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Early Identification of Clinically Relevant Drug Interactions With the Human Bile Salt Export Pump (BSEP/ABCB11)

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A comprehensive analysis was performed to investigate how inhibition of the human bile salt export pump (BSEP/ABCB11) relates to clinically observed drug-induced liver injury (DILI). Inhibition of taurocholate (TA) transport was investigated in BSEP membrane vesicles for a data set of 250 compounds, and 86 BSEP inhibitors were identified. Structure-activity modeling identified BSEP inhibition to correlate strongly with compound lipophilicity, whereas positive molecular charge was associated with a lack of inhibition. All approved drugs in the data set (n = 182) were categorized according to DILI warnings in drug labels issued by the Food and Drug Administration, and a strong correlation between BSEP inhibition and DILI was identified. As many as 38 of the 61 identified BSEP inhibitors were associated with severe DILI, including 9 drugs not previously linked to BSEP inhibition. Further, among the tested compounds, every second drug associated with severe DILI was a BSEP inhibitor. Finally, sandwich-cultured human hepatocytes (SCHH) were used to investigate the relationship between BSEP inhibition, TA transport, and clinically observed DILI in detail. BSEP inhibitors associated with severe DILI greatly reduced the TA canalicular efflux, whereas BSEP inhibitors with less severe or no DILI resulted in weak or no reduction of TA efflux in SCHH. This distinction illustrates the usefulness of SCHH in refined analysis of BSEP inhibition. In conclusion, BSEP inhibition in membrane vesicles was found to correlate to DILI severity, and altered disposition of TA in SCHH was shown to separate BSEP inhibitors associated with severe DILI from those with no or mild DILI.

Key Words: **biliary excretion; drug induced liver injury; hepatocytes; *in vitro* and alternatives; disposition; risk assessment; alternatives to animal testing; predictive toxicology.**

During the last decade, drug-induced liver injury (DILI) has been the most frequent cause of safety-related drug marketing withdrawals in the United States (Food and Drug Administration, 2009). In addition to the suffering of individual patients and the costs of the associated health care, DILI poses a major economic challenge to the pharmaceutical industry, as a result of decreasing marketing approval rates, postmarketing restrictions, and boxed warnings. Most drugs that cause DILI do so in an irregular and unpredicted fashion, also described as idiosyncratic events. Among those drugs withdrawn from the market due to severe liver injuries, hepatic failure typically occurred in fewer than 1 of 10 000 treated patients (Food and Drug Administration, 2009). Due to the low frequency, compounds causing severe DILI are challenging to identify in clinical trials and often remain unidentified until postmarketing monitoring when the drug has become available to a larger population (Bleibel *et al.*, 2007). The initial mechanism of hepatotoxicity for drugs and their resulting metabolites varies, but independently of the origin of the first insult, the mitochondria seem to play a major role in the initiation and progression of DILI (Russmann *et al.*, 2009; Xu *et al.*, 2008). Initial cell stress can be caused by a wide range of mechanisms including glutathione depletion, binding to intracellular structures, and inhibition of hepatocellular functions, eg, canalicular bile salt secretion through inhibition of the bile salt export pump (BSEP/ABCB11) (Lee, 2003; Mackay, 1999; Pauli-Magnus and Meier, 2006; Rashid *et al.*, 2004).

BSEP mediates the ATP-dependent saturable efflux of monovalent bile salts across the canalicular membrane of the hepatocyte. The transporter constitutes the rate-limiting step in the transport of bile salts from the blood into the bile and thereby acts as an important determinant of bile flow (Gerloff *et al.*, 1998). BSEP is almost exclusively expressed in the hepatocyte canalicular membrane, although low extrahepatic expression has been detected at the mRNA level (Hilgendorf *et al.*, 2007; Langmann *et al.*, 2003).

The essential physiological function of BSEP in hepatobiliary bile salt secretion is apparent from several BSEP

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mutations resulting in absent or defective function of the protein. In progressive familial intrahepatic cholestasis type 2, the most severe form of BSEP deficiency syndrome, most of the afflicted patients have undetectable levels of BSEP protein at the canalicular membrane (Jansen *et al.*, 1999; Strautnieks *et al.*, 2008). This deficiency results in symptoms of cholestasis that develops before 6 months of age and progresses to end-stage liver disease within the first decade of life (Shneider, 2004; Whitington *et al.*, 1994). Other mutations lead to milder forms of BSEP deficiency syndromes, such as benign recurrent intrahepatic cholestasis type 2. The symptoms observed from low function variants further indicate that low BSEP activity within normal interindividual variability can be a risk factor for an increased susceptibility to acquired cholestasis, eg, intrahepatic cholestasis during pregnancy or drug-induced cholestasis (Pauli-Magnus *et al.*, 2010; van Mil *et al.*, 2004). The close link between BSEP dysfunction and different severe pathophysiological conditions, in particular the increased susceptibility to DILI, highlights the importance of early identification of clinically relevant drug interactions with BSEP.

In this study, we performed a comprehensive analysis of the role that BSEP inhibition plays in DILI and the extent to which it can be used to predict such adverse events prior to large-scale clinical trials. Two *in vitro* systems of different complexity (inverted membrane vesicles and sandwich-cultured human hepatocytes [SCHH]) were used to determine the influence of drugs on BSEP-mediated taurocholate (TA) transport. Based on our experimental data, we developed a computational model that correctly classified 84% and 91% of the BSEP inhibitors and noninhibitors, respectively. The clinical impact of BSEP inhibition was evaluated by determining the association between BSEP inhibition and the severity of DILI warnings in drug labels issued by the Food and Drug Administration (FDA). We found BSEP inhibition in membrane vesicles to correlate with DILI severity, and altered disposition of TA in SCHH was shown to distinguish BSEP inhibitors associated with severe DILI from those associated with no or mild DILI.

MATERIALS AND METHODS

Materials. [^{3}H]-TA was obtained from PerkinElmer (Waltham, MA). Ko143 was a kind gift from Dr Gerrit-Jan Koomen (Van't Hoff Institute for Molecular Sciences, University of Amsterdam, the Netherlands). GF120918 was kindly provided by GlaxoSmithKline (Stevenage, UK). MK571 was purchased from A.G. Scientific (San Diego, CA) and astemizole from MP Biomedicals (Eschwege, Germany). Bosentan was purchased from Sequoia Research Products Limited (Pangbourne, UK). All other compounds were purchased from Sigma-Aldrich, St Louis, MO, at the highest purity available (> 95%). Inverted membrane vesicles from Sf9 cells expressing human BSEP (ABCB11) were purchased from SOLVO Biotechnology (Budapest, Hungary). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Invitrogen, Carlsbad, CA. Dexamethasone, L-glutamine, penicillin, streptomycin, insulin, transferring, and selenium were purchased from Sigma-Aldrich. Hepatocyte maintenance medium (HMM) was obtained from Lonza, Basel, Switzerland and BD Matrigel Basement Membrane Matrix, phenol red free, was purchased from BD Biosciences (Bedford, MA). Standard Hanks' balanced salt solution (HBSS) and Ca^{2+}- and Mg^{2+}-free HBSS were purchased from Invitrogen.

Data sets. In the membrane vesicle screen to identify BSEP inhibitors, compounds were selected for inclusion with the intention of optimizing the structural diversity of the data set. The final data set included 250 compounds that were evenly distributed throughout the physicochemical space of registered drugs (Fig. 1).

The DILI classifications were conducted on approved drugs using the U.S. FDA's drug labels; nondrug compounds were therefore not included in our analysis. Of the compounds investigated for BSEP inhibition, 180 were identified as the active component in FDA-approved drugs. Two withdrawn drugs (troglitazone and benzbromarone) for which FDA classification data could be retrieved from the literature were also included in the DILI analysis (Chen *et al.*, 2011).

A subset of 15 model compounds was selected for further investigation in SCHH. These compounds were chosen to cover the possible combinations of different degrees of BSEP inhibition (inhibitors and noninhibitors) and DILI potential (severe and mild/no DILI). BSEP inhibitors that increase the risk of severe DILI were represented by cyclosporine A, ritonavir, rosiglitazone, and troglitazone. BSEP inhibitors with no or mild reported DILI were exemplified by mifepristone, isradipine, budesonide, and glyburide. Representative BSEP noninhibitors for severe DILI were valproic acid, flutamide, and zidovudine, whereas BSEP noninhibitors with no or only mild reported DILI were represented by omeprazole, cimetidine, haloperidol, and chlorpromazine.

BSEP-dependent TA transport assay. Taurocholate transport was determined, in a 96-well plate format, in inverted membrane vesicles from Sf9 cells overexpressing human BSEP. Statistical experimental design, as implemented in Modde version 7.0 (Umetrics, Umeå, Sweden), was used to optimize experimental parameters with regard to (1) amount of membrane vesicles per well (10–50 µg/well), (2) TA concentration (1–10µM), and (3) incubation time (1–10 min), at 5 levels per evaluated parameter. On the basis of the experimental design optimization (data not shown), 10 µg vesicles were used in each well and were incubated with 2µM TA for 5 min.

All experiments were performed using a rapid filtration technique modified from Pedersen *et al.* (2008). Briefly, transport buffer (10mM Tris-HCl [pH 7.4], 250mM sucrose, 10mM $MgCl_2$, and 10mM phosphocreatine) was used

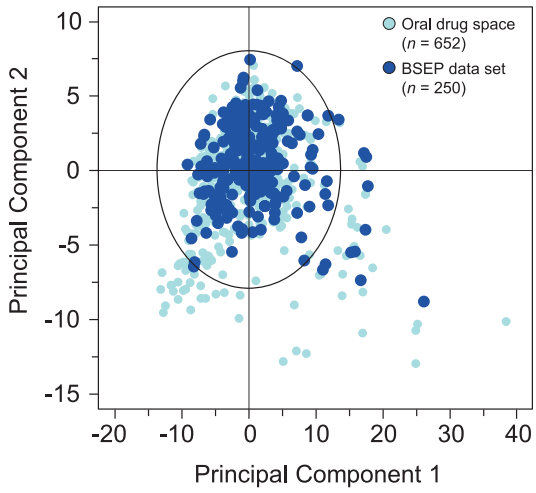

FIG. 1. Chemical diversity of the studied compounds. The chemical space of registered oral drugs (n = 652) was described using a principal component analysis (PCA) of 80 molecular descriptors. When projected onto this PCA, the BSEP data set (n = 250) was shown to evenly distribute through the chemical space of oral drugs.

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to dilute dimethyl sulfoxide (DMSO) stock solutions to final substrate concentrations of 2µM (0.8µM/0.1 µCi ^{3}H-TA). Final DMSO concentrations were consistently <0.3%, which in our laboratory has been shown to have negligible effects on the TA transport. BSEP membrane vesicles were quickly thawed from −85°C to 37°C and diluted in transport buffer to a final concentration of 0.2 µg/µl. Vesicle solution (50 µl) including substrate (2µM TA) was preincubated at 37°C for 10 min, after which transport was initiated by the addition of 4mM adenosine triphosphate (ATP) and 90 U/ml creatine kinase. All plates included control samples in which 4mM ATP was replaced by 4mM Adenosine monophosphate (AMP) to determine passive uptake of TA in the vesicles. After a 5-min incubation at 37°C on an orbital shaker (300 rpm), transport was terminated by adding 200 µl ice-cold stop solution (10mM Tris-HCl [pH 7.4], 250mM sucrose, 0.1M NaCl, and 100µM TA). The incubation solutions were immediately transferred to and filtered through a 96-well glass filter plate with a pore size of 0.65 µm (Millipore, Bedford, MA). Filters were rinsed 5 times with 200 µl ice-cold stop solution and dried before 100 µl Microscint 40 (PerkinElmer) was added. Radioactivity was measured in a TopCount NXT (PerkinElmer) scintillation counter. Measurements in cpm were converted to molar amounts by normalizing to the cpm measured in substrate solutions of known concentration. ATP-dependent transport was calculated by subtraction of passive permeability determined from AMP incubations. All experiments were performed in triplicate and positive inhibition controls with 50µM cyclosporine A or indocyanine green were included on each plate.

To determine transport kinetics, the BSEP-mediated TA transport was measured in 5-min incubations at concentrations between 0.8 and 100µM. The resulting transport rates were used to determine Michaelis-Menten kinetic parameters (K_m and V_{max}) by nonlinear regression in Prism version 5 (GraphPad, San Diego, CA).

BSEP inhibition screen. The optimized membrane vesicle assay was used to screen the selected 250 compounds for BSEP inhibition at a concentration of 50µM. The assay was performed as a normal transport experiment (described above) with the only difference being that TA in the inhibition studies was coincubated (during preincubation and incubation) with 50µM test compound.

The ATP-dependent TA transport rates were measured in the presence and absence of each test compound, and relative transport rates were expressed as the percentage of the uninhibited control. All compounds that significantly ($p < .05$) decreased the TA transport were classified as inhibitors, which resulted in an inhibition cutoff at 27.5%. Also, in order to rank the inhibition as either weak or strong, a second cutoff at 50% transport inhibition was included. Compounds with inhibitory effects between the 2 cutoffs (ie, resulting in TA transport between 72.5% and 50% that of the controls) were denoted weak inhibitors. Compounds inhibiting the TA transport by more than 50% were denoted strong inhibitors. Compounds with nonsignificant BSEP inhibition were denoted noninhibitors.

The inhibitory effects of the 2 positive controls, cyclosporine A and indocyanine green, were determined in concentration intervals of 0.1–100µM. IC_{50} values were calculated from the nonlinear regression of dose-response curves using Prism version 5 (GraphPad) according to equation 1:

$$\text{percentage of control} = \frac{100}{1+10^{\log IC_{50}-[I]}} \quad (1)$$

Computational structure-inhibition modeling. Computational models were developed to identify molecular properties associated with BSEP inhibition. A total of 249 compounds (53 strong inhibitors, 33 weak inhibitors, and 163 noninhibitors) were included in the computational analyses. Oxaliplatin was excluded from the analysis because its molecular surface areas were not possible to compute due to the presence of an unparameterized platinum atom. The data set was randomly split with a ratio of 2:1 into a training set of 163 compounds (34 strong inhibitors, 21 weak inhibitors, and 108 noninhibitors) for model development and a test set of 86 compounds (19 strong inhibitors, 12 weak inhibitors, and 55 noninhibitors) for validation of the final model. Chemical structures were obtained as 2D SD files from PubChem (Bolton *et al.,* 2008). These structures were further processed to calculate molecular descriptors using the Dragon version 6 (Talete, Italy), ADMETPredictor version 6.0 (SimulationsPlus, Lancaster, CA), and MAREA version 3.02 (in-house) software. The descriptors were related to the inhibition class using orthogonal partial least-squares projection to latent structures discriminant analysis (OPLS-DA), according to a previous protocol (Pedersen *et al.*, 2008).

Classifying DILI severity of drugs in the data set. The main goal of this investigation was to explore to what extent BSEP inhibition predicts clinically observed DILI. We identified the classification system of adverse drug reactions (ADR) implemented in the FDA drug labels as a suitable data source. The FDA drug labels represent a consensus of regulatory and industry experts who evaluate and balance data combined from controlled clinical trials, published literature case studies, spontaneous ADR reports, and postmarket monitoring. The 3 ADR sections within the drug labels categorize ADRs by increasing severity, ranging from the least severe "adverse reactions" (AR), through the intermediate "warnings and precautions" (WP), to the most severe "boxed warnings" (BW). According to the definitions in U.S. federal regulations 21 CFR 201.57, the AR section describes the overall adverse reaction profile of a drug and includes those adverse events believed to have a causal relationship with the drug. The WP section is required to describe clinically significant adverse reactions as soon as reasonable evidence of a causal association with the drug is established. The BW section contains certain contraindications or serious warnings, particularly those that may lead to death or serious injury, and is generally based on clinical data (U.S. Federal Regulations, 2012).

To assess the DILI potential of registered drugs within the data set, information on hepatic ADRs in FDA-approved drug labels was obtained from DailyMed (http://dailymed.nlm.nih.gov/), and the DILI potential was classified using the method of Chen *et al.* (2011). Briefly, FDA drug labels were reviewed for hepatic adverse reactions by searching for keywords related to liver injury (Supplementary Table S1). A compound was regarded as a DILI mediator if the keywords were identified in the BW, WP, or AR sections within a drug label. If a keyword was mentioned in several sections, the compound was classified in the most severe category (BW > WP > AR). In addition, we categorized drugs where no hepatic ADRs appeared in any of the ADR sections as "not mentioned" (NM). To verify that the classifications from the automated search were correct, a low threshold search using "liver" and "hepatic" as search keywords followed by manual inspection of matching drug labels was conducted. The ADR sections in FDA drug labels describe ADRs in a hierarchical way on the basis of their severity. To assure that the ADR sections described DILI severity in the same hierarchical way, without influence from other ADRs reported for the same drug, the severity of DILI reported in the different sections was evaluated using the approach described in Chen *et al.* (2011) and Supplementary Table S2.

Because FDA drug labels of registered drugs were used to investigate the correlation between BSEP inhibition and DILI, the impact on the data set composition from the exclusion of nondrug compounds ($n = 68$) was assessed. The frequency of BSEP inhibitors was comparable in the full data set ($n = 250$) investigated for BSEP inhibition in membrane vesicles (34% BSEP inhibitors) and in the subset of drugs ($n = 182$) in the DILI analysis (33% BSEP inhibitors). Furthermore, the exclusion of nondrugs did not skew the data set with regard to chemical diversity, as confirmed by a principal component analysis of molecular descriptors (data not shown).

Isolation and culture of human hepatocytes. Liver tissues free from metastases were obtained from human donors undergoing partial liver resections at the Department of Surgery, Uppsala University Hospital, Sweden (see Table 1 for patient demographics). All donors gave informed consent, in accordance with the approval from the Uppsala Regional Ethical Review Board (Ethical Approval no. 2009/028). Hepatocytes were isolated using the 2-step liver digestion technique of Lecluyse and Alexandre (2010). Primary hepatocytes, with a viability >85%, were seeded in collagen-1-coated 24-well plates (BD Biosciences) at a density of 3.75×10^5 cells per well and initially maintained at 37°C and 5% CO_2 in 500 µl DMEM supplemented with 5% (vol/vol) fetal bovine serum, 4 µg/ml insulin, 1µM dexamethasone, 4mM L-glutamine,

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TABLE 1
Demographic Data for Human Liver Donors

Sex	Age	Diagnosis	Medication	Cytostatic Treatment
F	59	GIST	Imatinib treatment until 7 days before resection	—
M	73	MM	Low dose acetylsalicylic acid	—
M	61	MM	Prednisolone, simvastatin, azathioprine, cholecalciferol	—
M	55	CRC	Bisoprolol, loratadine, zopiclone	Folfiri
M	67	CRC	Allopurinol, tenormin, amlidopine, enalapril, alfuzisin	Folfiri, cetuximab
F	45	CRC	—	—

Abbreviations: CRC, colorectal cancer; F, female; Folfiri, fluorouracil, leucovorin, and irinotecan; GIST, gastrointestinal stromal tumor; M, male; MM, malignant melanoma.

100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were allowed to attach to the plate for 2–3 h in a humidified culture chamber at 37°C and 5% CO_2, after which the medium was carefully aspirated and replaced with 500 µl culture medium (HMM supplemented with insulin 10 µg/ml, transferrin 5.5 µg/ml, selenium 5 ng/ml, 0.1µM dexamethasone, 100 U/ml penicillin, and 100 µg/ml streptomycin). After overnight incubation, the cells were overlaid with 500 µl ice-cold 0.25 mg/ml Matrigel in culture medium. To allow the formation of bile canaliculi, the cells were cultured for an additional 4–6 days during which the culture medium was refreshed every 24 h.

Determination of TA accumulation in sandwich-cultured hepatocytes. Taurocholate accumulation in SCHH was determined using a method modified from that described by Liu *et al.* (1999). In short, the SCHH were rinsed twice with either 400 µl of standard HBSS, to maintain the integrity of tight junctions and bile canaliculi, or with Ca^{2+}- and Mg^{2+}-free HBSS, to disrupt the tight junctions. Cultures were then preincubated for 15 min, with or without 10µM test compound, dissolved in either 200 µl standard or Ca^{2+}- and Mg^{2+}-free HBSS. DMSO was included at the same final concentration (0.1%) in control (compound-untreated) wells as that in the compound-treated wells. Preincubation medium was removed and TA uptake was initiated by the addition of 1µM substrate solution (0.75µM [^{3}H]-TA and 0.25µM TA) together with 10µM test compound in standard or Ca^{2+}- and Mg^{2+}-free HBSS. Transport was stopped after 10 min by removing the incubation medium and rinsing the cells twice with 400 µl ice-cold standard or Ca^{2+}- and Mg^{2+}-free HBSS. Cells were lysed with 100 µl 1M NaOH and kept at 4°C overnight before being analyzed for radioactivity in a TopCount NXT (PerkinElmer) scintillation counter. Total protein content was determined using the BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. All experiments were performed in triplicates on 3 separate occasions, using cells isolated from 6 different donors.

The measurements were used to calculate the amount of TA in the respective compartments, and the numbers were normalized to total protein content. Total accumulation ($TOTAL_{Acc}$; intracellular plus bile accumulation after incubation in HBSS) and intracellular accumulation (IC_{Acc}; after incubation in Ca^{2+}- and Mg^{2+}-free HBSS) of TA were used to calculate bile accumulation ($BILE_{Acc}$) according to equation 2:

$$BILE_{Acc} = TOTAL_{Acc} - IC_{Acc} \quad (2)$$

The biliary excretion index (BEI; equation 3), ie, the ratio describing the biliary accumulation of a compound in relation to its total accumulation (intracellular + bile) (Liu *et al.*, 1999), was calculated according to equation 3:

$$BEI = \frac{BILE_{Acc}}{TOTAL_{Acc}} \times 100 \quad (3)$$

In addition to BEI, we introduced the bile intracellular correlation (BIC; equation 4), which describes biliary accumulation in relation to the intracellular accumulation according to equation 4. Relating bile secretion to the intracellular accumulation is of interest because it is the intracellular substrate concentration that governs the efflux kinetics across the canalicular membrane:

$$BIC = \frac{BILE_{Acc}}{IC_{Acc}} \quad (4)$$

Finally, the *in vitro* biliary clearance (CL_{Bile}; equation 5) was calculated based on TA media concentrations. CL_{Bile} is dependent on the net substrate flux across the cell, ie, on the net basolateral uptake (including passive- and transporter-mediated basolateral uptake and efflux) and the net canalicular efflux:

$$CL_{Bile} = \frac{BILE_{Acc}}{AUC} \quad (5)$$

where AUC is the area under the concentration-time curve based on TA concentrations in the incubation medium.

The calculated parameters were presented as percentages of the corresponding value in control incubations (ie, TA transport measured in the absence of inhibitors), to allow comparison between different batches of SCHH.

Statistical analysis. Data are expressed as means with SDs or SEMs, as indicated in the text. In the BSEP inhibition screen, compounds that significantly decreased BSEP transport were identified by 1-way ANOVA with Bonferroni's *post hoc* test, as implemented in Prism 5 (GraphPad Software, Inc, La Jolla, CA). Differences in the proportions of DILI-inducing agents in each ADR class were determined using normality tests. The Marascuillo procedure was used to determine statistical differences of mild and severe DILI distribution in the different ADR sections. Analysis of SCHH accumulation data were conducted using 2-way ANOVA with Bonferroni's *post hoc* test.

RESULTS

TA Transport in Inverted Membrane Vesicles

To investigate the impact of drugs on BSEP-mediated TA transport, we used inverted membrane vesicles from cells overexpressing human BSEP. This system allows direct measurement of drug interactions with BSEP, thereby eliminating certain confounding factors that affect whole-cell systems (such as drug metabolism and the need for sufficient membrane

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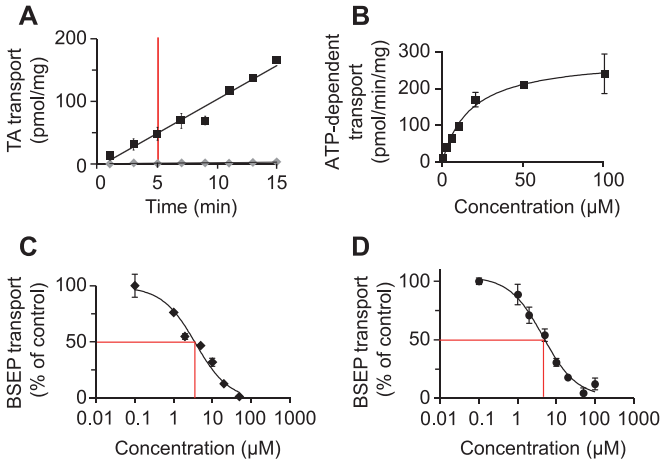

FIG. 2. Kinetics of taurocholate (TA) transport in bile salt export pump (BSEP)–overexpressing inverted Sf9 membrane vesicles. A, Linearity of TA transport. The TA transport kinetics was linear up to 15 min (squares). At 5-min incubation (used in the BSEP inhibition screen and marked with a red dashed line), the passive uptake determined in AMP incubations (diamonds) was approximately 5% of the active uptake. B, Concentration dependence of TA transport. Michaelis-Menten kinetic parameters for the TA transport were determined by nonlinear regression, resulting in a K_m of 17.8 ± 5.0μM and a V_{max} of 286.2 ± 28.2 pmol/mg protein/min. C and D, Inhibition of the ATP-dependent TA transport by the model inhibitors indocyanine green (ICG) and cyclosporine A, respectively. The IC_{50} (marked with red lines) was determined to be 3.7 ± 1.3 and 4.6 ± 1.2μM, respectively, using nonlinear regression to a sigmoidal dose-response relationship. The interday variability ($n = 45$) of ICG- and cyclosporine A–mediated inhibition was 6% and 7%. Data are presented as the mean ± SD. Experiments were run in triplicates on at least 2 separate occasions.

permeability to reach transporter binding sites). The ATP-dependent transport of TA into inverted membrane vesicles was linear for up to 15 min (Fig. 2A). The passive TA transport was approximately 5% of the active uptake under the conditions applied. The kinetics of the TA transport was determined from the data in Figure 2B using nonlinear regression. This resulted in a K_m of 17.8 ± 5.0μM and a V_{max} of 286.2 ± 28.2 pmol/mg protein/min, in good agreement with previously published data (Kis *et al.*, 2009; Yabuuchi *et al.*, 2008). Two inhibitors (cyclosporine A and indocyanine green) were used as controls throughout the experiments and gave low interday ($n = 45$) variability of 6% and 7%, respectively. Indocyanine green and cyclosporine A inhibited TA uptake with IC_{50} values of 3.7 ± 1.3 and 4.6 ± 1.2μM, respectively, as determined by fitting a sigmoidal dose-response relationship to the inhibition data (Figs. 2C and 2D).

Inhibition of BSEP Transport in Inverted Membrane Vesicles

Of the 250 compounds screened for inhibition of TA transport, 86 were identified to significantly ($p < .05$) inhibit BSEP at 50μM (Fig. 3). Thirty-seven of these have, to our knowledge, not been previously reported (Table 2). No transport stimulation was detectable at the statistical significance level used ($p < .05$). Among the 86 inhibitors, 53 reduced BSEP-mediated TA transport by more than 50% compared with the controls (without inhibitor) and were therefore defined as strong inhibitors (as described in the Materials and Methods section).

The number of BSEP inhibitors varied in the different therapeutic classes. For example, strong BSEP inhibitors were frequently found among antiviral drugs (7 out of 14 investigated), statins (5 out of 8), and antidiabetic drugs (5 out of 11). In contrast, only fusidic acid, out of the 20 antibacterial compounds studied, was identified to inhibit TA transport by more than 50%. The lower incidence of BSEP inhibitors among antibacterial drugs may, in part, result from the fact that antibacterials tend to have different physicochemical properties than most other drugs (eg, they are larger and more hydrophilic). It may also reflect the considerable structural diversity in this therapeutic class, where many compounds derive from natural products.

Molecular Properties Important for BSEP Inhibition

Common molecular descriptors previously identified to be important for the inhibition of BSEP (Warner *et al.*, 2012) and other ATP-binding cassette (ABC) transporters (Matsson *et al.*, 2007; Pedersen *et al.*, 2008) were different for the inhibitors and noninhibitors. Eight of 10 evaluated molecular descriptors, describing lipophilicity/hydrophobicity, size, ionization state, and charge, showed significant differences between the 53 strong inhibitors and the noninhibitors ($p < .01$) (Figs. 4A and 4B–K). The weak inhibitors generally had intermediate values in the examined molecular properties, with statistically significant differences to noninhibitors ($p < .01$) only observed for molecular weight and the surface area of saturated nonpolar atoms (Figs. 4A and 4B–K). This observation suggests that weak inhibitors may not be easily distinguished from noninhibitors in the vesicle assay. To investigate this issue further, we developed 2 OPLS-DA models to describe differences between strong inhibitors and noninhibitors and between all inhibitors (strong and weak) and noninhibitors, respectively (see Supplementary Figures S1 and S2). The best model had a total prediction accuracy of 89% in the test set and correctly classified 84% and 91% of the strong inhibitors and noninhibitors, respectively (Supplementary Figure S1).

The final models both indicated BSEP inhibition to be positively correlated with lipophilicity, hydrophobicity, and the number of halogen atoms in the molecule, whereas a negative correlation was seen for descriptors of positive charge, hydrophilicity, and hydrogen bond acceptors. Differences between strong and weak inhibitors were also indicated by the models. For instance, strong BSEP inhibitors were suggested to be larger and more flexible than the weak inhibitors; the latter were more similar to the noninhibitors. This is in agreement with the analysis of the differences in individual molecular descriptors (Fig. 4).

DILI Classification of Drugs Included in the Data Set

As described in detail in the Materials and Methods section, the DILI potential of the drugs in the data set was classified on

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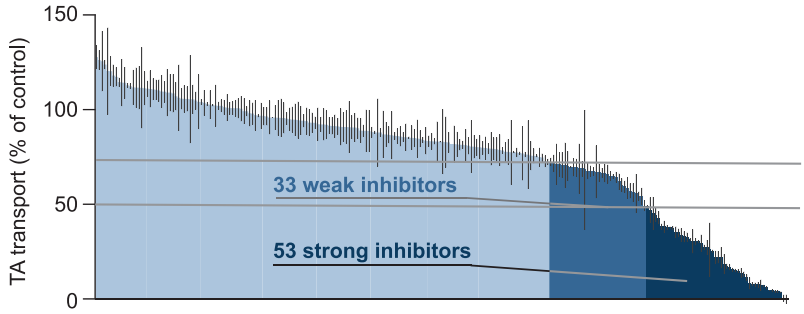

FIG. 3. Inhibition of bile salt export pump (BSEP)-mediated taurocholate (TA) transport in inverted membrane vesicles. The 250 drugs and drug-like compounds were screened at 50μM concentration, identifying 86 compounds that significantly ($p < .05$) inhibited BSEP transport. Compounds inhibiting the TA transport by more than 50% were denoted as strong inhibitors ($n = 53$) and those that inhibited less than 50% of the TA transport, but still maintained significant inhibition, were denoted as weak inhibitors ($n = 33$). The data are presented as the mean ± SE. Experiments were run in triplicates, repeated 1–3 times.

the basis of the information on hepatic ADRs extracted from the FDA drug labels. Briefly, depending on the ADR severity, the FDA drug labels report the ADRs in different sections (BW, WP, or AR, ordered by decreasing severity). Drugs were assigned to classes according to the drug label section reporting a hepatic ADR. If hepatic ADRs were reported in several sections, the drug was assigned to the class representing the most severe drug label section. Drugs were assigned to the NM class if no hepatic ADRs were reported.

FDA drug labels were available for 182 of the 250 investigated compounds, enabling their classification according to DILI severity. For 73 of these 182 drugs, hepatic ADRs were only reported in the adverse reaction section of the FDA drug label, and the compound was assigned to the AR class. In the same manner, 61 drugs were classified as WP and 14 as BW. The remaining 34 drugs were classified to have no hepatic adverse reactions (NM) (Table 2).

Confirming this classification, keywords that define severe DILI (eg, acute liver failure and liver necrosis) were more often reported in the BW or WP sections than in the AR section (Supplementary Figure S3). By contrast, milder DILI (eg, increased liver aminotransferases and liver steatosis) were more frequently reported in the AR section than in the WP and BW sections (Supplementary Figure S3). This indicates that classifying DILI severity according to the FDA drug label sections was applicable for the purpose of our study.

BSEP Inhibition as a Predictor of DILI

To explore to what extent BSEP inhibition in membrane vesicles predicts DILI, the experimental results were compared with the DILI severity classification. The frequency of drugs with BW-class DILI was significantly higher for strong BSEP inhibitors than for both weak inhibitors and noninhibitors ($p < .05$, Fig. 5). WP-classified DILI was significantly more frequent among both strong and weak inhibitors compared with the noninhibitors ($p < .05$ and .01, respectively, Fig. 5). In contrast, 60 of the 121 noninhibitors were included in the AR class, resulting in a significantly higher frequency of AR-classified drugs among noninhibitors compared with inhibitors ($p < .01$, Fig. 5). The frequency of drugs with no reported hepatic adverse events (NM) was similar for inhibitors and noninhibitors (Fig. 5). In total, 38 out of the 61 drugs (62%) identified as inhibitors of BSEP-mediated transport were reported to increase the risk of the more severe BW- or WP-class DILI, in comparison to 37 out of the 121 (31%) BSEP noninhibitors (Figs. 6A and 6B). The orthogonal analysis showed that 51% of the compounds associated with severe DILI were BSEP inhibitors, compared with 21% BSEP inhibitors among the compounds that result in mild or no DILI (Fig. 6C). Notably, 9 compounds that are associated with severe DILI have not been identified as BSEP inhibitors before (amiodarone, atazanavir, celecoxib, clarithromycin, dipyridamole, erythromycin, ezetimibe, lovastatin, and tipranavir), suggesting that this mechanism can contribute to their observed clinical toxicity.

DILI Prediction in SCHH Compared With BSEP Vesicles

Many cellular factors that influence the ability of a compound to inflict DILI cannot be properly assessed in the relatively simple, BSEP-expressing membrane vesicles. We therefore used SCHH to assess the relationship between DILI and TA transport inhibition in more detail (Fig. 7). A subset of 15 model compounds with different effects in the vesicle assay (inhibitors or noninhibitors) and DILI potential (BW/WP or AR/NM) was selected for the SCHH studies, as illustrated in Figure 6D (see "Data sets" in the Materials and Methods section). Untreated SCHH showed stable TA disposition in all 6 batches used with BEI of 72 ± 3%, BIC of 2.9 ± 1.2, and CL_{Bile} of 17 ± 3 ml/mg/min.

In good agreement with the vesicle data, the 4 BSEP inhibitors reported to give BW- or WP-classed DILI (cyclosporine A, ritonavir, rosiglitazone, and troglitazone), significantly decreased the bile accumulation of TA compared with the untreated controls ($p < 2 \times 10^{-5}$; Table 3 and Fig. 7A). These compounds all decreased TA accumulation in bile to a much greater extent than the decrease in the intracellular compartment, which indicates that the main effect of the compounds was their inhibition of the canalicular efflux. The observation that their intracellular accumulation also decreased, although

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TABLE 2
BSEP-Mediated TA Transport of 250 Test Compounds (50μM) and DILI Classifications

BSEP Inhibition Class[a]	Compound (n = 250)	TA Transport (% of control)			DILI (n = 182) Group[c]
		Average	SEM	Significance[b]	
●	**Chelerythrine**	–0.1	2.3	**	—
●	**Tipranavir**	0.5	1.7	**	BW
●	Taurolithocholate	3.6	0.2	**	—
●	MK571	3.8	0.5	**	—
●	Telmisartan	4.0	0.7	**	WP
●	Clotrimazole	4.4	0.5	**	WP
●	**Indocyanine green**	4.7	0.0	**	NM
●	Nefazodone	5.9	0.7	**	BW
●	Saquinavir	7.0	0.4	**	WP
●	Lopinavir	7.2	2.9	**	WP
●	Glyburide	7.4	1.3	**	AR
●	Ketoconazole	7.6	1.0	**	BW
●	Ritonavir	7.9	1.0	**	BW
●	Cyclosporine A	8.2	3.1	**	WP
●	**Mifepristone**	11.3	1.6	**	NM
●	**Loratadine**	13.2	1.3	**	AR
●	**Bromosulfophthalein**	13.7	2.4	**	—
●	**Ergocristine**	14.5	0.8	**	—
●	Benzbromarone	15.0	0.8	**	WD
●	Fusidic acid	15.4	0.9	**	—
●	Troglitazone	15.7	1.3	**	WD
●	Nicardipine	16.7	1.5	**	AR
●	Bosentan	17.1	2.9	**	BW
●	Reserpine	17.4	1.1	**	WP
●	**Lovastatin**	18.4	5.1	**	WP
●	**Felodipine**	21.7	5.4	**	AR
●	**Bromosulfalein**	22.0	3.1	**	—
●	**Diethylstilbestrol**	22.1	3.3	**	—
●	**GF120918**	25.3	0.6	**	—
●	**Ko143**	26.0	14.2	**	—
●	Losartan	27.2	3.8	**	AR
●	Glimepiride	27.4	1.3	**	AR
●	Disulfiram	29.5	4.3	**	WP
●	Simvastatin	30.2	0.7	**	WP
●	**Spironolactone**	30.3	1.8	**	AR
●	Atorvastatin	31.3	0.7	**	WP
●	Cerivastatin	32.7	2.4	**	—
●	**Isradipine**	32.8	2.8	**	NM
●	**Hoechst 33342**	33.7	3.5	**	—
●	**Ezetimibe**	34.4	3.8	**	WP
●	**Dipyridamole**	35.0	2.0	**	WP
●	Pitavastatin	35.4	2.9	**	WP
●	Fenofibrate	37.3	1.0	**	WP
●	**Silymarin**	37.7	0.5	**	—
●	**Atazanavir**	37.8	1.2	**	WP
●	**Repaglinide**	38.2	3.0	**	AR
●	**Sulfinpyrazone**	38.2	1.4	**	—
●	**Ranolazine**	40.7	3.2	**	NM
●	Midazolam	42.7	3.4	**	NM
●	Rosiglitazone	44.3	3.7	**	WP
●	Nelfinavir	45.1	8.5	**	AR
●	**Budesonide**	48.6	0.6	**	NM
●	Indinavir	49.1	2.6	**	WP
○	**Celecoxib**	54.2	4.4	**	WP
○	**Cefaclor**	54.3	2.3	**	AR
○	**Dehydroisoandrosterone 3-sulfate**	55.0	2.4	**	—
○	Rifampicin	55.7	10.1	**	WP

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TABLE 2—*Continued*

BSEP Inhibition Class[a]	Compound (n = 250)	TA Transport (% of control) Average	SEM	Significance[b]	DILI (n = 182) Group[c]
○	Vinblastine	56.2	4.8	**	NM
○	Astemizole	57.1	3.9	**	—
○	**Amiodarone**	58.6	5.6	**	BW
○	**Tinidazole**	59.2	3.2	**	AR
○	Progesterone	60.1	3.5	**	WP
○	**Pilsicainide**	61.9	4.2	**	—
○	Nitrofurantoin	64.4	2.6	**	WP
○	Imatinib	64.6	1.2	**	WP
○	Indomethacin	64.9	2.4	**	WP
○	**Fluvoxamine**	65.9	4.9	**	AR
○	Ofloxacin	66.4	4.7	**	—
○	Cholate	66.5	2.5	**	—
○	Glipizide	66.7	4.5	**	AR
○	**Olmesartan**	67.1	2.5	**	NM
○	**Atenolol**	67.2	2.1	**	NM
○	Efavirenz	67.6	2.8	**	WP
○	Terfenadine	67.6	4.2	**	—
○	**Clarithromycin**	68.1	4.4	**	WP
○	**Glycyrrhizic acid**	68.8	4.0	*	—
○	Atropine	68.8	13.1	*	NM
○	Cholic acid	69.4	4.6	*	—
○	Nitrendipine	69.5	4.7	*	—
○	Diclofenac	69.7	0.5	*	WP
○	Sulfamethoxazole	70.2	7.7	*	WP
○	Naproxen	70.7	6.7	*	WP
○	Nifedipine	71.1	6.4	*	WP
○	**Erythromycin**	71.6	4.5	*	WP
○	**Morin**	72.4	2.1	*	—
○	Flucloxacillin	72.4	1.5	*	—
-	Thioridazine	72.6	2.9	—	AR
-	Vincristine	74.7	2.0	—	NM
-	Amlodipine	74.9	4.9	—	WP
-	Sulindac	75.5	0.7	—	WP
-	Estradiol	75.5	3.8	—	AR
-	Doxorubicin	75.6	7.5	—	NM
-	Valsartan	76.2	4.9	—	AR
-	Thiotepa	76.3	1.3	—	NM
-	Dehydroisoandrosterone	76.9	14.2	—	—
-	Glycylproline	77.0	0.7	—	—
-	Acetylsalicylic acid	77.3	2.4	—	NM
-	Chrysin	77.4	0.3	—	—
-	Topotecan	77.4	17.0	—	NM
-	Flupentixol	77.6	6.7	—	—
-	N-methylnicotinamide	77.7	5.0	—	—
-	Buspirone	78.4	1.6	—	AR
-	Gemfibrozil	78.6	8.2	—	WP
-	Naringin	79.0	6.2	—	—
-	Berberine	79.2	5.8	—	—
-	Bumetanide	79.3	8.7	—	AR
-	Theophylline	79.4	2.8	—	NM
-	Hydrocortisone	79.7	6.0	—	NM
-	Testosterone	79.9	3.5	—	WP
-	1-Methyl-4-phenylpyridinium	80.1	6.2	—	—
-	Eletriptan	80.2	4.3	—	AR
-	Irinotecan	80.4	6.0	—	AR
-	Fexofenadine	80.5	13.8	—	NM
-	Coumestrol	80.9	4.5	—	—

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TABLE 2—*Continued*

BSEP Inhibition Class[a]	Compound (n = 250)	TA Transport (% of control)			DILI (n = 182) Group[c]
		Average	SEM	Significance[b]	
-	Tetraethylammonium	81.0	7.7	—	—
-	Moclobemide	81.2	5.3	—	—
-	Azathioprine	81.2	8.4	—	WP
-	Mesalazine	81.4	2.0	—	AR
-	Ticlopidine	81.4	2.8	—	WP
-	4-Methylumbelliferone glucuronide	81.7	6.5	—	—
-	Nicotine	81.8	9.6	—	NM
-	Fluoxetine	81.9	0.0	—	AR
-	Cefamandole	82.7	5.3	—	—
-	Phenacetin	82.8	13.4	—	—
-	Itraconazole	83.0	17.0	—	WP
-	Nevirapine	83.0	0.2	—	BW
-	Cholchicine	83.1	10.6	—	AR
-	Metformin	83.2	1.5	—	NM
-	Sildenafil	83.3	4.3	—	NM
-	Dexamethasone	83.4	2.9	—	AR
-	Fluconazole	83.5	7.1	—	WP
-	Methoxsalen	83.9	1.4	—	NM
-	Tamoxifen	84.6	4.3	—	WP
-	Verapamil	84.8	9.9	—	WP
-	Cephalexin	85.0	4.7	—	AR
-	Procyclidine	85.1	6.4	—	NM
-	Cimetidine	85.3	0.5	—	AR
-	Pravastatin	85.4	7.1	—	WP
-	Sparfloxacin	85.5	2.4	—	—
-	Carbamazepine	85.8	5.2	—	WP
-	Fumitremorgin C	86.0	0.6	—	—
-	Fendiline	86.0	4.0	—	—
-	Lisinopril	86.2	2.0	—	WP
-	Amantadine	86.5	12.6	—	WP
-	Dextromethorphan	86.6	4.3	—	NM
-	Diazepam	86.9	6.5	—	AR
-	Biochanin A	87.9	2.1	—	—
-	Rosuvastatin	87.9	4.5	—	WP
-	Prazosin	88.0	0.8	—	AR
-	Loperamide	88.1	2.7	—	NM
-	*p*-Acetamidophenyl glucuronide	88.8	11.0	—	—
-	Famotidine	88.8	11.1	—	AR
-	Genistein	88.8	3.1	—	NM
-	Tetracycline	88.9	6.6	—	WP
-	Adefovir	90.2	5.5	—	WP
-	Propafenone	90.6	7.6	—	AR
-	Chloroquine	90.6	6.1	—	AR
-	Varenicline	90.7	13.5	—	AR
-	Quercetin	90.8	8.7	—	—
-	Caffeine	90.8	7.8	—	NM
-	Naringenin	90.9	9.6	—	—
-	*p*-Aminohippuric acid	91.2	4.3	—	—
-	5-Carboxyfluorescein	91.3	6.0	—	—
-	Pioglitazone	91.4	4.5	—	WP
-	Baicalin	91.5	4.4	—	—
-	Estrone	92.0	7.5	—	WP
-	Chlorzoxazone	92.2	8.4	—	WP
-	Phenylethyl isothiocyanate	92.2	3.9	—	—
-	Gliclazide	92.3	8.6	—	—
-	Atomoxetine	93.0	2.6	—	WP
-	Disopyramide	93.1	3.1	—	AR
-	Oxaliplatin	93.1	5.2	—	WP
-	Tolbutamide	93.2	7.1	—	AR
-	Noscapine	93.3	1.6	—	—

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TABLE 2—*Continued*

BSEP Inhibition Class[a]	Compound (n = 250)	TA Transport (% of control)			DILI (n = 182) Group[c]
		Average	SEM	Significance[b]	
-	Cefadroxil	93.9	7.0	—	AR
-	Isoniazid	94.1	3.4	—	BW
-	Dofetilide	94.2	7.5	—	AR
-	Digoxin	94.5	9.6	—	NM
-	Daidzein	94.7	8.4	—	—
-	Ibuprofen	94.7	6.7	—	WP
-	Antipyrine	94.9	6.0	—	NM
-	Enalapril	95.3	3.2	—	AR
-	Mephenytoin	95.6	8.0	—	AR
-	Ivermectin	95.6	10.0	—	AR
-	Ranitidine	95.7	9.3	—	AR
-	Hydrochlorothiazide	95.8	6.5	—	AR
-	Metoprolol	95.9	0.1	—	AR
-	Amoxicillin	95.9	8.8	—	WP
-	Haloperidol	96.4	4.7	—	AR
-	Pantoprazole	96.5	4.8	—	AR
-	Emtricitabine	96.7	6.8	—	AR
-	Medroxyprogesterone	96.7	0.7	—	AR
-	Amiloride	97.0	8.0	—	AR
-	Phenytoin	97.9	5.8	—	WP
-	Sulfasalazine	98.0	6.8	—	WP
-	Valproic acid	98.9	16.1	—	BW
-	Clomipramine	99.1	2.5	—	WP
-	Sotalol	99.1	6.7	—	AR
-	Coumarin	99.2	2.0	—	—
-	Metronidazole	100.4	6.6	—	NM
-	Warfarin	100.7	5.1	—	AR
-	Estradiol-17-β-glucuronide	100.7	4.3	—	—
-	Prednisolone	100.8	3.6	—	AR
-	Etoposide	100.8	3.2	—	AR
-	Probenecid	101.0	6.9	—	AR
-	Phenobarbital	101.2	3.8	—	WP
-	Cetirizine	101.9	3.2	—	NM
-	Flutamide	102.0	2.1	—	BW
-	Zidovudine	102.1	10.9	—	BW
-	Acyclovir	102.3	0.5	—	AR
-	Amodiaquine	102.6	7.4	—	—
-	Quinidine	102.8	0.9	—	AR
-	Maprotiline	103.6	6.7	—	AR
-	Bupropion	103.9	1.5	—	AR
-	Paroxetine	104.1	14.3	—	AR
-	Ouabain	104.8	4.6	—	—
-	Pindolol	105.4	7.6	—	NM
-	Bestatin	105.6	6.5	—	—
-	Trimethoprim	105.6	7.2	—	NM
-	Tranylcypromine	105.7	5.5	—	AR
-	N-methylpyridinium	106.3	17.4	—	—
-	Prednisone	107.0	7.3	—	AR
-	Mitoxantrone	108.5	10.0	—	AR
-	Novobiocine	109.0	12.4	—	—
-	Valacyclovir	109.0	9.7	—	AR
-	5-Carboxyfluoresceindiacetate	109.4	5.6	—	—
-	Propranolol	109.5	8.7	—	WP
-	Nootkatone	110.1	5.8	—	—
-	Omeprazole	110.4	5.3	—	AR
-	Procainamide	110.7	5.6	—	AR
-	Diltiazem	110.9	9.6	—	WP
-	Amitriptyline	111.1	4.1	—	AR
-	Estrone-3-sulfate	111.2	8.8	—	—
-	Triazolam	111.5	3.2	—	AR
-	Furosemide	111.6	11.2	—	AR

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TABLE 2—*Continued*

BSEP Inhibition Class[a]	Compound (n = 250)	TA Transport (% of control) Average	SEM	Significance[b]	DILI (n = 182) Group[c]
-	Methotrexate	111.6	10.5	—	BW
-	Phenformin	111.7	9.2	—	—
-	Captopril	112.3	1.4	—	AR
-	Lansoprazole	112.8	1.1	—	AR
-	Carnitine	114.1	8.3	—	NM
-	Sulfaphenazole	114.3	12.4	—	—
-	Desipramine	114.5	2.4	—	AR
-	Paclitaxel	117.3	5.0	—	AR
-	Ciprofloxacin	117.9	5.8	—	WP
-	Clindamycin	120.3	7.8	—	AR
-	Apigenin	121.2	11.8	—	—
-	Chlorpromazine	123.8	2.4	—	AR
-	Chlorprothixene	125.5	15.7	—	—
-	Quinine	126.2	5.0	—	AR
-	Furafylline	128.2	17.2	—	—

Inhibitors (n = 37), that to our knowledge, not have been previously published are marked with bold text.
[a]-/○/●classified as BSEP noninhibitor, weak inhibitor, and inhibitor, respectively.
[b]—/*/** describe significance with $p > .05$, .05–.01, and < .01, respectively.
[c]DILI groups for the 182 drugs according to FDA labeling sections BW, WP, and AR as described and published by DailyMed (http://dailymed.nlm.nih.gov/).

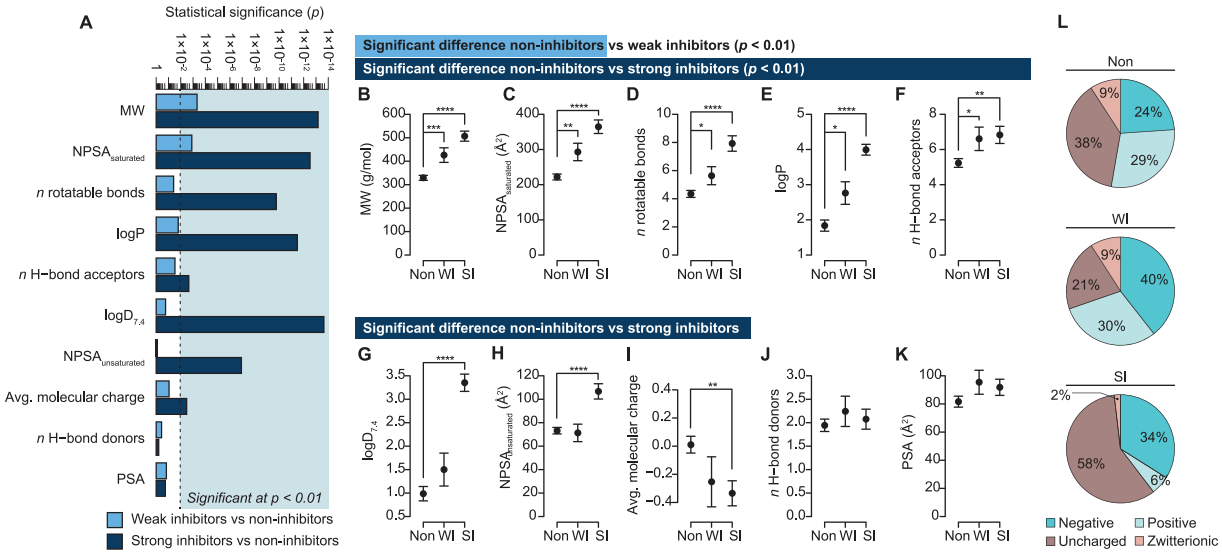

FIG. 4. Molecular properties of the studied compounds. A, Statistical significance of differences between noninhibitors and weak and strong bile salt export pump (BSEP) inhibitors (27%–50% and > 50% inhibition, respectively), for 10 commonly used molecular properties: molecular weight (MW), octanol-water partition coefficient (logP and $logD_{7.4}$, respectively), the surface areas of polar (PSA), saturated nonpolar ($NPSA_{saturated}$) and unsaturated nonpolar atoms ($NPSA_{unsaturated}$), the number of rotatable bonds, hydrogen bond acceptors and hydrogen bond donors, and the molecular net charge at pH 7.4. Bars over figure parts B to I denote the p values from t tests of strong inhibitors versus noninhibitors and weak inhibitors versus noninhibitors, respectively. B–K, Average values of the 10 selected molecular properties for strong inhibitors (SI), weak inhibitors (WI), and noninhibitors (Non) of BSEP-mediated taurocholate (TA) transport. Data are presented as the mean ± SE. Statistical significance, as determined by 1-way ANOVA and Bonferroni's *post hoc* test, is depicted by * for $p < .05$, ** for $p < .01$, *** for $p < .001$, and **** for $p < .0001$. L, The distribution of negative, positive, uncharged, or zwitterionic net molecular charges at pH 7.4 in the Non, SI, and WI groups.

more modestly, indicates that the compounds also decreased the basolateral net uptake of TA. Notably, rosiglitazone and ritonavir had similar effects in the SCHH assay, whereas markedly different levels of BSEP inhibition were observed in the vesicles (8% and 44% activity remaining, respectively; Fig. 7A).

In comparison, the 4 BSEP inhibitors with mild or no reported DILI (mifepristone, isradipine, budesonide, and glyburide) displayed significantly lower influence on the TA canalicular efflux in SCHH (Table 3 and Fig. 7B), despite showing similar degrees of BSEP inhibition in the vesicular assay (Table 3 and Figs. 7A and 7B). Within this group of compounds, the results also differed between the SCHH and the vesicular assays; glyburide and isradipine showed similarly modest effects on the canalicular efflux in SCHH, whereas

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the vesicular assay indicated mifepristone to be a more potent BSEP inhibitor (Fig. 7B). In the same group of compounds, budesonide resulted in comparable decreases in both intracellular and bile accumulation, suggesting that budesonide only inhibited the basolateral uptake of TA in SCHH without influencing the canalicular efflux. Regardless of their DILI severity classification, the 7 compounds that lacked inhibitory effect on BSEP-mediated TA transport in the membrane vesicles also lacked inhibitory effects in SCHH (Table 3 and Figs. 7C and 7D). Notably, the group of BSEP inhibitors associated with severe (BW or WP) DILI (Fig. 7A) had significantly greater impact on the biliary TA excretion than any of the other groups investigated ($p < .0001$; Fig. 7E). These results thus suggest that SCHH is a suitable *in vitro* model to differentiate BSEP inhibitors that may result in relatively mild DILI from those that carry the risks of more severe DILI.

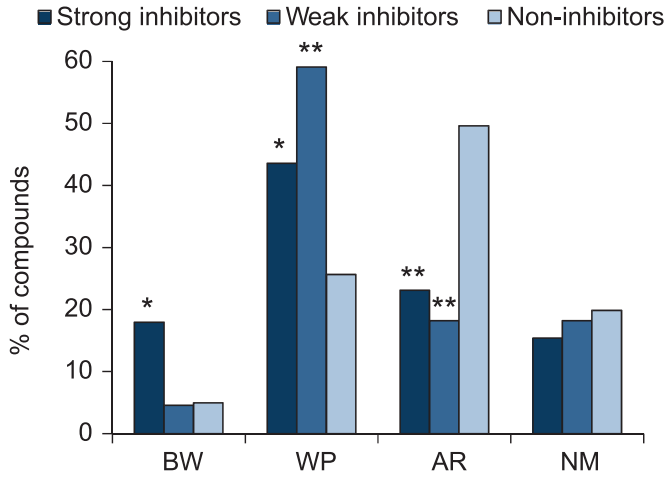

FIG. 5. Drug-induced liver injury (DILI) potential of drugs associated with different levels of bile salt export pump (BSEP) inhibition. The Food and Drug Administration drug label sections, boxed warnings (BW), warning and precautions (WP), and adverse reactions (AR), were used to classify DILI potential of the drugs ($n = 182$) in the data set also screened for BSEP inhibition in membrane vesicles. Drugs were assigned to classes according to the most severe drug label section reporting a hepatic ADR (BW > WP > AR), or to the NM class if no hepatic ADRs were reported. Bars show the fraction of all compounds in the same BSEP inhibition class (strong/weak/noninhibitor) that are associated with DILI of a certain severity. BSEP inhibition was shown to significantly increase the frequencies of hepatic ADRs reported in the BW and WP section. Strong inhibitors are described by dark columns, weak inhibitors by intermediate, and noninhibitors by light columns. Normality tests, using BSEP noninhibitors as control, were used to test the significance of the frequency differences, illustrated by * if $p < .05$ and ** if $p < .01$.

DISCUSSION

Several drugs reported to cause DILI have been identified as BSEP inhibitors (Kis *et al.*, 2009; Morgan *et al.*, 2010; Stieger *et al.*, 2000) and, in a recent publication based on 85 compounds, the incidence of DILI was found to be higher among BSEP inhibitors than among noninhibitors (Dawson *et al.*, 2012). In the present investigation, BSEP inhibition in inverted membrane vesicles was correlated to DILI in human subjects, using a larger, structurally diverse series of compounds, without selection bias toward DILI-associated compounds. We investigated the role that BSEP inhibition may play in DILI and analyzed to what extent BSEP inhibition in vesicles is a predictor of DILI. In general, the results confirm the conclusions from previous investigations (Dawson *et al.*, 2012; Morgan *et al.*, 2010) with regard to the utility of BSEP-inverted membrane vesicles as a screening tool to DILI-risk evaluation within drug discovery. However, we also found that the vesicle assay results in a significant number of false positives, ie, BSEP inhibitors that do not appear to increase the risk of severe DILI in humans.

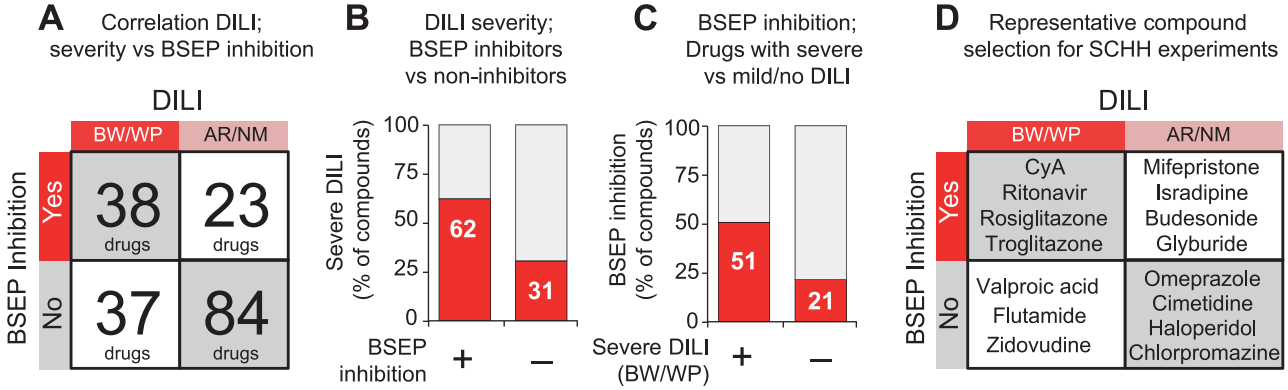

FIG. 6. Correlation between drug-induced liver injury (DILI) severity and bile salt export pump (BSEP) inhibition. A, Classification of the registered drugs in the data set ($n = 182$) with respect to BSEP inhibition in inverted membrane vesicles and DILI severity. B, Frequency of severe (boxed warnings/warnings and precautions, BW/WP) DILI among BSEP inhibitors and noninhibitors. Severe DILI was twice as common among the BSEP inhibitors as among the noninhibitors in the data set. C, Frequency of BSEP inhibitors among drugs that are associated with severe DILI, compared with drugs with mild or no DILI. Every second (51%) compound that was associated with BW/WP DILI inhibited BSEP-mediated taurocholate (TA) transport, whereas only every fifth (21%) compound associated with mild or no (adverse reactions/not mentioned, AR/NM) DILI decreased the TA transport. D, Selection of representative compounds for sandwich-cultured human hepatocytes (SCHH) experiments. Fifteen model compounds with different BSEP inhibition profiles (inhibitor or noninhibitor) and DILI potential (BW/WP or AR/NM) were selected from the 182 drugs for further investigation in SCHH.

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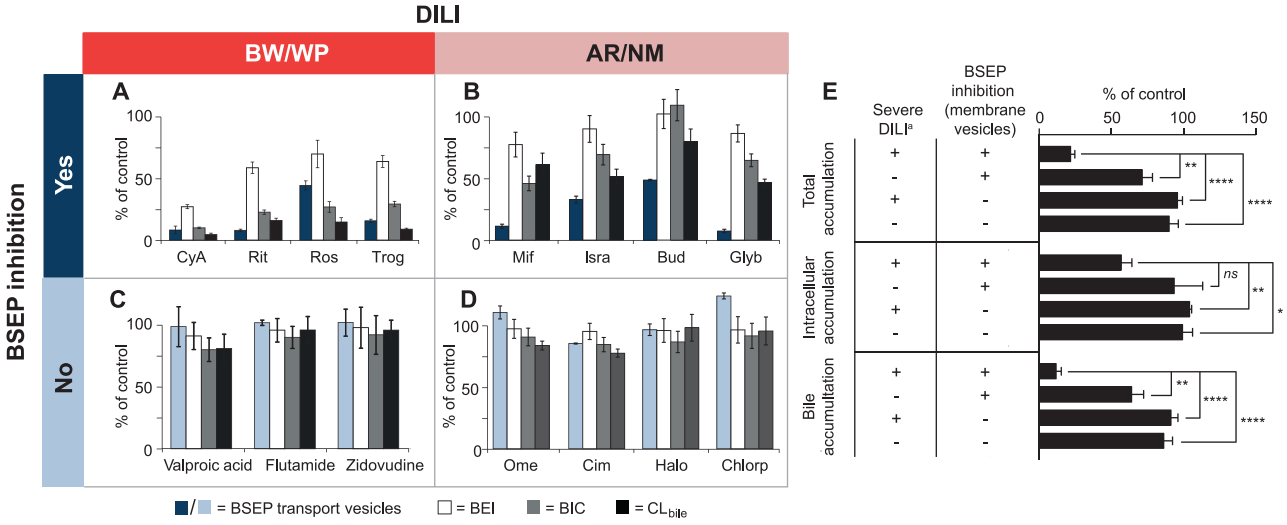

FIG. 7. The impact on taurocholate (TA) transport in sandwich-cultured human hepatocytes (SCHH) of 15 drugs with different bile salt export pump (BSEP) inhibition and drug-induced liver injury (DILI) potential. Drug impact on TA disposition is shown for each of the 4 combinations of BSEP inhibition (yes or no) in inverted membrane vesicles and DILI classifications obtained from the Food and Drug Administration drug labels (severe or mild/no), as described in Figure 6D: A, BSEP inhibitors associated with severe (boxed warnings/warnings and precautions, BW/WP) DILI; B, BSEP inhibitors associated with mild or no DILI (adverse reactions/not mentioned, AR/NM); C, BSEP noninhibitors associated with BW/WP DILI; and D, BSEP noninhibitors associated with AR/NM DILI. Each panel shows, from left to right, the effect of the compounds on BSEP transport in inverted membrane vesicles (dark columns for inhibitors and light columns for noninhibitors) and on the canalicular efflux of TA transport in SCHH using 3 complementary parameters: biliary excretion index (BEI), bile intracellular correlation (BIC), and *in vitro* biliary clearance (CL_{Bile}), the latter of which describes the effect of the basolateral net TA uptake in addition to the canalicular efflux. Each compound was tested in triplicate experiments at 3 different occasions. Data are presented as the mean ± SE. E, Differences in TA distribution patterns between the different compound groups in (A–D). Statistical significance, as determined by 1-way ANOVA and Bonferroni's *post hoc* test, is depicted by *ns* for $p \geq .05$, * for $p < .05$, ** for $p < .01$, *** for $p < .001$, and **** for $p < .0001$. Pairwise comparisons that are not shown in the figure were nonsignificant ($p >> .05$). The BSEP inhibitors associated with severe (BW/WP) DILI were found to cause significantly greater changes in the hepatocellular TA distribution than any of the other compound groups.

TABLE 3
Accumulation of TA in SCHH Treated With Compounds With Different Effects in the Vesicle Assay (Inhibitors or Noninhibitors) and DILI Potential (BW/WP or AR/NM)

			$TOTAL_{Acc}$[c] (% of control)		IC_{Acc}[d] (% of control)		$BILE_{Acc}$[e] (% of control)	
DILI[a]	BSEP Inhibition[b]		Average	SEM	Average	SEM	Average	SEM
BW/WP	Yes	Cyclosporine A	16.6	0.7	45.3	1.9	4.5	2.6
BW/WP	Yes	Ritonavir	27.3	2.1	70.7	1.6	16.0	9.2
BW/WP	Yes	Rosiglitazone	21.0	3.0	54.5	3.7	14.6	8.5
BW/WP	Yes	Troglitazone	14.0	0.6	30.4	1.9	8.9	0.7
AR/NM	Yes	Mifepristone	78.9	7.7	132.9	11.1	61.0	7.9
AR/NM	Yes	Isradipine	57.2	4.7	74.4	6.6	51.5	6.2
AR/NM	Yes	Budesonide	78.0	7.6	72.9	4.6	79.6	9.2
AR/NM	Yes	Glyburide	54.1	1.1	72.3	5.6	46.7	3.7
BW/WP	No	Valproic acid	89.1	6.0	101.4	10.1	81.3	9.8
BW/WP	No	Flutamide	100.2	5.9	106.6	8.5	96.1	9.5
BW/WP	No	Zidovudine	98.0	4.7	104.2	16.9	96.0	16.2
AR/NM	No	Omeprazole	86.1	1.6	92.4	7.1	83.6	6.6
AR/NM	No	Cimetidine	81.5	1.7	91.8	6.0	77.5	5.3
AR/NM	No	Haloperidol	102.3	7.5	113.3	7.7	98.0	9.8
AR/NM	No	Chlorpromazine	99.1	5.3	104.3	10.1	95.4	10.6

[a]DILI groups according to FDA labeling sections BW, WP, and AR as described and published by DailyMed (http://dailymed.nlm.nih.gov/).
[b]BSEP inhibition—Yes: strong BSEP inhibitors and No: compounds without significant effect on BSEP transport.
[c]$TOTAL_{Acc}$, total accumulation (intracellular and bile) of TA determined in incubations with standard HBSS.
[d]IC_{Acc}, intracellular accumulation of TA determined in incubations with Ca^{2+}- and Mg^{2+}-free HBSS.
[e]$BILE_{Acc}$, calculated from the total and intracellular accumulations ($TOTAL_{Acc} - IC_{Acc} = BILE_{Acc}$).

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This finding prompted us to explore whether SCHH (Hewitt *et al.*, 2007; Nakakariya *et al.*, 2012; Smith *et al.*, 2012) were better suited for identification of BSEP inhibitors causing DILI. Although a limited series of 15 compounds were studied in the SCHH, we propose that the SCHH assay is more effective than the vesicular assay in distinguishing compounds that may cause severe DILI due to BSEP inhibition from those that are less likely to do so. Additional preliminary results from 17 compounds studied in SCHH support these findings and confirm the capacity of SCHH to discriminate BSEP inhibitors associated with severe DILI from those less likely to induce DILI (data not shown).

The major molecular properties related to BSEP inhibition were charge, lipophilicity, hydrophobicity, and size. Similar observations have previously been reported for the inhibition of BSEP (Warner *et al.*, 2012) and other ABC transporters (Matsson *et al.*, 2007; Pedersen *et al.*, 2008), suggesting that the molecular interactions may be similar, and also that inhibition of additional canalicular transporters is likely for compounds that inhibit BSEP. The strong inhibitors (ie, those that decreased TA transport by more than 50%) were found to differ from noninhibitors in 8 of 10 common molecular properties, whereas weaker (yet statistically significant) inhibitors were indistinguishable from noninhibitors in all properties except molecular weight and the surface area of saturated nonpolar atoms. These results support the use of the 50% inhibition cutoff for separation of BSEP inhibitors from noninhibitors in the OPLS-DA models.

Transported BSEP substrates are preferably monovalent, negatively charged bile acids. Of the few nonbile acid substrates identified to date, all carry a negative net charge at physiological pH (Hirano *et al.*, 2005; Matsushima *et al.*, 2008). This is in agreement with our results on BSEP inhibition, where positive charge was strongly associated with a lack of inhibition (Supplementary Figure S1). However, unlike substrate interactions with BSEP, which are clearly disfavored by the lack of a negative charge (Gerloff *et al.*, 1998), the majority of the BSEP inhibitors (58%) were unionized (Fig. 4L). This highlights an important difference in inhibitor and substrate molecular interactions with BSEP.

It should be noted that our data set was selected for structural diversity and can be regarded as a snapshot of the chemical space of marketed drugs. The compound selection was performed before, and hence not biased by, the DILI analysis and the overall incidence of BW (8%) was comparable with the 10% BW incidence among all approved drugs between 1975 and 1999 (Lasser *et al.*, 2002). In contrast, the 85 compounds studied by Dawson *et al.* (2012) were selected on the basis of their liver-related ADRs and therefore their data set had a higher BW incidence (21%). The differences in data set selection explain the differences in ADR frequencies—including those for BW DILI—for the 2 data sets. It may also explain why we observed a relationship between BSEP inhibition and DILI severity, which the investigation by Dawson *et al.* did not.

Our investigation of the association between BSEP inhibition and DILI found that the majority (62%) of the BSEP inhibitors increased the risk of more severe (BW and WP) DILI (Fig. 6B), and a significant proportion (18%) were associated with potentially life-threatening, BW-classified DILI (Fig. 5). Interestingly, only the strong inhibitors increased the risk of BW-classified DILI, whereas the weaker inhibitors only increased the risk of intermediate severity (WP) DILI (Fig. 5). This is in agreement with the observed differences in molecular properties between the strong and weak inhibitors. The association of strong and weak inhibitors with BW and/or WP DILI supports our assay design and shows that the vesicle assay can be used for preliminary classification of the severity of DILI associated with BSEP inhibition.

SCHH were used to further assess how BSEP inhibition correlates with DILI. To enable a mechanistic interpretation of how the hepatocellular TA disposition was affected by the added compounds, we monitored the accumulation of TA in both the intracellular and bile compartments, separately as well as in combination. We also analyzed 3 derived parameters (CL_{Bile}, BEI, and BIC) that describe complementary aspects of the cellular TA disposition. CL_{Bile} describes the rate of transport across the hepatocyte from the media compartment to the bile canaliculi (equation 5). Therefore, CL_{Bile} decreases if a test compound inhibits the basolateral net uptake or the canalicular efflux. BEI describes the accumulation of TA in relation to the total accumulation (ie, the sum of the intracellular and bile accumulation; equation 3) and will decrease if a test compound inhibits the canalicular efflux. By definition, the BEI parameter includes the bile accumulation in both the numerator and denominator, and a decrease in BEI will thus not be directly proportional to a decrease in canalicular efflux caused by an added transporter inhibitor (Supplementary Table S3). As a complement to CL_{Bile} and BEI in investigations of canalicular efflux inhibition, we therefore introduced a third, easily calculated parameter—BIC—to facilitate the interpretation of SCHH data. BIC describes the canalicular efflux in relation to the intracellular accumulation (equation 4) and will, similar to BEI, decrease if a test compound inhibits the canalicular efflux. Because BIC is calculated using the accumulation in the intracellular and bile compartments (ie, the 2 compartments that are separated only by the canalicular membrane), any decreases in BIC will be directly proportional to decreases in the canalicular efflux (Supplementary Table S3). In this respect, the BIC parameter is similar to the $CL_{Bile,int}$ parameter that was recently shown to significantly improve predictions of human *in vivo* biliary clearance (Nakakariya *et al.*, 2012). $CL_{Bile,int}$ is analogous to CL_{Bile} but uses the intracellular concentration instead of that in the media. When combined, these parameters can be used to separate effects on transport processes in the basolateral membrane from those in the canalicular membrane. For instance, if a compound inhibits the basolateral net uptake (eg, Sodium-taurocholate cotransporting polypeptide [NTCP] inhibition)

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but not the canalicular efflux, CL_{Bile} decreases, whereas BIC remains unchanged (Supplementary Table S3).

Our studies on TA transport and accumulation in SCHH supported and extended the observations obtained in the membrane vesicles. As expected from the vesicle results, the 3 BSEP inhibitors associated with severe DILI (BW or WP) resulted in major decreases in the canalicular efflux of TA in SCHH (Fig. 7A). Notably, this group of compounds had a significantly greater impact on the biliary TA excretion than any of the other groups investigated ($p < .0001$). In contrast, despite showing similar degrees of inhibition in the vesicle assay, the BSEP inhibitors with less severe or no reported DILIs (AR or NM) only marginally affected the canalicular TA efflux in SCHH (Fig. 7B). These results thus support the utility of SCHH in separating BSEP inhibitors associated with severe DILI from those with no or mild DILI. Importantly, compounds that inhibit BSEP but which are not reported to cause severe DILI (Fig. 7B) still resulted in modest alterations of the canalicular efflux. Such compounds may contribute to an increased ADR incidence, if coadministered with other drugs. Caution is thus warranted, although further analyses are needed to fully explore such combination effects.

None of the BSEP noninhibitors (Figs. 7C and 7D) affected the TA disposition in SCHH. The increased risk of severe DILI with valproic acid, flutamide, and zidovudine must therefore derive from mechanisms other than BSEP inhibition. Valproic acid is one of the drugs most frequently implicated in acute liver failure (Bjornsson and Olsson, 2006). Its toxicity mechanisms include an increased formation of reactive oxygen species, the inhibition of the mitochondrial membrane potential, and an increased leakiness of lysosomal membranes (Pourahmad *et al.*, 2012; Tong *et al.*, 2003). Reactive metabolites have been implicated in the hepatotoxicity of flutamide, resulting in the formation of intracellular protein adducts and depletion of hepatic glutathione (Ohbuchi *et al.*, 2009). The mechanism by which zidovudine causes hepatotoxicity is not yet determined, but the compound has been associated with mitochondrial toxicity (Lynx *et al.*, 2006). The diversity of cellular mechanisms that can result in clinical DILI clearly demonstrates that multiple endpoints must be monitored to fully predict the risk of DILI for new drugs (Opar, 2012); the results here show that inhibition of BSEP is one valuable piece of such a panel of toxicity measurements.

In conclusion, the present study showed that BSEP inhibition doubles the risk of severe DILI and identified half of the drugs reported to inflict severe DILI as BSEP inhibitors. This demonstrates the importance of BSEP inhibition determination in early drug discovery. We show that BSEP-expressing inverted membrane vesicles are suitable as a primary screening assay to evaluate the risk of liver injury associated with BSEP inhibition. However, it results in many false positives and therefore needs to be complemented with a physiologically more relevant method such as the SCHH. To facilitate the interpretation of such SCHH experiments, we introduced BIC as a parameter to describe canalicular efflux in relation to the intracellular substrate concentration. When used in combination with traditional parameters like CL_{Bile} and BEI, BIC can improve the mechanistic understanding of compound transport through the hepatocyte. Finally, we showed that an SCHH assay can be used to differentiate BSEP inhibitors associated with severe DILI from those less associated with DILI, thereby greatly improving the prediction of BSEP-associated DILI.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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