amount of GCA or GCDCA in extracellular medium was used for the transport activity.

Fig. 5. Inhibition of troglitazone (left panel) and glimepiride (right panel) on bile salt transport activity in mouse, rat, dog, monkey, and human hepatocytes. Glycocholic acid (GCA) was measured for rat, human, and monkey, and taurocholic acid (TCA) was used for mouse and dog (N = 3).

Fig. 6.

Plots of log (BSEP activity AUC) against log (normalized ROS/ATP ratio) (a) and log (ATP activity AUC) (b). The cut-off value is indicated in dash line of each axis. The category of each drug group is labeled with colors.

IC₅₀ values for inhibition of bile salt transport activity by drugs associated with DILI in human hepatocytes.

Table 1

Drug	Severity	BSEP IC ₅₀ (μM) ^a	Log(BSEP AUC) ^b	Log(ROS/ATP AUC) ^c	Log(ATP AUC) ^c	Category	Cmax (μM)	Daily dose (mg)	DILI annotation
Atorvastatin	SD	2.6	1.4	1.2	2.0	1	0.008	20	Mixed
Benzbromarone	SD	2.8	1.1	2.0	1.7	1	4.3	100	HCN
Benziodarone	SD	0.76	0.8	1.8	1.2	1	1.8	600	HCN
Bicalutamide	SD	22.4	1.6	1.8	2.0	1	2.0	50	HCN
Bosentan	SD	9.2	1.8	0.7	2.1	1	7.4	250	CHL
Cerivastatin	SD	3.9	1.3	1.1	2.1	1	0.03	0.2	Mixed
Cyclofenil	SD	10.7	1.6	1.0	2.0	1	0.21	140	HCN
Deferasirox	SD	11.9	1.7	0.9	2.0	1	170.0	125 to 500	HCN
Disulfiram	SD	12.0	1.3	1.1	2.2	1	5.4	200	HCN
Fipexide	SD	4.9	1.5	1.4	2.1	1	N/A	600	HCN
Fluxetine	SD	5.5	1.3	1.2	1.5	1	0.05	20	HCN
Flutamide	SD	21.4	1.6	1.4	2.0	1	0.36	750	Mixed
Itraconazole	SD	Atypical inhibition	1.9	0.9	1.9	1	0.40	200	CHL
Ketoconazole	SD	3.0	1.1	2.0	1.6	1	7.0	200	HCN
Ritonavir	SD	0.2	0.9	0.8	2.1	1	7.6	1200	CHL
Saquinavir	SD	0.4	0.8	1.2	1.9	1	5.4	1800	CHL
Troglitazone	SD	0.5	0.8	1.4	1.9	1	6.4	400	CHL
Abacavir	SD	>100	2.0	1.3	1.9	2	14.9	600	HCN
Amiodarone	SD	>100	2.0	1.6	1.7	2	0.85	200	HCN
Azathioprine	SD	>100	2.0	1.4	2.0	2	0.34	150	Mixed
Azithromycin	SD	>100	2.0	0.8	2.2	2	0.53	500	CHL
Bromfenac	SD	93.3	2.0	0.8	2.1	2	18.0	150	HCN
Carbamazepine	SD	>100	2.0	0.5	2.2	2	25.8	1000	Mixed
Ciprofloxacin	SD	>100	2.0	0.6	2.2	2	6.6	N/A	
Clarithromycin	SD	>100	1.7	0.6	2.2	2	4.0	500	CHL
Diclofenac	SD	31.8	1.9	0.7	2.1	2	6.6	100	HCN
Ibuprofen	SD	>100	2.0	0.6	2.2	2	250.0	1200	Mixed
Imipramine	SD	>100	1.8	1.3	2.0	2	0.29	100	Mixed

Drug	Severity	BSEP IC50 (μM) ^a	Log(BSEP AUC) ^b	Log(ROS/ATP AUC) ^c	Log(ATP AUC) ^d	Category	Cmax (μM)	Daily dose (mg)	DILI annotation
Isoniazid	SD	>100	2.0	0.7	2.1	2	76.6	300	HCN
Labetalol	SD	>100	2.0	0.8	2.1	2	2.7	600	HCN
Leflunomide	SD	50	2.0	1.3	1.9	2	24.8	20	HCN
Lumiracoxib	SD	45.6	2.0	0.9	2.2	2	31.5	100	CHL
Ciglitazone	SD	0.78	1.1	NA	NA	NA	N/A	N/A	N/A
Fialuridine	SD	>100	2.0	NA	NA	NA	0.60	N/A	HCN
Indinavir	SD	2.8	1.6	NA	NA	NA	13.2	2400	Mixed
Chlorpromazine	NSD	6.4	1.7	1.7	1.9	3	0.94	300	CHL
Clotrimazole	NSD	1.1	1.0	0.6	2.2	3	0.09	100	CHL
Cyclosporine	NSD	0.1	0.8	0.4	1.9	3	0.78	250	CHL
Glimepiride	NSD	14.1	1.6	0.1	2.3	3	1.1	2	Mixed
Haloperidol	NSD	25.3	1.7	1.3	2.0	3	0.05	8	CHL
Lovastatin	NSD	1.7	1.2	0.4	2.1	3	0.05	45	Mixed
Pioglitazone	NSD	1.4	1.4	0.6	2.2	3	2.9	30	Mixed
Piperacillin	NSD	>100	2.0	0.3	2.3	3	N/A	14,000	CHL
Rofecoxib	NSD	>100	1.8	0.5	2.2	3	0.69	25	CHL
Rosiglitazone	NSD	0.2	0.9	1.1	2.2	3	1.0	6	Mixed
Trazadone	NSD	43	1.7	0.3	2.1	3	5.1	300	Mixed
Amiloride	NSD	>100	2.0	1.4	2.0	4	0.09	10	HCN
Aminosalicylic acid	NSD	>100	2.0	0.4	2.3	4	5.5	1500	HCN
Atropine	NSD	>100	2.0	0.5	2.2	4	0.02	1.5	NM
Benzazac acid	NSD	>100	2.0	0.5	2.2	4	N/A	N/A	HCN
Biotin	NSD	>100	1.9	0.4	2.3	4	0.001	Supplement	NM
Bisoprolol	NSD	>100	2.0	0.4	2.3	4	0.13	10	HCN
Bupropion	NSD	>100	2.0	0.5	2.2	4	0.50	300	HCN
Cyclophosphamide	NSD	>100	2.0	0.2	2.3	4	264.3	50 to 350	HCN
Cytarabine	NSD	>100	2.0	0.4	2.3	4	N/A	IV injection	HCN
Dacarbazine	NSD	>100	2.0	0.3	2.2	4	20.6	N/A	HCN
Emtricitabine	NSD	>100	2.0	0.4	2.3	4	17.2	200	NM
Etodolac	NSD	83.9	2.0	0.3	2.3	4	68.5	400	Mixed
Etoposide	NSD	>100	2.0	0.5	2.3	4	17.0	50	HCN

Drug	Severity	BSEP IC ₅₀ (μM) ^a	Log(BSEP AUC) ^b	Log(ROS/ATP AUC) ^c	Log(ATP AUC) ^c	Category	C _{max} (μM)	Daily dose (mg)	DILI annotation
Exifone	NSD	>100	2.0	0.5	2.2	4	N/A	N/A	
Fluconazole	NSD	>100	2.0	0.6	2.2	4	8.8	200	CHL
Folic acid	NSD	>100	2.0	0.1	2.3	4	0.04	0.4	NM
Furosemide	NSD	>100	1.9	0.3	2.3	4	3.3	40	NM
Ganciclovir	NSD	>100	2.0	0.6	2.2	4	4.6	3000	NM
Gemfibrozil	NSD	>100	2.0	0.5	2.3	4	360.7	1200	HCN
Isocarboxazid	NSD	>100	2.0	1.0	2.1	4	N/A	15	HCN
Leuporelin	NSD	>100	2.0	0.5	2.3	4	N/A	1	NM
Captopril	NSD	>100	2.0	0.4	2.2	5	8.9	50	CHL
Chlorpropamide	NSD	>100	2.0	0.5	2.3	5	130.1	375	CHL
Cimetidine	NSD	>100	1.9	0.2	2.3	5	4.5	800	Mixed
Enalapril	NSD	>100	2.0	0.5	2.3	5	0.40	10	Mixed
Erythromycin (free base)	NSD	>100	1.9	0.3	2.3	5	11.0	1000	CHL
Ethambutol	NSD	>100	2.0	0.6	2.3	5	24.5	1200	CHL
Fenofibrate	NSD	61	1.8	0.5	2.2	5	4.1	200	HCN
Griseofulvin	NSD	>100	1.9	0.4	2.3	5	4.5	500	CHL
Methimazole	NSD	>100	1.9	0.5	2.3	5	2.6	15–60	CHL
Norfloxacin	NSD	>100	1.9	0.5	2.2	5	8.0	800	Mixed
Penicillamine	NSD	>100	2.0	0.4	2.2	5	27.5	500	CHL
Phenobarbital	NSD	>100	2.0	0.5	2.2	5	174.0	100	Mixed
Piroxicam	NSD	>100	1.9	0.5	2.3	5	6.0	20	CHL
Sulindac	NSD	>100	1.7	0.4	2.3	5	32.0	400	CHL
Ticlopidine	NSD	82	1.7	0.5	2.1	5	8.1	500	CHL
Tolbutamide	NSD	>100	1.8	0.3	2.3	5	233.0	1500	Mixed
Buspirone	NSD	19	1.9	NA	NA	NA	N/A	N/A	NM
Didanosine	NSD	>100	2.0	NA	NA	NA	N/A	400	HCN
Entacapone	NSD	74	2.0	NA	NA	NA	N/A	N/A	CHL

SD: severe DILI; NSD: non-severe DILI; CHL: cholestatic; HCN: hepatocellular necrosis; Mixed: mixed cholestatic and hepatocellular injury; NM: DILI not mentioned.

^aIC₅₀ values were calculated using the mean values of triplicate experiments.

^bAUC: area under the dose-response curve, the AUC value was approximated to be 100 for drug with no inhibition at 100 μM.

The results were reported elsewhere [25].

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Table 2

IC₅₀ values for bile salt transport activity by drugs associated with DILI in human, monkey, dog, rat, and mouse hepatocytes.^a

Drug	Human ^b	Monkey	Dog	Rat	Mouse
Haloperidol	25.3	64	73	54	>100
Ticlopidine	82.0	>100	>100	>100	>100
Saquinavir	0.4	3.1	3.5	5.1	>100
Atorvastatin	2.6	0.6	0.9	0.2	31.7
Azathioprine	>1000	>100	>100	>100	>100
Cerivastatin	3.9	0.99	8.8	1.9	26.5
Chlorpromazine	44.3	7.28	29.4	13.1	136.6
Cyclosporine	0.1	0.71	2.4	0.5	4.69
Enalapril	>1000	>100	>100	>100	>100
Etidolac	83.9	>100	>100	>100	>100
Fenofibrate	61	126.5	89	178.6	>100
Glimepiride	14.1	5.3	12.3	1.5	>100
Griseofulvin	756.9	43.5	85.8	45.7	>100
Imipramine	185.2	106.1	171.3	169.3	>100
Ketoconazole	1.5	4.4	7.5	4.1	>100
Lovastatin	1.7	14	15.9	14.2	>100
Methimazole	>1000	>100	>100	>100	>100
Norfloxacin	>1000	>100	>100	>100	>100
Piroxicam	151.4	182.4	>100	>100	>100
Ritonavir	0.21	1.2	1.7	0.5	5.7
Rosiglitazone	0.23	5	24.4	9.5	>100
Sulindac	122.9	>100	>100	>100	>100
Trazodone	42.9	134	>100	186	>100
Troglitazone	0.48	1.4	4.6	4.9	65.4

^a Glycocholic acid (GCA) was measured for rat, human and monkey, taurocholic acid (TCA) for mouse and dog, and IC₅₀ values were calculated using the mean values of triplicate experiments.

^b Same values as those in Table 1.