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Interference with Bile Salt Export Pump Function Is a Susceptibility Factor for Human Liver Injury in Drug Development

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The bile salt export pump (BSEP) is an efflux transporter, driving the elimination of endobiotic and xenobiotic substrates from hepatocytes into the bile. More specifically, it is responsible for the elimination of monovalent, conjugated bile salts, with little or no assistance from other apical transporters. Disruption of BSEP activity through genetic disorders is known to manifest in clinical liver injury such as progressive familial intrahepatic cholestasis type 2. Drug-induced disruption of BSEP is hypothesized to play a role in the development of liver injury for several marketed or withdrawn therapeutics. Unfortunately, preclinical animal models have been poor predictors of the liver injury associated with BSEP interference observed for humans, possibly because of interspecies differences in bile acid composition, differences in hepatobiliary transporter modulation or constitutive expression, as well as other mechanisms. Thus, a BSEP-mediated liver liability may go undetected until the later stages of drug development, such as during clinical trials or even postlicensing. In the absence of a relevant preclinical test system for BSEP-mediated liver injury, the toxicological relevance of available *in vitro* models to human health rely on the use of benchmark compounds with known clinical outcomes, such as marketed or withdrawn drugs. In this study, membrane vesicles harvested from BSEP-transfected insect cells were used to assess the activity of more than 200 benchmark compounds to thoroughly investigate the relationship between interference with BSEP function and liver injury. The data suggest a relatively strong association between the pharmacological interference with BSEP function and human hepatotoxicity. Although the most accurate translation of risk would incorporate pharmacological potency, pharmacokinetics, clearance mechanisms, tissue distribution, physicochemical properties, indication, and other drug attributes, the additional understanding of a compound's potency for BSEP interference should help to limit or avoid BSEP-related liver liabilities in humans that are not often detected by standard preclinical animal models.

Key Words: **bile salt export pump; BSEP; liver; bile salt transport; hepatobiliary transport; hepatotoxicity.**

Hepatobiliary transporters maintain liver homeostasis by regulating intracellular exposure to endobiotic and xenobiotic chemicals. As a polar cell type, hepatocytes have specialized transport systems located at the blood or sinusoidal domain (also referred to as the basolateral domain) or at the canalicular/apical domain (Fig. 1). Transporters at the basolateral domain are responsible for hepatocellular uptake of various substrates from the blood/sinusoid, elimination to the blood/sinusoid, or both depending on the transporter. At the canalicular domain, however, these pumps are exclusively efflux transporters, mediating the elimination of various substrates into the bile (Alrefai and Gill, 2007; Borst *et al*., 2007; Byrne *et al*., 2002; Dawson *et al*., 2009; Geier *et al*., 2007; Klaassen and Aleksunes, 2010; Nies and Keppler, 2007; Pauli-Magnus and Meier, 2005, 2006; Stieger *et al*., 2007; Trauner and Boyer, 2003; Zollner *et al*., 2006; Zollner and Trauner, 2008). Of the canalicular transporters, the bile salt export pump (BSEP) is responsible for the elimination of monovalent, conjugated bile salts into the bile canaliculi (Gerloff *et al*., 1998; Strautnieks *et al*., 1998). In addition, BSEP—formerly referred to as sister of permeability-glycoprotein—has been shown to transport some xenobiotics, such as pravastatin, vinblastine, and possibly others (Hirano *et al*., 2005; Sakurai *et al*., 2007). Bile acids are amphipathic, steroidal compounds produced from cholesterol in hepatocytes or returning via enterohepatic circulation and secreted into bile across the canalicular membrane. Bile acids are required for intestinal absorption of dietary fat and hydrophobic vitamins and return with high efficiency to the liver through enterohepatic circulation. Interference in BSEP function can result in the hepatocellular accumulation of bile salts and the development of liver injury (Alrefai and Gill, 2007; Chiang, 2009; Davit-Spraul *et al*., 2009; Dawson *et al*., 2009; Fattinger *et al*., 2001; Feng *et al*., 2009; Hofmann, 1999; Keitel *et al*., 2009; Kostrubsky *et al*., 2006; Pauli-Magnus and Meier, 2006; Stieger, 2009;

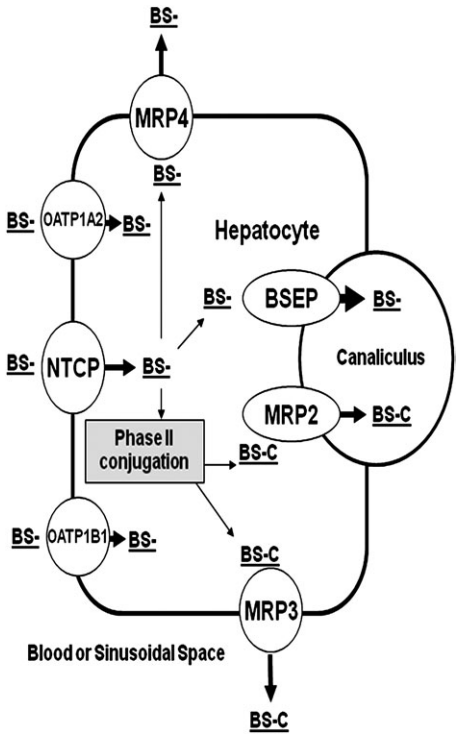

FIG. 1. Major transporters involved in hepatocellular bile acid homeostasis. Illustration of a polarized primary hepatocyte and the localization of basolateral and apical/canalicular transporters. BSEP is the canalicular transporter responsible for efflux of monovalent bile salts. The NTCP is the primary basolateral transporter responsible for uptake of bile salts from the blood and/or sinusoid, whereas the sodium-independent uptake transporters organic anion transporting polypeptides (OATPs) 1A and 1B play a lesser role in this regard. The multidrug resistance–associated protein 2 (MRP2) excretes divalent bile salts. BS, monovalent bile salts, BS-C, divalent bile salts (e.g., glucuronide-conjugated bile salts).

Stieger *et al*., 2000, 2007; Trauner and Boyer, 2003; Zollner and Trauner, 2008). In the case of progressive familial intrahepatic cholestasis type 2 (PFIC2), where one or more polymorphisms exist in the genetic code for BSEP (ATP-binding cassette, subfamily B, member 11, or ABCB11), inadequate BSEP function is associated with liver injury (Alissa *et al*., 2008; Davit-Spraul *et al*., 2009; Kagawa *et al*., 2008; Pauli-Magnus and Meier, 2005; Stieger, 2009; Stieger *et al*., 2007; Trauner and Boyer, 2003; Wang *et al*., 2001). In fact, human BSEP mutations are the molecular basis for at least three clinical forms of liver disease, PFIC2, benign recurrent intrahepatic cholestasis type 2 (BRIC2), and intrahepatic cholestasis of pregnancy (Alissa et al., 2008; Byrne *et al*., 2009; Davit-Spraul *et al*., 2009; Dixon *et al*., 2009; Kagawa *et al*., 2008; Pauli-Magnus and Meier, 2005; Stieger, 2009; Stieger et al., 2007; Strautnieks *et al*., 2008; Trauner and Boyer, 2003). The phenotypes of PFIC2 and BRIC2 differ although both are caused by mutations in ABCB11. PFIC2 is characterized by progressive liver damage usually requiring transplantation. In contrast, BRIC2 is manifested by intermittent and usually nonprogressive cholestasis (Alissa *et al*., 2008; Byrne *et al*., 2009; Davit-Spraul *et al*., 2009; Kagawa *et al*., 2008; Lam *et al*., 2006; Pauli-Magnus and Meier, 2005; Stieger, 2009; Stieger *et al*., 2007; Strautnieks *et al*., 2008). Several different mutations in BSEP have been reported in patients having PFIC2 (Byrne *et al*., 2009; Davit-Spraul *et al*., 2009; Stieger, 2009; Stieger *et al*., 2007; Strautnieks *et al*., 2008). Kagawa *et al*. (2008) showed through *in vitro* studies that taurocholate transport activity corresponded to BSEP protein levels in most PFIC2 and BRIC2 mutants, indicating that the impaired function is derived from decreased protein expression. Their observation that a representative mutant had a shorter biochemical half-life than the wild type suggested that rapid degradation of Bsep protein may be responsible for impaired function. The variance in expression levels and activity of BSEP, through natural mutations, correlates with the associated liver disease severity, such that a greater decrease in BSEP abundance and function corresponds to a more severe disease outcome (Kagawa *et al*. 2008). This establishes a dose-response relationship for these phenomena, one of the basic tenets of toxicology, thus strengthening the hypothesis that BSEP is an important toxicological target. Further evidence of the deleterious effects associated with nonfunctional BSEP is found in two case series described by Jara *et al*. (2009) and Keitel *et al*. (2009), where patients with PFIC2 developed antibodies to BSEP and following liver transplantation (with subsequent *de novo* exposure to BSEP) resulted in prolonged cholestasis. These case studies offer a unique example of how inhibition of BSEP transport can result in a disease state resembling that which is derived from BSEP dysfunction through genetic mutations.

Therapeutics shown to interfere with BSEP function are often associated with liver liabilities in humans (Fattinger *et al*., 2001; Feng *et al*., 2009; Funk *et al*., 2001; Iwanaga *et al*., 2007; Kostrubsky *et al*., 2003, 2006; McRae *et al*., 2006; Pauli-Magnus and Meier, 2006; Sakurai *et al*., 2007; Snow and Moseley, 2007; Stieger, 2009). Examples of therapeutics having BSEP interference implicated as at least a contributor to liver injury include bosentan (an endothelin antagonist for pulmonary arterial hypertension [PAH]), erythromycin estolate (a macrolide antibiotic), nefazodone (5-HT_2 receptor antagonist for depression), CI-1034 (an experimental endothelin antagonist for PAH), and CP-724,714 (an experimental HER2 kinase inhibitor for oncology) (Fattinger *et al*., 2001; Feng *et al*., 2009; Kostrubsky *et al*., 2003, 2006; Stieger, 2009). It is likely that BSEP may be one of many susceptibility factors in these cases. A recurring observation in the literature is that compounds shown to interfere with BSEP function are often not associated with significant liver injury in standard preclinical models, yet have been associated with liver injury when administered to humans (Fattinger *et al*., 2001; Feng *et al*., 2009; Kostrubsky *et al*., 2003, 2006; Leslie *et al*., 2007; Pauli-Magnus and Meier, 2006; Sakurai *et al*., 2007; Stieger,

2009). The seemingly inability of preclinical test species to reliably predict liver liabilities in humans is a concern for the pharmaceutical industry (Kostrubsky *et al*., 2003, 2006). A few *in vitro* models have been established to interrogate the potential for compounds to interfere with bile salt transport, such as the primary hepatocyte, sandwich culture model (Kemp *et al*., 2005; Kostrubsky *et al*., 2003, 2006; Leslie *et al*., 2007; Liu *et al*., 1998, 1999a,b; McRae *et al*., 2006; Xia *et al*., 2007), the BSEP or Bsep membrane vesicle assay (Byrne *et al*., 2002; Fattinger *et al*., 2001; Saito *et al*., 2009; Sakurai *et al*., 2007; Xia *et al*., 2007), cytoplasmic membrane vesicle preparations from whole liver (Horikawa *et al*., 2003), and the doubly transfected BSEP/sodium taurocholate cotransporting polypeptide (NTCP) model for vectorial, bile salt transport (Mita *et al*., 2005, 2006a,b; Xia *et al*., 2007). Each of these models has demonstrated its relative merits and challenges in identifying chemical entities with the potential to disrupt bile salt transport (Xia *et al*., 2007). We have chosen BSEP-expressing membrane vesicles to evaluate the relationship between BSEP inhibition potency, as described by an IC_{50} (the concentration at which 50% inhibition of transport is achieved) and clinical liver injury using a compendium of more than 200 approved drugs with well-described clinical experiences.

The primary objective of this work was to investigate the potential correlation between a compound's ability to inhibit BSEP function and liver injury in humans using a large selection of marketed or withdrawn drugs, in addition to other chemical entities. Technical performance of the assay, details about the benchmark compounds, and a comparison of the assay results with known human outcomes will be presented. Finally, this work will offer preliminary guidance on how best to use a BSEP transport assay to estimate the risk of liver liabilities in humans in the absence of a relevant preclinical toxicity model.

MATERIALS AND METHODS

Materials. Human BSEP vesicles were purchased from Solvo (Budapest, Hungary), and rat Bsep vesicles from BD-Gentest (Woburn, MA). The membrane vesicles were harvested from transiently transfected Sf9 insect cells and processed for inside-out vesicles. Where available, test articles were purchased through various commercial sources, including Sigma (St Louis, MO), Biomol (Plymouth Meeting, PA), and others. For test articles not available through commercial means, the synthesis of such compounds was performed at external contract laboratory organizations. All test articles were solubilized in dimethyl sulfoxide (DMSO) to a top concentration of 10mM and then stored in a freezer set to maintain −20°C until ready for use. Human toxicity information for select benchmark drugs was collated from product labels or inserts and/or Pharmapendium version 2.5 (database version 2010.1) (Elsevier Properties, SA; New York, NY), which included Mosby's Drug Consult and Meyler's Side Effect of Drugs.

Membrane vesicle transport assay. In this model, plasma membrane vesicles expressing human BSEP (or other species of interest) are harvested from transfected Sf9 insect cells. Vesicles in the inside-out configuration allow BSEP, in the presence of ATP, to transport a radiolabeled bile salt (^{3}H-taurocholate or ^{3}H-T) from the reaction buffer and trap it inside the vesicle (Fig. 2). A decrease in the amount of vesicle-associated ^{3}H-T as a function of test article concentration indicates interference in BSEP transport (Byrne *et al*., 2002; Saito *et al*., 2009; Sakurai *et al*., 2007; Xia *et al*., 2007). Test articles were solubilized in DMSO and evaluated for human BSEP or rat Bsep interference at a concentration range of 0–133μM (final DMSO content in the reaction was 1.3%) in a 96-well format (10 concentrations per compound, no replicates, and eight compounds per plate—column 6 was reserved for ATP controls and column 12 for no-ATP controls). Vesicles were maintained frozen at approximately −80°C. On the day of the assay, the vesicles were placed in a room temperature water bath (approximately 24°C) for 10–15 min until completely thawed and then stored on ice until needed. Reagents were prepared according to a modified Solvo Assay Protocol for BSEP membrane vesicles (product number SB-BSEP-Sf9-VT). Briefly, an assay mix was prepared containing 2mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-Tris, pH 7.4, 100mM KNO_3, 10mM $Mg(NO_3)_2$, and 50mM sucrose and ultrapure water, and a wash mix was prepared containing 10mM Tris-HCl, pH 7.4, 100mM KNO_3, 50mM sucrose, and 0.1mM sodium taurocholate and ultrapure water (refer to the Solvo Assay Protocol for details). For a 96-well plate, the reaction mix was formulated as follows: 1 ml of membrane vesicles (5 mg/ml total protein for both human BSEP and rat Bsep vesicles) was combined with 4 ml of assay mix, 5 μl of 200μM unlabeled taurocholate, and 5 μl of 5 Ci/mmol (200μM) ^{3}H-T. A Titertek Multidrop was used to dispense 50 μl of the above reaction mix to each well of a 96-well plate. Then, 1 μl of test article or DMSO alone was added to each well, and the reaction was incubated on an orbital shaker for 10 min at room temperature. A 12mM ATP mix was then prepared by combining 150 μl of 0.2M magnesium-ATP with 2.35 ml of the assay mix. Following the 10-min preincubation, 25 μl of ATP mix was added to each well of the 96-well reaction plate, and for the no-transport/no-ATP controls, 25 μl of assay mix alone was added instead. The reaction plate was then returned to the orbital shaker and incubated for approximately 15 min at room temperature. Following the final incubation, transport was stopped by rapidly filtering the reaction mix through a PerkinElmer 96-well Unifilter GF/C filter plate (preblocked for 30 min with 30 μl 0.5% polyethylenamine solution) using a PerkinElmer FilterMate cell harvester and washing the reaction plate 4× with 200 μl ice-cold wash buffer. The intact membrane vesicles were trapped on the filter bed while unbound radiolabel was washed away, thereby leaving only vesicle-associated radioactivity available for measurement on the filter bed. The Unifilter plate was dried for 1 h in a 65°C vacuum oven and then allowed to cool to room temperature prior to the addition of 40 μl per well of PerkinElmer Microscint 20. Radioactivity was measured using a Packard TopCount.

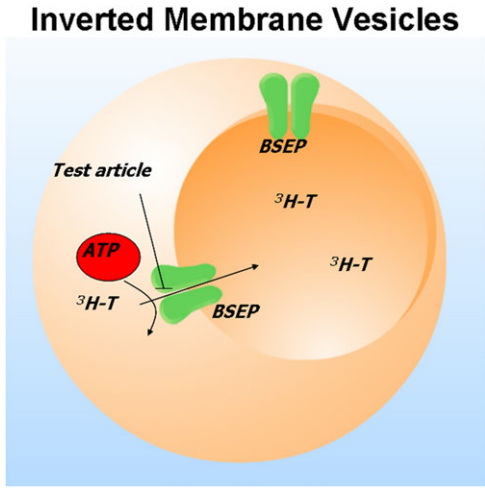

FIG. 2. Illustration of an inside-out membrane vesicle expressing BSEP. The vesicles are prepared from Sf9 insect cells transfected with human BSEP. After processing for membranes, approximately 20% of the vesicles have an inside-out configuration. This inside-out or inverted configuration allows BSEP transport to mediate internalization of a radiolabeled substrate ^{3}H-T. A decrease in ^{3}H-T internalization as a result of test article exposure indicates interference with BSEP function. This system is not metabolically competent.

Curve fitting. Curve fitting and quality control (QC) of the percentage of control (POC) values were performed using the Condoseo module of Genedata Screener software suite (Genedata AG, Basel, Switzerland). An M-estimation (maximum likelihood estimation)–based nonlinear regression method (Fomenko *et al*., 2006), implemented within Condoseo for fitting concentration-response data to a four-parameter logistic equation, was used to determine the IC_{50} values and corresponding fit quality metrics. M-estimation is a robust and unbiased technique for estimating each of the four parameters to fit the concentration-response curve of the experimental data.

Apart from the four parameters of the logistic equation, the POC value at the highest included concentration, termed as "max activity," was evaluated to estimate the activity of the tested compounds. The 95% confidence intervals of the four Hill parameters were used to assess the quality of the curve fit using the M-estimation method. To facilitate consistent analysis and reporting, the curve fitting and QC workflow was automated in Condoseo based on specifications for each parameter of the logistic equation. Additionally, visual review of the data and the fitted curves was performed, and in a few cases, data were flagged for manual intervention. Manual data point exclusion was allowed only for data with solubility issues at higher tested concentrations. Manual locking of a parameter was allowed only if a curve failed QC because of unacceptable estimates of the top or the bottom parameters as indicated by a wide 95% confidence interval for that parameter.

If a curve failed QC because of an incomplete bottom portion of the curve and if the max activity was greater than −80 POC, then the bottom parameter was locked to −100 POC, and a three-parameter Hill fit was performed using the M-estimation method. In the present study, the bottom parameter was locked to −100 POC for approximately 70% of the compounds. If a curve failed QC because of an incomplete bottom portion of the curve and if the max activity was between −50 and −80 POC, then an absolute estimate of the IC_{50} called "Flank" was determined instead of the IC_{50} value calculated by the Hill fit. A 95% confidence interval was also calculated for the Flank value to assess its quality. The Flank value was calculated using a "Flank fit" algorithm that took the concentration-POC data set and split it into two segments to perform linear least-square fits. The algorithm scanned across the data set, breaking it into segments of different sizes with the constraint that a segment must have at least four data points. It then selected the set of fits with the minimum error across both fits and split the data into a "plateau" part and a "slope" part. The concentration at which the segment fit across the slope part crossed the −50 POC level was termed as Flank. In the present study, the Flank estimate was used to generate IC_{50} values for less than 10% of the compounds.

IC_{50} values were used as the primary metric to assess the potency of the tested compounds. In cases when a curve fit failed QC and an IC_{50} value was not derived, Flank value was used as an estimate of the potency. For the purposes of this work, IC_{50} values generated using M-estimation or Flank fit were considered equivalent. Therefore, the IC_{50} values presented here may be because of one or the other fit methodologies. When a data set failed QC for both IC_{50} and Flank calculations, only the max activity value was reported as an estimate of a compound's potency for BSEP interference (max activity data not shown). Although max activity values for BSEP interference may aid in prioritizing developmental therapeutics, this work focuses on the use of IC_{50} values for this purpose, and compounds with insufficient activity to derive IC_{50} values via M-estimation or Flank fit are described here as being negative for BSEP interference, despite the fact that some compounds may have had some activity at the top one or more concentrations. For illustration purposes, charts may represent these compounds as having an IC_{50} value equal to or greater than the top concentration evaluated (133μM).

Assay precision analysis. An evaluation of the variability associated with the human BSEP vesicle transport assay as presented here was performed to assess the intra- and interplate precision. Six compounds were selected with previously determined IC_{50} concentrations for BSEP inhibition ranging from 5 to 100μM. Each compound was run in at least four separate trials per day. For trial 1, all compounds were run on a single 96-well plate, one row for each compound. This allowed for the calculation of one IC_{50} concentration for each compound. For the remaining three trials, each compound was run on its own 96-well plate. This allowed for the calculation of eight IC_{50} concentrations for each compound per trial.

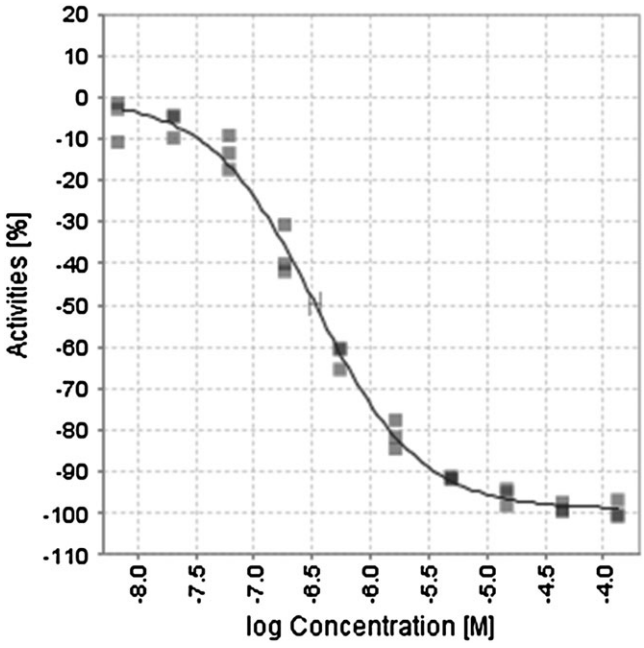

FIG. 3. A 10-point titration of cyclosporine A was performed using a 1:3 dilution scheme and then assayed in the BSEP filter-binding assay. The compound concentration-activity response coordinates were plotted, and the data were then fit using a standard four-parameter logistical Hill model. Data represent three separate trials with cyclosporine A, with a mean IC_{50} value of 0.88μM.

Curve fitting and QC were performed following the methods used within the Condoseo module of Genedata Screener software suite for each row of the 96-well plate. The intraplate variability was evaluated by computing the % coefficient of variation (CV) of the calculated IC_{50} concentrations for each plate by compound. The trial 1 data did not contribute to this evaluation because there were no replicates for a given compound during that trial. The interplate variability was evaluated by computing the %CV of the calculated IC_{50} across all observations for each compound. The range of %CV values for the observed IC_{50} concentrations for the intraplate evaluation and the range of %CV values for the observed IC_{50} concentrations for the interplate evaluation were tabulated to demonstrate the assay's intra- and interplate precision, respectively.

RESULTS

Assay Performance

The vesicle manufacturer's methods were adapted to a higher throughput screening paradigm, and the reproducibility/robustness of this assay was demonstrated in that setting. Cyclosporine A was employed as a positive control, evaluated on at least one or more plates during every BSEP vesicle transport experiment. As can be seen in Figure 3, the effect of cyclosporine A on BSEP is quite potent, with an average IC_{50} in the nanomolar range. Given this level of potency, the dose-response curve is well sculpted, allowing for a good IC_{50} estimation. To better understand the variability of the IC_{50}

TABLE 1
BSEP Vesicle Transport Assay Precision

	Intraplate %CV				Interplate %CV
Compound	Trial 2	Trial 3	Trial 4	Trial 5	All observations
Norethindrone	26	17	8	*NA	29
Bosentan	7	19	25	NA	20
Tolcapone	8	14	10	NA	20
Indomethacin	10	28	24	14	27
Compound X	9	28	22	NA	29
Nefazodone	23	55	15	NA	30

Note. Intraplate %CV calculated for each trial and interplate %CV across all trials for each compound. The %CV was not calculated for trial 1 because there were no replicates. Only indomethacin was subjected to a fifth trial. NA, not applicable.

estimates, a series of precision experiments were conducted using six compounds of varying potencies in the human BSEP assay. The intraplate and interplate %CV for each compound in each trial is included in Table 1. Figure 4 illustrates the observed IC_{50} values for each compound in each trial, along with the average IC_{50} for each trial and across trials.

Benchmark Compounds

The literature was reviewed for compounds known to interfere with BSEP activity (Fattinger *et al*., 2001; Feng *et al*., 2009; Funk *et al*., 2001; Iwanaga *et al*., 2007; Kostrubsky *et al*., 2003, 2006; McRae *et al*., 2006; Pauli-Magnus and Meier, 2006; Saito *et al*., 2009; Sakurai *et al*., 2007; Snow and Moseley, 2007; Stieger, 2009; Stieger *et al*., 2000; Trauner and Boyer, 2003; Zollner and Trauner, 2008). Benchmark compounds thus identified were used as positive controls to establish the assay's sensitivity. Then, drugs (marketed or withdrawn), and prototypical toxicants not known to be associated with BSEP-mediated liver injury in humans, were randomly selected for evaluation in the BSEP vesicle transport assay. Compounds selected here included the following: therapies with no known liver liability, compounds associated with drug-induced liver injury, and other toxicants or experimental chemicals. This collection of benchmarks was used to evaluate the specificity and sensitivity of the BSEP vesicle transport assay. Because traditional preclinical animal models have been demonstrated to be poor predictors of BSEP-mediated liver injury (Fattinger *et al*., 2001; Kostrubsky *et al*., 2003, 2006), it was critical that most of the selected benchmark compounds had known effects in humans. A comparison was then made between BSEP potency and clinical outcome, thus benchmarking the assay's correlation with human liver injury.

The potencies for greater than 200 benchmark compounds, as represented by the IC_{50} values derived from their respective concentration-response curves, are illustrated in Figure 5. Compounds for which IC_{50} values could not be derived are designated as having an $IC_{50} > 133\mu M$ and considered to be negative in the assay. Although binned as negative for BSEP interference, some of these compounds did demonstrate modest decreases in BSEP transport over the selected concentration range (0–133μM); however, the effect was so minimal that the nonlinear regression models (M-estimation and Flank fit) could not fit a curve suitable to derive an IC_{50}. The concentration range of 0–133μM was selected primarily because of solubility limits for a number of test articles. We recommend this concentration range for routine screening based on our experience with the compounds presented in this report. If reassessed at higher concentrations, it is likely that IC_{50} values could be derived for a number of the negative compounds presented here. Based on these criteria, as shown in Figure 6, approximately 75% of the benchmark compounds were negative in the assay. On the other

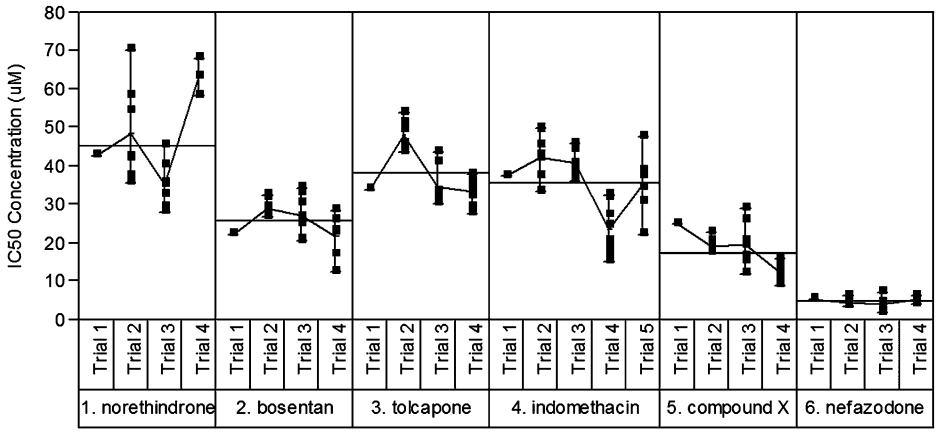

FIG. 4. The observed IC_{50} values determined for each compound (black squares) over each trial. Lines connect the means of each trial, and a horizontal line illustrates the mean across all trials for each compound.

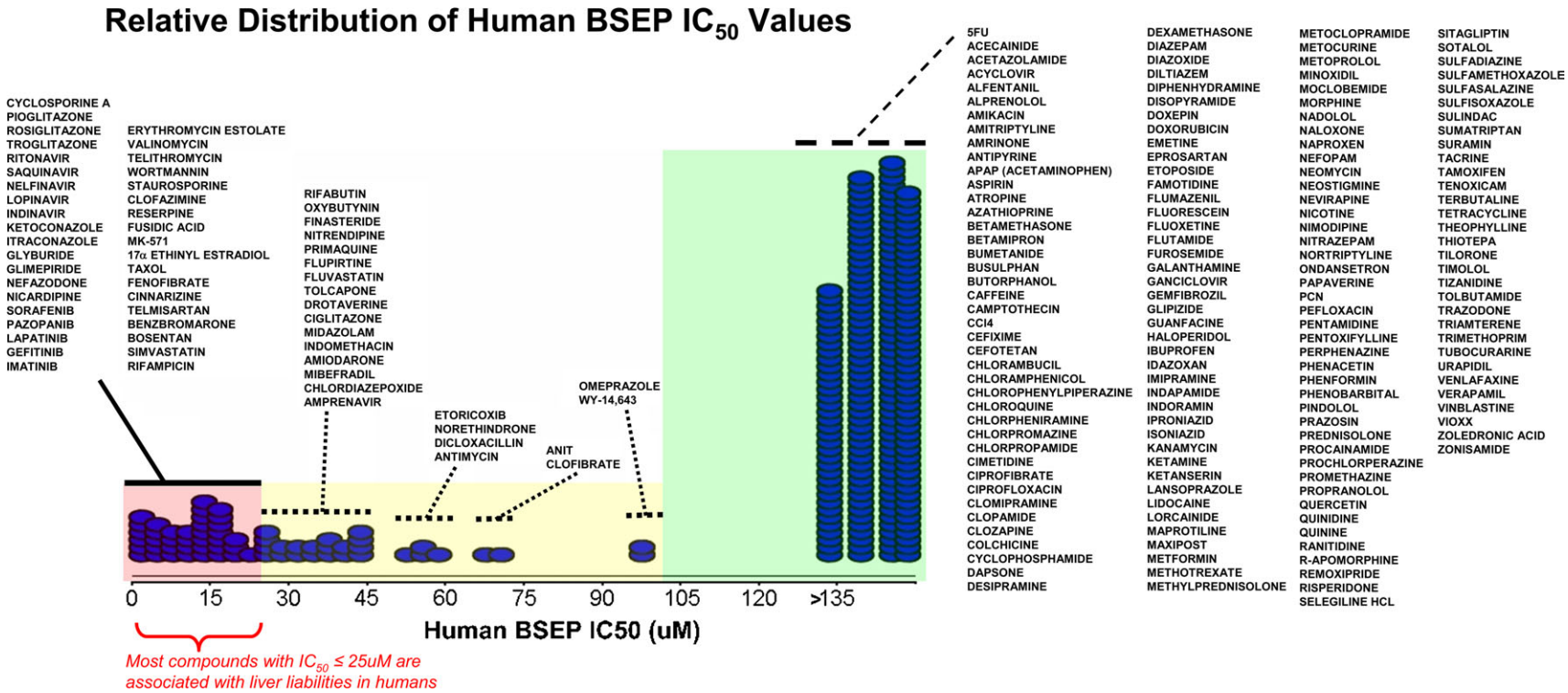

FIG. 5. Relative distribution of human BSEP IC_{50} values. Dot plot representation of the IC_{50} distribution across benchmark compounds evaluated in the human BSEP assay. Each blue dot represents one compound—solid or broken lines identify the list of compounds represented by each series of dots. As is evident with some of the compounds with IC_{50} values in the ''negative'' range (IC_{50} > 135μM), such as acetaminophen (APAP), CCl_4, zonisamide, and others, the BSEP assay is not predictive of liver injury related to other mechanisms. Actual IC_{50} values were not generated for compounds categorized as having an IC_{50} of > 135μM, rather this designation was used for illustration purposes only. Compounds in this category had insufficient effect on BSEP transport in the given concentration range to generate an actual IC_{50} value.

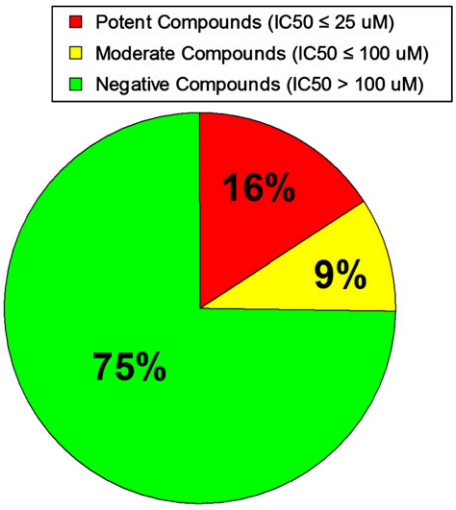

FIG. 6. A pie chart illustrates the percentage of compounds binned as potent, moderate, or negative for over 200 compounds evaluated in the human BSEP assay. The majority of marketed drugs were negative for BSEP.

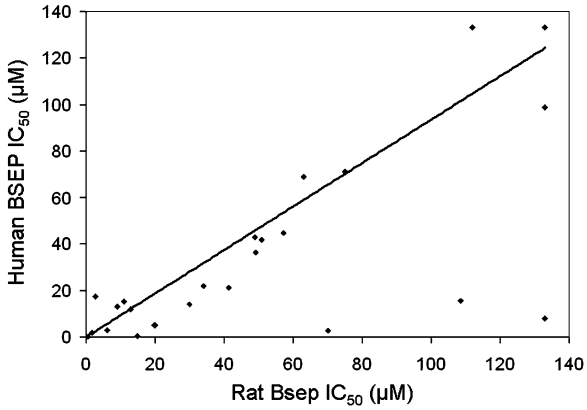

FIG. 7. A subset of randomly selected benchmark compounds were evaluated for their effect on human BSEP and rat Bsep function. In general, human IC_{50} values are similar to those obtained in rat Bsep vesicles, with only a few exceptions. Note that 32 compounds were ascribed an IC_{50} value of 133μM in human and rat BSEP/Bsep in this plot (depicted as a single dot at these coordinates). An IC_{50} was not actually generated for these compounds because of insufficient activity; however, for illustration purposes of compounds having little or no activity in either the human BSEP or the rat Bsep assays, they were designated with an IC_{50} of the top concentration evaluated (133μM).

hand, about 16% of the compounds had a potency of $\leq$ 25μM, the majority of which are associated with liver injury (Fattinger *et al.*, 2001; Feng *et al.*, 2009; Funk *et al.*, 2001; Iwanaga *et al.*, 2007; Kostrubsky *et al.*, 2003, 2006; McRae *et al.*, 2006; Pauli-Magnus and Meier, 2006; Saito *et al.*, 2009; Sakurai *et al.*, 2007; Snow and Moseley, 2007; Stieger, 2009; Stieger *et al.*, 2000; Trauner and Boyer, 2003; Zollner and Trauner, 2008). Compounds with IC_{50} values between 25 and 100μM accounted for approximately 9% of the benchmark test set. The correlation between BSEP inhibition in this range and clinical liver injury is less convincing, although some of the compounds populating this range of BSEP potency are associated with liver injury in humans, such as in the case of tolcapone, rifabutin, and indomethacin (Benedetti, 1995; Boelsterli *et al.*, 2006; Boelsterli and Lim, 2007; Griffith *et al.*, 1995; Ramachandran and Kakar, 2009; Spahr *et al.*, 2000).

Correlation of Rat Bsep and Human BSEP

Although typical preclinical models for drug safety assessments are not believed to be suitable for evaluating liver injury associated with Bsep interference, animals such as the rat may be useful for investigating topics ranging from target coverage to biomarker validation relating to Bsep function. Thus, we evaluated a set of compounds for their propensity to interfere with either rat Bsep or human BSEP transport in the Sf9 membrane vesicle system described earlier. The results show a relatively high degree of concordance between rat Bsep and human BSEP interference across the 56-compound data set (Fig. 7). This suggests that the rat could be a suitable model for conducting functional studies to better understand pharmacokinetic and pharmacodynamic relationships and to help realize the time course and potency of BSEP interference with the end goal of being able to use drug concentration as a direct predictor of response. These data also show that although most compounds affect human BSEP and rat Bsep with similar potencies, some compounds may have greater potency in one species than the other. This information could be useful in trying to determine a relevant *in vivo* model in which to conduct such functional assessments of Bsep interference. A point of caution when evaluating such *in vivo* models is that interference in bile salt uptake transporters should also be considered.

DISCUSSION

The BSEP vesicle transport assay, as presented here, appears robust and reproducible. The potency range for BSEP interference, as represented by an IC_{50} value derived from a 10-point concentration-response curve, dictated assay variability for given compounds (Table 1 and Fig. 4). These data suggest that the assay can be reliably employed in a high-throughput manner to identify compounds that interfere with BSEP function.

Greater than 200 benchmark compounds, mostly comprised of marketed or withdrawn drugs, were used to assess how well the BSEP vesicle transport assay correlated with liver injury in humans. The majority of drugs (~75%) showed little or no effect on BSEP transport. The vesicle transport assay appears specific as evidenced by the fact that compounds such as acetaminophen (Hinson *et al.*, 2010), zonisamide (Vuppalanchi *et al.*, 2006), carbon tetrachloride (Manibusan *et al.*, 2007), and others with reasonably accepted mechanisms of liver injury,

that are independent from BSEP inhibition, were negative for BSEP interference. A few of the compounds with BSEP IC_{50} values of approximately 30–100μM have been associated with liver injury in humans, such as rifabutin, tolcapone, indomethacin, and fluvastatin (Benedetti, 1995; Boelsterli *et al*., 2006; Boelsterli and Lim, 2007; Griffith *et al*., 1995; Ramachandran and Kakar, 2009; Spahr *et al*., 2000) and (Pharmapendium); however, the role of BSEP inhibition has not been described. It is conceivable that BSEP interference may influence the liver liability associated with these therapeutics, particularly if they can accumulate in liver as has been seen with other compounds (Feng *et al*., 2009; Hamadeh *et al*., 2010).

Of particular interest are the compounds with an IC_{50} value of $\leq$ 25μM. Although non–BSEP mediated mechanisms of liver injury have been proposed for several of these compounds (Fouassier *et al*., 2002; Julie *et al*., 2008; Lee, 2003; Maddrey, 2005)—such as troglitazone and its association with mitochondrial dysfunction (Julie *et al*., 2008)—almost all compounds with this level of potency for BSEP interference are associated with liver injury in humans, and the effect of some of these compounds on BSEP transport has been well described by others (Fattinger *et al*., 2001; Feng *et al*., 2009; Funk *et al*., 2001; Kostrubsky *et al*., 2003, 2006; Leslie *et al*., 2007; McRae *et al*., 2006; Mita *et al*., 2006a; Sakurai *et al*., 2007; Snow and Moseley, 2007; Stieger, 2009). The potential role of BSEP in the liver injury associated with therapies such as thiazoladinediones, protease inhibitors, endothelin antagonists, sulfonylureas, and antibiotics has been previously described (Fattinger *et al*., 2001; Feng *et al*., 2009; Funk *et al*., 2001; Kostrubsky *et al*., 2003; McRae *et al*., 2006; Snow and Moseley, 2007; Stieger, 2009). However, to the best of our knowledge, only one recent publication has implicated BSEP inhibition as a contributor to liver injury associated with a kinase inhibitor (Feng *et al*., 2009). Our data indicate that several kinase inhibitors interfere with BSEP transport with relatively high potencies, including the oncology therapies pazopanib, lapatinib, sorafenib, imatinib, and gefitinib, as well as the research compounds staurosporine and wortmannin. The therapeutic kinase inhibitors shown here are associated with varying degrees of liver injury in humans (Pharmapendium), and we hypothesize that BSEP interference may be one underlying mechanism that contributes to their respective liver liabilities.

The majority of approved drugs on the market were not found to inhibit the function of BSEP, suggesting that natural attrition, because of liver injury during the clinical stages of drug development, might have eliminated drug candidates with this property. In fact, several published reports describe drug-induced BSEP inhibition as either the cause or a likely contributing factor for liver injury observed in late stage clinical trials (Fattinger *et al*., 2001; Feng *et al*., 2009; Funk *et al*., 2001; Kostrubsky *et al*., 2003, 2006; McRae *et al*., 2006; Stieger, 2009). Thus, active evaluation of compounds for their potential to interfere with BSEP function is recommended at the earliest stages of drug development. The membrane vesicle assay can be conducted in an expedited manner to aid medicinal chemists in their iterative compound design efforts. This is especially important because BSEP-associated liabilities in humans often fail to result in a liver injury signal during the preclinical phases of development. This may be because of a variety of reasons ranging from the increased efficiency by which some preclinical models, such as the rat, can metabolize and/or eliminate bile acids from hepatocytes as compared with human (Borst *et al*., 2007; Lee *et al*., 2001; Stieger, 2009; Wang *et al*., 2001, 2009; Zelcer *et al*., 2006) to the fact that rodent bile acid composition is less hydrophobic than humans and therefore less cytotoxic (Fattinger *et al*., 2001; Lundell and Wikvall, 2008; Palmeira and Rolo, 2004; Rolo *et al*., 2003, 2004; Setchell *et al*., 1997), even if it does accumulate. In fact, Abcb11 knockout mice show a relatively mild form of cholestasis, whereas humans with PFIC2 display a severe form of the disease, demonstrating a clear interspecies difference in sensitivity to BSEP interference (Stieger, 2009; Wang *et al*., 2001). The seemingly inability of preclinical species to predict this potential liability further underscores the need for early identification. Circulating bile acids in the blood are rarely included in clinical trial protocols, yet are likely the best hallmark for BSEP dysfunction. By identifying BSEP interference in the early stages of drug development, compounds thus chosen for evaluation in humans could have serum bile acids added to their clinical monitoring protocol, such that treatment regimens could be altered or discontinued as appropriate—such as in the case of bosentan. Early signs of BSEP-mediated hepatotoxicity can manifest in elevated transaminases as a consequence of bile acid–related hepatocyte injury instead of prototypical cholestatic liver enzymes, such as alkaline phosphatase and gamma glutamyl transpeptidase (Marschall *et al*., 2007; Stieger, 2009; Stieger *et al*., 2007). Given that bile acids are not often measured during clinical development, such an early indicator of BSEP-related hepatotoxicity may be missed. This type of clinical information could prove valuable in resolving a mechanism of toxicity in support of backup clinical candidate therapies that might otherwise go unconfirmed. However, changes in circulating bile acid levels could be the result of interference with other transporters, such as NTCP and the organic anion transporting polypeptides, as well as bile acid signaling pathways. These non–BSEP mediated perturbations to bile acid homeostasis should also be considered when interpreting clinical bile acid measures.

Although mutations in BSEP have been associated with liver disease in a univariate manner (Alissa *et al*., 2008; Byrne *et al*., 2009; Davit-Spraul *et al*., 2009; Dixon *et al*., 2009; Kagawa *et al*., 2008; Pauli-Magnus and Meier, 2005; Stieger, 2009; Stieger *et al*., 2007; Strautnieks *et al*., 2008; Trauner and Boyer, 2003), it is not yet fully understood how pharmacological inhibition of BSEP in humans *in vivo* relates to the familial dysfunction of this transporter. The case examples where autoantibodies to BSEP led to posttransplant

liver failure in patients with PFIC2 (Jara *et al.*, 2009; Keitel *et al.*, 2009) offer a glimpse at how complete shutdown of BSEP might manifest when exposed to an unlimited challenge. However, this is an example of extreme pharmacology and not necessarily representative of what occurs with small molecules.

It is not clear whether pharmacological BSEP interference by small molecules has a univariate relationship with a liver injury outcome or whether it constitutes a relatively strong susceptibility factor. The activities of a compound on other related transporters, such as the multidrug resistance–associated proteins (MRPs) MRP2, MRP3, MRP4, and potentially others, may factor into the overall liver injury outcome. Additional susceptibility factors that may contribute to the overall risk associated with pharmacological inhibition of BSEP function in humans are as follows: exposure, route of excretion, metabolism, drug-drug interactions, nuclear receptor activation, formation of reactive metabolites, interference with subcellular organelles such as the mitochondria, as well as other drug-related interactions. For this reason, it is most appropriate to consider BSEP inhibition as a susceptibility factor with the understanding that other toxicological and dispositional drug attributes will ultimately determine the risk for liver liabilities in humans. In fact, some marketed or withdrawn therapeutics are associated with liver injury during phase 1 clinical trials, during which time patient populations are smaller (Fattinger *et al.*, 2001; Feng *et al.*, 2009; Kostrubsky *et al.*, 2003), whereas others fail to show a signal for liver injury until patient populations are much larger, such as in postmarketing (Lee, 2003; Maddrey, 2005). Compounds in both categories, such as the endothelin antagonists with liver liabilities identified in clinical trials (Fattinger *et al.*, 2001; Kostrubsky *et al.*, 2003), versus the thiazoladinediones where liver signals were not appreciated until postmarketing (Funk *et al.*, 2001; Lee, 2003; Maddrey, 2005; Snow and Moseley, 2007) have comparable effects on human BSEP (Table 2 and Fig. 5).

The translation of an *in vitro* potency of a small molecule on BSEP to human risk of liver injury is likely multifactorial. However, in early screening of drug candidates, dosing and exposure data are not often available, so the vesicle transport assay should be used to prioritize compounds with the least amount of BSEP interference as possible to decrease the likelihood of BSEP-mediated liver injury in people versus being used as a "go/no-go" decision tool. A good example cited frequently is bosentan, which is a frontline therapy for PAH. Routine liver monitoring limits the possibility of catastrophic liver failure while reaping the therapeutic benefits of this drug. Because PAH carries with it a grievous prognosis, a BSEP-mediated liver liability is tolerable under such circumstances.

The BSEP vesicle transport assay appears to be a useful tool in evaluating therapeutic candidates for their potential interaction with BSEP function. By evaluating a compendium of marketed or withdrawn drugs for their ability to interfere with BSEP in this assay, we have provided a benchmark against which therapeutic candidates can be compared for their relative risk for clinical liver liabilities. The BSEP membrane vesicle assay has a technical advantage over alternative *in vitro* models in that the transporter-expressing vesicles can be maintained frozen for extended periods and thawed just prior to each use. In contrast, cell-based systems require routine maintenance to ensure viability and consistent performance, and primary cultures have the additional requirement of specialized harvest techniques from the organism of interest. The vesicle system also offers a specific measurement of interference in BSEP function, independent of other transporters, such as the NTCP. However, limitations of this system should be considered. For example, the vesicle transport assay as presented here is not metabolically competent and lacks other subcellular organelles, whereas cell-based systems have this capacity. It is well established that hepatotoxic potential is not always because of parent compounds but may be caused by metabolites formed via hepatic metabolism (Giri *et al.*, 2010). The use of S9 fractions from microsomes may be investigated in the future for use in conjunction with this assay to circumvent this limitation. An understanding of the limitations of this model, such as lack of metabolic capacity, should still help glean meaningful data about the parent compound, and follow-up studies can be performed to evaluate metabolites and their effect, or lack thereof, on BSEP activity. Specific methods for the BSEP vesicle transport assay described in this work do not discriminate competitive substrates from inhibitors; however, these methods can be manipulated to resolve the two. In addition, the relatively poor performance of nonradiolabeled bile acid probes in the vesicle transport system necessitates the use of radioactivity, which may deter some laboratories from using this model.

In the cluster of compounds represented in Figure 5 as having a potency of > 133μM, to the best of our knowledge, only one (flutamide) has been reported by others to interfere with BSEP transport (Iwanaga *et al.*, 2007; Kostrubsky *et al.*, 2007). According to Iwanaga *et al.* (2007), flutamide had an IC_{50} value in the BSEP vesicle transport assay of approximately 50μM; however, only three concentrations of flutamide were used to generate this IC_{50} value (1, 10, and 100μM), and at a concentration of 100μM, the maximum effect of flutamide was approximately 40–45% of controls. The curve-fitting methods employed in our work would likely not derive an IC_{50} value from such a concentration-response relationship, so it is not surprising that flutamide was undefined (IC_{50} > 133μM) in our assay and had a maximum inhibitory effect of approximately 50% of controls at the top concentration (data not shown). Kostrubsky *et al.* (2007) generated an IC_{50} for biliary excretion of ^{3}H-T in human primary hepatocyte cultures of about 75μM. Again, a limited number of concentrations of flutamide were evaluated (10, 25, 50, and 100μM), with a maximum inhibition of around 20% of controls. The 10 and 25μM concentrations had no effect in their model (Kostrubsky *et al.*, 2007). The example of flutamide being negative in our model, yet positive for BSEP

TABLE 2
Clinical Details for Benchmark Compounds Shown to be Potent in the BSEP Vesicle Transport Assay

Compound name	Human BSEP IC_{50} (μM)	Pharmacology	Acute or chronic therapy	Primary route of excretion	Clinical dose levels	Known effects on liver	Comment on clinical relevance of BSEP finding[a]
Pioglitazone	0.4	Diabetes (PPARγ)	Chronic	Biliary	15–60 mg QD	Known association with liver injury, liver monitoring recommended	Low incidence of liver injury
Cyclosporine A	0.9	Transplant rejection	Acute	Biliary	20–600 mg/kg	Associated with drug-induced cholestasis	Likely
Valinomycin	1.6	Research substance produced by streptomyces bacteria	Not applicable	Not applicable	Not applicable	*In vitro* BSEP interference has been shown by others	None
Ritonavir	2.2	Protease inhibitor for HIV	Chronic	Biliary	600 mg Q12 h	Known association with liver injury	Low incidence of liver injury
Ketoconazole	3.4	Antifungal	Acute	Biliary	200 mg QD (oral)	10–15% elevated liver enzymes; liver monitoring recommended	Likely
MK-571	3.5	Inflammation	Not found	Not found	Not found	Not found	None
Telithromycin	4.0	Ketolide antibiotic	Acute	Biliary	400 mg BID for 5–10 days	Known association with liver injury	Likely
Rosiglitazone	4.4	Diabetes (PPARγ)	Chronic	Biliary	2–8 mg QD	Known association with liver injury, liver monitoring recommended	Low incidence of liver injury; typically dosed at low levels
Saquinavir	4.9	Protease inhibitor for HIV	Chronic	Biliary	400–1200 mg TID	Known association with liver injury	Low incidence of liver injury
Troglitazone	5.9	Diabetes (PPARα and γ)	Chronic	Biliary	200–600 mg QD	Withdrawn from market because of liver injury	Likely
Glyburide	6.1	Diabetes (sulfonylurea)	Chronic	Biliary/urinary (50/50)	1.25–25 mg QD	Associated with drug-induced cholestasis	Low incidence of liver injury
Nefazodone	6.1	Antidepressant	Chronic	Urinary	300–600 mg QD	Known association with liver injury; sales discontinued in Canada in 2003	Likely
Lapatinib	6.5	Oncology (HER2 kinase inhibitor)	Acute	Biliary	1250 mg QD	Black box warning of severe and fatal hepatotoxicity	Likely
Nicardipine	7.9	Hypertension (Ca++ channel blocker)	Chronic	Biliary/urinary (50/50)	60–120 mg QD	Known association with liver injury	Likely
Sorafenib	8.0	Oncology (multikinase inhibitor)	Acute/chronic	Biliary	400 mg BID	Known association with liver injury; tansient liver enzyme elevations are common (1–10% of patients)	Likely

TABLE 2—*Continued*

Compound name	Human BSEP IC_{50} (μM)	Pharmacology	Acute or chronic therapy	Primary route of excretion	Clinical dose levels	Known effects on liver	Comment on clinical relevance of BSEP finding[a]
Reserpine	8.4	Antihypertensive and antipsychotic	Chronic	Biliary	0.1–1.0 mg QD (though doses up to 40 mg QD have been used)	No convincing evidence for liver injury	Rarely prescribed
Fusidic acid	10.1	Gram-positive antibiotic	Acute	Biliary	1500 mg QD (osteomyelitis)	Known association with liver injury; not sold within the US	Likely
Pazopanib	10.3	Oncology (multityrosine kinase inhibitor)	Acute/chronic	Biliary	200–800 mg QD	Black box warning of severe and fatal hepatotoxicity	Likely
Gefitinib	10.9	Oncology (tyrosine kinase inhibitor)	Acute/chronic	Biliary	250–500 mg QD	Known association with liver injury, liver monitoring recommended	Likely
Nelfinavir	11.8	Protease inhibitor	Chronic	Biliary	750 mg TID to 1250 mg BID	Known association with liver injury	Low incidence of liver injury
Clofazimine	12.9	Anti-*Mycobacterium leprae* (lepromatous leprosy)	Acute/chronic	Biliary	100 mg QD	No convincing evidence for liver injury	Therapeutic effect on lepromatous leprosy-induced liver injury appears more significant than liver injury because of drug alone
Erythromycin estolate	13.0	Macrolide antibiotic	Acute	Biliary	400 mg Q6 h (up to 4 g/day) for up to 15 days	Black box warning of cholestatic liver injury	Likely
Wortmannin	13.6	Kinase inhibitor (research substance)	Not applicable	Not applicable	Not applicable	Not applicable	None
17α ethinyl estradiol	14.0	Birth control (synthetic estrogen)	Chronic	Biliary/urinary (50/50)	0.025 mg QD	Associated with drug-induced cholestasis	Likely; typically dosed at low levels
Taxol	15.0	Tubulin polymerizer	Acute/subchronic	Biliary	135–175 mg/m^2 infusions for 3 or 24 h	Known association with liver injury	Likely
Fenofibrate	15.3	LDL cholesterol lowering (PPARα)	Chronic	Urinary	43–130 mg QD	Known association with liver injury, liver monitoring recommended	Likely
Cinnarizine	15.7	Motion sickness, anti-emetic	Acute/chronic	Urinary	10–20 mg QD	Associated with drug-induced cholestasis	Likely
Glimepiride	15.7	Diabetes (sulfonylurea)	Chronic	Urinary	1–4 mg QD	Associated with drug-induced cholestasis	Likely
Telmisartan	16.2	Hypertension (angiotensin II antagonist)	Chronic	Biliary	20–80 mg QD	No convincing evidence for liver injury	Though rare, liver dysfunction associated with Micardis HCT has been reported in AERS

TABLE 2—*Continued*

Compound name	Human BSEP IC_{50} (μM)	Pharmacology	Acute or chronic therapy	Primary route of excretion	Clinical dose levels	Known effects on liver	Comment on clinical relevance of BSEP finding[a]
Lopinavir	17.3	Protease inhibitor	Chronic	Biliary	400–800 mg QD (formulated with ritonavir, sold as Kaletra)	Known association with liver injury	Low incidence of liver injury
Benzbromarone	17.5	Antigout	Chronic	Biliary	100–200 mg QD	Known association with liver injury; withdrawn from market in 2003	Likely
Itraconazole	18.0	antifungal	Acute	Biliary	200 mg QD for 1–2 weeks	Known association with liver injury; liver monitoring recommended if taking itraconazole > 1 month	Likely
Staurosporine	18.7	Kinase inhibitor (research substance)	Not applicable	Not applicable	Not applicable	Model inducer of apoptosis	None
Indinavir	21.2	Protease inhibitor	Chronic	Biliary	800 mg Q8 h	Known association with liver injury	Low incidence of liver injury
Bosentan	22.0	PAH (endothelin antagonist)	Chronic	Biliary	62.5–125 mg BID	Associated with drug-induced cholestasis	Likely
Simvastatin	24.7	LDL cholesterol lowering (HMG CoA reductase inhibitor)	Chronic	Biliary	5–80 mg QD	Known association with liver injury; liver monitoring recommended	Likely
Imatinib	25.1	Oncology (tyrosine kinase inhibitor)	Acute/chronic	Biliary	400–600 mg QD	3–6% have severe ALT/AST or bilirubin elevations; liver monitoring recommended	Likely
Rifampicin	25.3	Antibiotic	Acute	Biliary	150–600 mg QD	Known association with liver injury; liver monitoring recommended for some patients	Likely
Rifabutin	26.7	Antimycobacterial (inhibitors DNA-dependent RNA polymerase in bacteria)	Acute	Urinary	300–900 mg QD	Known association with liver injury	Likely
Oxybutynin	27.4	Incontinence; over active bladder (anticholinergic)	Chronic	Biliary	5–30 mg QD	No convincing evidence of liver injury	Typically dosed at low levels
Finasteride	28.2	Benign prostate hyperplasia and alopecia (antiandrogen, 5α reductase inhibitor)	Chronic	Biliary	1 mg QD (alopecia); 5 mg QD (benign prostate hyperplasia)	No convincing evidence of liver injury	Typically dosed at low levels
Primaquine	32.7	antiprotozoal (vivax malaria)	Acute	Biliary	15 mg QD for 14 days	Known association with liver injury	Acute therapy typically dosed at low levels
Tolcapone	34.5	Parkinson's disease	Chronic	Urinary	100–200 mg TID	Known association with liver injury	Likely

TABLE 2—*Continued*

Compound name	Human BSEP IC_{50} (μM)	Pharmacology	Acute or chronic therapy	Primary route of excretion	Clinical dose levels	Known effects on liver	Comment on clinical relevance of BSEP finding[a]
Flupirtine	35.5	Nonnarcotic analgesic	Acute/chronic	Biliary	100–600 mg QD	Known association with liver injury	Likely
Fluvastatin	36.1	Hyperlipidemia (HMG-CoA reductase inhibitor)	Chronic	Biliary	20–80 mg QD	Known association with liver injury; liver monitoring recommended for some patients	Likely
Drotaverine	37.0	Antispasmodic (inhibits PDE4)	Acute	Biliary/urinary (50/50)	40–80 mg TID oral	No convincing evidence of liver injury	Not enough information to comment
Ciglitazone	37.8	Research substance (PPARγ agonist)	Chronic	Biliary	Not applicable	Never marketed	None
Midazolam	41.7	Benzodiazepine (CNS depressant)	Acute	Urinary	0.01–0.04 mg/kg	No convincing evidence of liver injury	Acute therapy typically dosed at low levels
Indomethacin	42.0	NSAID	Acute/chronic	Urinary	25–50 mg TID	Known association with liver injury	Likely

Note. A table of all benchmark compounds presented here that potently interfered with BSEP function ($IC_{50} \leq 25\mu M$) and a few compounds with IC_{50} values near 25μM. Most compounds with IC_{50} values in the 25μM range are associated with drug-induced liver injury in humans. Although it is not certain what role BSEP plays in the hepatotoxicity associated with many of these compounds, the correlation between *in vitro* BSEP potency and liver injury indicates that BSEP is a possible contributor or susceptibility factor. Also included in the table is information on therapy duration, primary route of excretion, and typical dose regimens—important factors to consider when predicting risk in humans. All clinical information were collated from pharmapendium and/or individual product labels or inserts. AERS, adverse events reporting system; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BID, two times a day; CNS, central nervous system; HCT, hydrochlorothiazide; HIV, human immunodeficiency virus; LDL, low-density lipoprotein; NSAID, non-steroidal anti-inflammatory; PPAR, peroxisome proliferator-activated receptor; QD, once a day; Q6h, once every 6 hours; Q12h, once every 12 hours; TID, three times a day.

[a]Comments on the clinical relevance of the BSEP finding are subjective.

interference in the hands of others, demonstrates the importance of study design and interpretation. Whereas we have chosen to only report IC_{50} values as a measure of a compound's potency, there may be value in using the maximum inhibitory effect of a compound at the top concentration.

In this study, we have presented a relatively extensive survey of the potential for marketed and withdrawn drugs to inhibit BSEP function. Based on these data, we show evidence that BSEP inhibition may be associated with unforeseen liver injury liabilities during the clinical phases of drug development. In addition, we generated *in vitro* rat Bsep data (using Sf9 membrane vesicles) that suggest the rat may be a suitable model for evaluating the pharmacokinetic/pharmacodynamic (PK/PD) relationship for *in vivo* Bsep inhibition because in general compounds that interfere with human BSEP appear to do so in the rat. The importance of having such a model is to better understand toxicokinetic parameters that can influence the translation of BSEP IC_{50} values to *in vivo* risk in humans. However, examples of compounds showing potent BSEP interference with limited rat Bsep potency were identified (Fig. 7), suggesting that there may be value in evaluating rat Bsep interference *in vitro* prior to pursuing the rat as a PK/PD model. One should also consider a compound's effect on rat Ntcp when evaluating such *in vivo* models as the measurement of bile acid elevations in blood following exposure to test compound (Fattinger *et al*., 2001; Kostrubsky et al., 2003, 2006) or biliary excretion of taurocholate in a bile duct-cannulated rat (Akashi *et al*., 2006; Iwanaga *et al*., 2007) because interference in Ntcp could complicate data interpretation.

In summary, BSEP interference can be reliably measured in membrane vesicles harvested from transfected Sf9 insect cells, and with the evaluation of greater than 200 marketed or withdrawn drugs, there appears to be a strong correlation between potency for BSEP interference (expressed in IC_{50} values) and liver injury in humans. Although arbitrary in lieu of exposure data, compounds with an IC_{50} value for BSEP interference of $\leq 25\mu M$ showed the strongest correlation with liver liabilities in humans. For the purposes of early screening, binning compounds based on their relative potency could help limit the possibility of BSEP-mediated liver liabilities in humans. These data suggest that compounds with an IC_{50} of $\leq 25\mu M$ should be categorized as of high risk, of medium risk are compounds with IC_{50} values of ~26–133μM, and of low risk are compounds for which IC_{50} values could not be derived because of insufficient BSEP interference. Of course, the best liability assessments will include exposure levels in humans, will account for metabolism, and will integrate other risk factors. But as an early screen, this assay provides a means of addressing

what could be a significant susceptibility factor in humans that might otherwise go undetected during preclinical development.

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