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A Multifactorial Approach to Hepatobiliary Transporter Assessment Enables Improved Therapeutic Compound Development

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The bile salt export pump (BSEP) is expressed at the canalicular domain of hepatocytes, where it serves as the primary route of elimination for monovalent bile acids (BAs) into the bile canaliculi. The most compelling evidence linking dysfunction in BA transport with liver injury in humans is found with carriers of mutations that render BSEP nonfunctional. Based on mounting evidence, there appears to be a strong association between drug-induced BSEP interference and liver injury in humans; however, causality has not been established. For this reason, drug-induced BSEP interference is best considered a susceptibility factor for liver injury as other host- or drug-related properties may contribute to the development of hepatotoxicity. To better understand the association between BSEP interference and liver injury in humans, over 600 marketed or withdrawn drugs were evaluated in BSEP expressing membrane vesicles. The example of a compound that failed during phase 1 human trials is also described, AMG 009. AMG 009 showed evidence of liver injury in humans that was not predicted by preclinical safety studies, and BSEP inhibition was implicated. For 109 of the drugs with some effect on *in vitro* BSEP function, clinical use, associations with hepatotoxicity, pharmacokinetic data, and other information were annotated. A steady state concentration (C_{ss}) for each of these annotated drugs was estimated, and a ratio between this value and measured IC_{50} potency values were calculated in an attempt to relate exposure to *in vitro* potencies. When factoring for exposure, 95% of the annotated compounds with a C_{ss}/BSEP IC_{50} ratio ≥ 0.1 were associated with some form of liver injury. We then investigated the relationship between clinical evidence of liver injury and effects to multidrug resistance-associated proteins (MRPs) believed to play a role in BA homeostasis. The effect of 600+ drugs on MRP2, MRP3, and MRP4 function was also evaluated in membrane vesicle assays. Drugs with a C_{ss}/BSEP IC_{50} ratio ≥ 0.1 and a C_{ss}/MRP IC_{50} ratio ≥ 0.1 had almost a 100% correlation with some evidence of liver injury in humans. These data suggest that integration of exposure data, and knowledge of an effect to not only BSEP but also one or more of the MRPs, is a useful tool for informing the potential for liver injury due to altered BA transport.

Key Words: **bile salt export pump; bile acid; transporter; ATP-binding cassette transporter; multidrug resistance-associated protein; drug-induced liver injury.**

Drug-induced liver injury (DILI) is a major concern for the pharmaceutical industry, accounting for clinical drug failures, market withdrawals, as well as black box warnings (Olson *et al.*, 2000; Stine and Lewis, 2011). Such late stage attrition or use limitations due to liver injury is a clear sign that traditional preclinical models can be poor predictors of human DILI. Indeed, the efforts of a consortium comprised of several pharmaceutical companies published by Olson *et al.* (2000) evaluated the concordance between preclinical animal safety data and human clinical outcomes for 150 development compounds and found that hepatotoxicity (< 60% concordance) and cutaneous/hypersensitivity reactions (< 40% concordance) were the 2 poorest predicted adverse events (Olson *et al.*, 2000). Hepatotoxicity was responsible for the second highest termination rate for these compounds (Olson *et al.*, 2000). As they pointed out, there is a need to discover the underlying mechanisms for DILI in order to develop tools (eg, *in vitro*, *ex vivo*, or other models) to better predict hepatotoxicity in humans (Olson *et al.*, 2000). The often low incidence of DILI seen during the postmarketing phase of drug development adds to the challenge of identifying the underlying mechanisms of hepatotoxicity, compounded by a diverse patient population exposed to almost innumerable confounding variables (Stine and Lewis, 2011). Several reviews have focused on DILI and the possible contributing mechanisms, such as reactive metabolite formation and covalent binding, immune-mediated toxicity, mitochondria toxicity in its various forms, and hepatobiliary transporter inhibition (Amacher, 2012; Corsini *et al.*, 2012; Daly, 2010; Kubitz *et al.*, 2012; Stepan *et al.*, 2011; Tujios and Fontana, 2011). In the case of hepatobiliary transporter inhibition, rodents have

been demonstrated to be an unreliable model for hepatotoxicity due to inhibition of bile salt export pump (Bsep) function (Fattinger *et al.*, 2001; Feng *et al.*, 2009; Kostrubsky *et al.*, 2003, 2006). An additional example is provided in the present work—AMG 009. This compound showed no evidence of liver injury in rats, mice, hamsters, rabbits, or nonhuman primates; however, 5 of 8 healthy volunteers showed significant elevations in transaminases that returned to normal upon cessation of AMG 009 administration (data not shown). And, the exposures in blood achieved in the preclinical animal models were greater than those observed in humans. AMG 009 was subsequently discovered to interact with multiple hepatocellular transporters, including BSEP. Experience with this compound adds to the examples published by others, demonstrating how BSEP inhibition can be a liability for DILI that goes undetected during preclinical testing.

Although an essential component of bile, bile acids (BAs) are detergent-like molecules capable of damaging cellular membranes and organelles (Lundell and Wikvall, 2008; Palmeira and Rolo, 2004; Rolo *et al.*, 2004). To mitigate potential cytotoxicity, BA homeostasis is tightly regulated by metabolic, excretion, absorption, and feedback mechanisms to limit their intracellular accumulation (Trauner and Boyer, 2003). Several works describe in detail these processes and their relationship to health and disease (Stieger, 2010; Stieger *et al.*, 2000; Trauner and Boyer, 2003). An understanding of the association between BSEP dysfunction and liver injury through genetic mutations is what led to the hypothesis that some forms of DILI may be due to a BSEP-mediated mechanism (Fattinger *et al.*, 2001). Indeed, several drugs or drug candidates have been associated with liver injury in humans and with BSEP interference implicated as a possible contributing factor (Fattinger *et al.*, 2001; Feng *et al.*, 2009; Kostrubsky *et al.*, 2003, 2006; Morgan *et al.*, 2010). However, it is challenging to ascribe sole causality to BSEP interference given the possibility of other drug- or host-mediated mechanisms.

BSEP is an ATP-dependent transporter that manages the excretion of monovalent BAs into the bile canaliculi, with greater affinity for amidated (amino acids glycine or taurine) BAs (Stieger *et al.*, 2007). At physiological pH, BAs exist in their salt form; however, they are referred to as BAs throughout this work (Lundell and Wikvall, 2008). BAs are efficiently recycled through either enterohepatic circulation (the major pathway) or the chol-hepatic shunt pathway, where approximately 95% of the human BA pool is recycled daily (Lundell and Wikvall, 2008). BAs are reclaimed into blood through either the intestines (enterohepatic circulation) or cholangiocytes (chol-hepatic shunt) and then make their way back to the liver. Hepatic uptake transporters, predominantly the sodium-dependent taurocholate cotransporting polypeptide, then complete the BA recycling process. Under conditions such as progressive familial intrahepatic cholestasis type 2 or BSEP deficiency, the hepatocyte loses its primary route of BA excretion into the bile canaliculi and results in the hepatocellular accumulation of BAs up to cytotoxic concentrations (Palmeira and Rolo, 2004; Rolo *et al.*, 2003, 2004). Under such cholestatic conditions, the multidrug resistance-associated proteins-2, -3, and -4 (MRP2/*ABCC2*, MRP3/*ABCC3*, and MRP4/*ABCC4*) are described as "emergency safety valves" to help manage the canalicular (MRP2) or basolateral (MRP3 and MRP4) elimination of BAs (Keppler, 2011a,b). An illustration of this is provided in Figure 1.

MRP2 is responsible for the canalicular secretion of phase 2 conjugated BAs. Unlike BSEP, MRP2 has a long list of substrates aside from BAs, including various glucuronidated or sulfated drugs and/or their metabolites, and endogenous compounds such as conjugated bilirubin and leukotriene C4 (LTC4) (Nies and Keppler, 2007; Zhou *et al.*, 2008). In the case of conjugated bilirubin, genetic mutations in ABCC2 that render MRP2 dysfunctional are the cause of Dubin-Johnson syndrome, which presents as jaundice due to hyperbilirubinemia (Nies and Keppler, 2007). At the basolateral domain, MRP3 is primarily responsible for the elimination of glucuronidated endogenous and xenobiotic compounds. An important substrate for MRP3 is conjugated bilirubin, allowing for its elimination into the blood. In fact, increased MRP3 expression has been observed in individuals with Dubin-Johnson syndrome (Keppler, 2011b). Significant interspecies differences have been observed between rodent Mrp3 transport of BAs and human MRP3. Rodent Mrp3 transports BAs with high affinity, but human MRP3 with relatively low affinity (Keppler, 2011b). The other basolateral efflux pump capable of transporting BAs is MRP4, however, only in the presence of glutathione (GSH) (Keppler, 2011b). Other substrates of MRP4 include cyclic nucleotides, such as 3′-5′-cyclic adenosine monophosphate, leukotrienes, prostaglandins, and many others (Keppler, 2011b). Similar to MRP4 BA transport, several of its other substrates require GSH cotransport. MRP3 and MRP4 are likely less involved in the elimination of BAs under normal, homeostatic conditions but play an important compensatory role during cholestasis.

In a survey of over 200 benchmark drugs using the human BSEP membrane vesicle assay, the majority of compounds with an IC_{50} value of < 25μM were associated with liver injury in humans (Morgan *et al.*, 2010). Good concordance was shown in a subsequent study, also using BSEP membrane vesicles (Dawson *et al.*, 2012). These works, and others, support that deployment of an early screening paradigm around BSEP may add to a weight-of-evidence risk assessment for hepatotoxicity. However, some compounds were identified in Morgan *et al.* (2010) or Dawson *et al.* (2012) as BSEP inhibitors with no convincing evidence of liver injury. Further, the assays could not discriminate severity or frequency of DILI. It was therefore hypothesized that knowledge of a compound's effect on the other major BA efflux transporters (MRP2, MRP3, and MRP4), in addition to BSEP, may help further discriminate compounds and limit false positives (compounds with no convincing evidence of DILI). In addition to the 200+ benchmark compounds reported in Morgan *et al.* (2010), more than 400

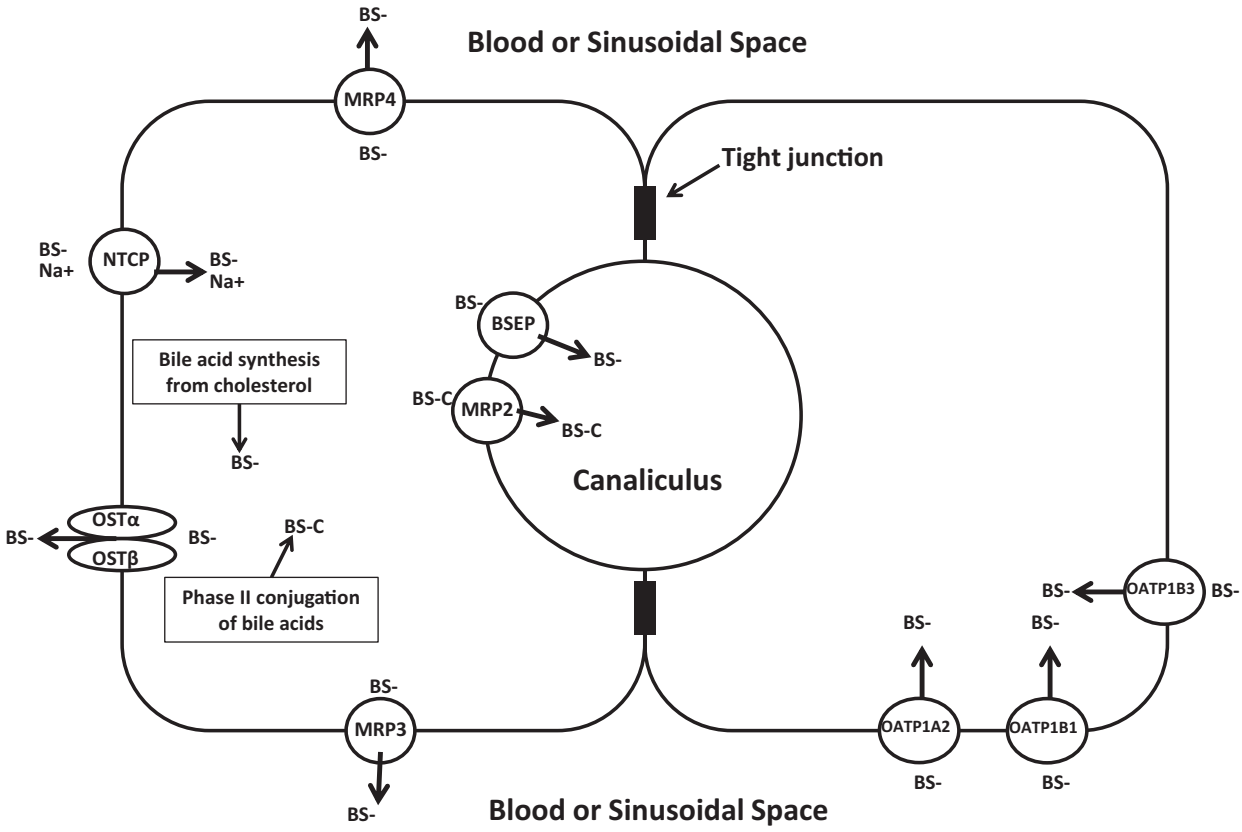

FIG. 1. Illustration of the hepatocellular transporters predominantly involved in bile acid (BA) homeostasis. BAs are synthesized from cholesterol within the hepatocyte. Additional processing of the BAs include conjugation to the amino acids glycine or taurine (amino acid conjugated and unconjugated BAs depicted as BS−). Other reactions may render BAs as phase 2 metabolized species—such as glucuronidated or sulfated BAs (represented as BS-C). BAs are then transported into the bile canaliculi via BSEP, or if a BS-C species, they are transported by MRP2. Under cholestatic conditions, MRP3 and MRP4 play a compensatory role in managing the elimination of BAs into the blood, whereby limiting the hepatocelllar accumulation of BAs. NTCP is the primary route by which hepatocytes reclaim BAs from blood; however, the organic anion-transporting polypeptide (OATPs) do represent a non-sodium-dependent means for BA uptake. The organic solute transporter subunits α and β (OSTα and OSTβ) also play a role in the basolateral elimination of BAs; however, less is known about their function within hepatocytes. Not all transporters or substrates are depicted in this figure. Abbreviations: BSEP, bile salt export pump; MRP, multidrug resistance-associated protein; NTCP, Sodium-dependent taurocholate cotransporting polypeptide; OST, organic solute transporter.

additional drugs were evaluated in the human BSEP membrane vesicle assay and similar human MRP2, MRP3, and MRP4 assays. Potency values for over 600 benchmark drugs were generated for the 4 transporters in the form of IC_{50} values. Where available through such resources as Pharmapendium (www.pharmapendium.com), the LiverTox database (www.livertox.nih.gov), literature searches via PubMed (www.ncbi.nlm.nih.gov/pubmed), and/or product labels, toxicology, pharmacology, pharmacokinetic, and other information are provided for 109 drugs, focused mainly on those with an effect on BSEP transport. The Dawson *et al.* (2012) work attempts to move beyond the use of a cutoff potency value to identify potentially hazardous compounds based on *in vitro* BSEP findings by incorporating *in vivo* exposure data into the assessment. To expand on their effort, *in vitro* potency values for the 109 drugs with annotated toxicology and pharmacokinetic data were related to exposure to provide a better extrapolation to human outcome.

The overall objectives of this work are (1) to demonstrate the utility of evaluating BSEP and the MRPs by surveying the effects of a large number of marketed or withdrawn drugs in functional *in vitro* transporter assays; (2) to demonstrate the utility of incorporating blood exposure data into a risk assessment for transporter inhibition by curating relevant information on a subset of the marketed or withdrawn drugs; and (3) to provide a recommendation on how to deploy a transporter panel to improve therapeutic compound development.

MATERIALS AND METHODS

Materials. Inverted membrane vesicles harvested from Sf9 insect cells overexpressing human BSEP, MRP2, MRP3, or MRP4 (catalog numbers GM0005, GM0001, GM0021, or GM0012, respectively) were manufactured by Genomembrane (Kanagawa, Japan) and purchased through Life Technologies (Grand Island, New York). Radioactive substrates for the membrane vesicle assays ^{3}H-taurocholate (^{3}H-T) for BSEP or ^{3}H-estradiol-17β-D-glucuronide

(^{3}H-$E_2$17βG) for the MRPs were purchased from Perkin Elmer (Waltham, Massachusetts). All other reagents and buffers for the membrane vesicle assays were of the highest grade possible and were exactly as described in van Staden *et al.* (2012). Where available, 635 test articles (mostly comprised of marketed or withdrawn drugs) were purchased through Sigma (St Louis, Missouri), Biomol (Plymouth Meeting, Pennsylvania), and Sequoia Research Products (Pangbourne, United Kingdom). AMG 009 was synthesized at Amgen Inc (Newbury Park, California). Some test articles were selected based on previous reports of BSEP inhibition; however, the majority of test articles were acquired based solely on their availability, with no knowledge of their potential effect on BSEP, MRP2, MRP3, or MRP4. 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.

Membrane vesicle transport assay. The authors of the present work recently published a detailed protocol for BSEP, MRP2, MRP3, and MRP4 membrane vesicle assays in *Current Protocols in Toxicology* (van Staden *et al.*, 2012). The methods and data analyses performed in the present work were exactly as described in van Staden *et al.* (2012). The van Staden *et al.* publication also provides a detailed overview of the assays, diagrams of the workflow, and helpful illustrations. Briefly, plasma membrane vesicles expressing human BSEP, MRP2, MRP3, or MRP4 were incubated with a radiolabeled substrate (^{3}H-T for BSEP or ^{3}H-$E_2$17βG for the MRP assays) in the presence or absence of 4mM ATP. The absence of ATP served as the negative control, and resulting radioactivity when exposed to vehicle alone (1.3% DMSO) was considered background or noise. The with-ATP controls and 1.3% DMSO represented true signal. For the MRP2 and MRP3 assays, 2mM GSH was also added to the reaction. The BSEP assay was performed at room temperature, with an incubation time of 15–20 min. The MRP2, MRP3, and MRP4 assays were performed at 37°C. The incubation time for MRP2 and MRP4 was 20 min, and for MRP3, it was 10 min. All test articles were evaluated at 10 concentrations, in 1/3 increments, spanning 0–133μM. Nonlinear regression analysis was performed, and IC_{50} values generated as an estimate of potency as described elsewhere (Morgan *et al.*, 2010; van Staden *et al.*, 2012).

Annotation of select drugs. One hundred and nine of the benchmark drugs evaluated in this work were annotated for their known association with hepatotoxicity, pharmacokinetic data in the form of area under the concentration versus time curve (AUC), indication/pharmacology, route of excretion, dose levels and frequencies, as well as other information to explore the relationship between *in vitro* transporter effects and evidence of liver injury in humans. An additional 21 compounds have partial annotations. The basis for selecting compounds to annotate was *in vitro* evidence of some level of BSEP inhibition in the present test system. Annotations were collated from the literature, product labels, the LiverTox database (http://livertox.nih.gov/), and/or Pharmapendium version 2.5–2.7 (database version 2010.1–2012.6) (Elsevier Properties, SA; New York, New York), which included Mosby's Drug Consult and Meyler's Side Effect of Drugs. Where available, links to the LiverTox database are provided. For the purposes of this work, the definition for convincing evidence of liver injury is the following: black box warning, one or more literature references to liver injury in humans, evidence of liver injury was summarized in the LiverTox database, and/or mention of liver injury in Mosby's Drug Consult or Meyler's Side Effect of Drugs. The AUC parameter was annotated from Pharmapendium, drug labels, and/or the literature. The top clinically relevant dose used to derive AUC data was selected, when possible. In some instances, median values were estimated. Where annotated, AUC data were divided by dose interval to estimate a concentration at steady state (C_{ss}). C_{ss} values were compared with transporter IC_{50} potency values by calculating the ratio of C_{ss}/transporter IC_{50} as a means of relating exposure to *in vitro* potencies.

Liver and plasma exposure of AMG 009 in rats. Male Sprague Dawley rats, 10–11 weeks of age (weight appropriate to age), were acquired from Charles River Laboratories (Wilmington, Massachusetts). All animals were cared for in accordance to the *Guide for the Care and Use of Laboratory Animals, 8th Edition* (National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research (U.S.), National Academies Press (U.S.), 2011). Animals were group housed (3 per cage) at an AAALAC, Intl-accredited facility in nonsterile ventilated microisolator housing with corn cob bedding. All research protocols were approved by the Institutional Animal Care and Use Committee. Animals had *ad libitum* access to pelleted feed and water (reverse osmosis purified) via an automatic watering system. Animals were maintained on a 12:12-h light:dark cycle in rooms with controlled temperature and humidity and had access to enrichment opportunities.

Three rats were assigned to each of 8 groups, for a total of 24 animals. Animals either received a single PO administration (gavage) of vehicle alone (water, pH 9.0 with NaOH) or 1500 mg/kg of AMG 009. The vehicle group was euthanized approximately 0.5 h postadministration, at which time blood was collected in lithium heparin tubes and processed for plasma, and the livers frozen in liquid nitrogen for determination of AMG 009 concentrations. The remaining 21 animals received 1500 mg/kg AMG 009 and were euthanized at 0.5, 1, 2, 4, 6, 24, or 48 h postdose, with plasma and liver collected as described above. Liquid chromatography/tandem mass spectrometry using electrospray ionization and multiple reaction monitoring in the positive ion mode was used for the bioanalysis of rat plasma and liver samples. The lower limit of quantitation for AMG 009 was 0.5 ng/ml for plasma and 20 ng/g for liver (Watson, Non-GLP PROD, version 7.0.0.1, Thermo Electron Corporation).

RESULTS

Over 600 drugs were selected for evaluation in a membrane vesicle transporter panel, IC_{50} values were generated, and clinical information was annotated for a subset of these drugs to assess the predictivity of liver injury outcome. IC_{50} values for the annotated compounds were normalized to C_{ss} to determine whether or not an appreciation for exposure further improved the prediction of liver injury outcome.

As seen in Figure 2, most of the 635 benchmark compounds evaluated in the BSEP membrane vesicle assay had little or no effect. This result is similar to what was published earlier

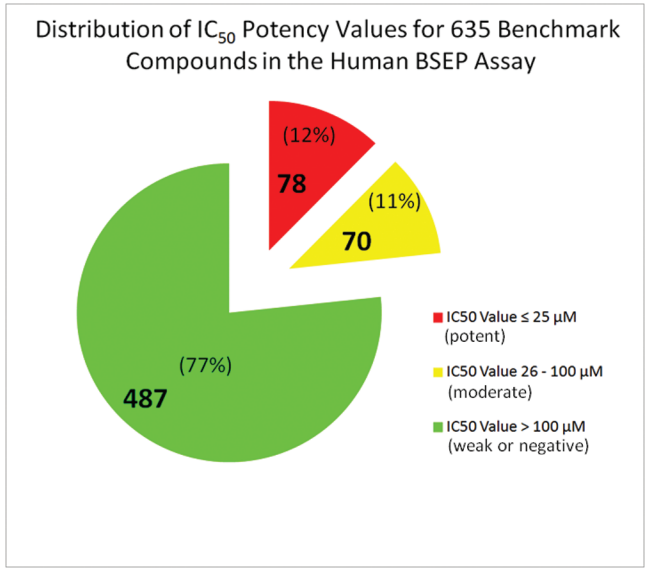

FIG. 2. A pie chart illustrates the percentage of compounds binned as either potent, moderate, or negative for 635 compounds evaluated in the human bile salt export pump (BSEP) assay. The majority of marketed drugs were negative for BSEP.

following evaluation of approximately 200 benchmark compounds, where 75% had little or no effect, 9% had an IC_{50} value 26–100μM, and 16% of the compounds had an IC_{50} value ≤ 25μM (Morgan *et al.*, 2010). All of the compounds included in the previous publication are included in the present 635 compound data set.

Of the 635 compounds evaluated for BSEP activity, 623 were evaluated for activity in the MRP2, MRP3, and MRP4 assays. Figure 3 is the hierarchical cluster analysis of IC_{50} values derived from all 623 compounds in the BSEP, MRP2, MRP3, and MRP4 assays. This illustration clearly shows how compounds can be distinguished into 3 major bins: no effect on all 4 transporters (the largest bin), an effect on one of the transporters, and an effect on more than one transporter. In Figure 3, the white cells in the MRP2 column indicate compounds that stimulated MRP2 transport of the reporter substrate, or stimulated at lower concentrations then inhibited at higher concentrations. It is known that transport of the MRP2 reporter substrate, $E_2$17βG, can be stimulated in the presence of some compounds, stimulated at lower concentrations but inhibited at higher concentrations for other compounds, and just inhibited by yet others (Nies and Keppler, 2007; Pedersen *et al.*, 2008; Zhou *et al.*, 2008). These phenomena are likely due to multiple binding sites on MRP2 that contribute to differential transport kinetics (Gerk *et al.*, 2004). There is substantial overlap and good concordance between compounds presented in this data set and those reported by others in a survey of over 200 drugs on MRP2 transport, with few exceptions (Pedersen *et al.*, 2008). However, 2 compounds described as classic MRP2 inhibitors (benzbromarone and MK-571) were less potent in the MRP2 assay as presented here (Pedersen *et al.*, 2008). These 2 compounds were evaluated several times, and the results varied little. Either these compounds are not as potent MRP2 inhibitors as has been described previously, or differences in experimental conditions may explain the different results. For example, GSH in the assay system can alter the effect of MK-571 on MRP2 transport (Letourneau *et al.*, 2005). Only 3 of the 623 compounds evaluated in the transporter panel

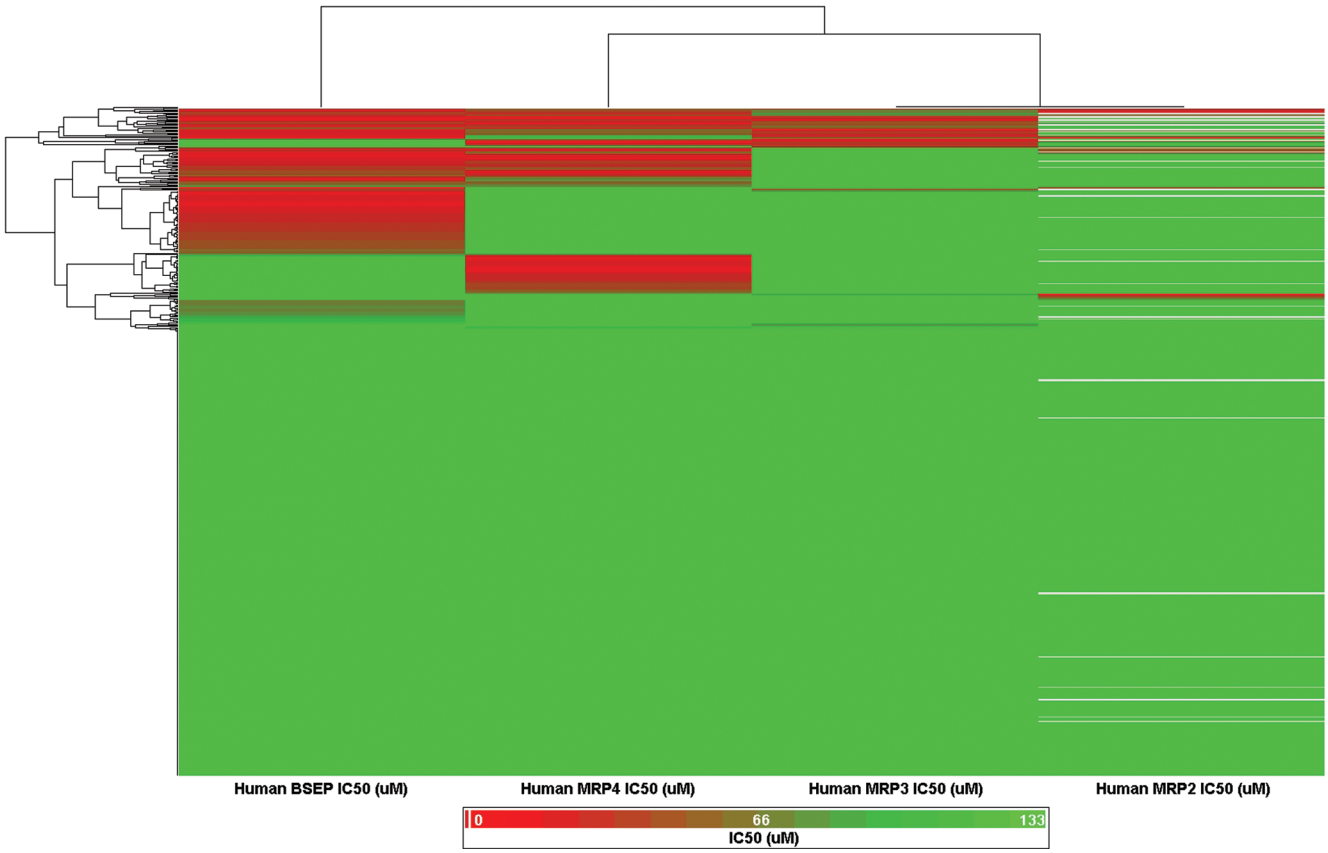

FIG. 3. Hierarchical cluster analysis (Euclidean row and column dissimilarity, average linkage row and column methods, agglomerative clustering method) performed in Partek Discovery Suite 6.4. The more potent a compound inhibits a transporter, the more red the cell, and therefore the lower the IC_{50} value. The less potent a compound inhibits a transporter, the more green the cell, and therefore the higher the IC_{50} value. This figure illustrates the effect of 623 compounds on all 4 transporters. Gray areas in the MRP2 column indicate that the compound either stimulated or stimulated then inhibited MRP2 transport of $E_2$17βG. Abbreviations: BSEP, bile salt export pump; MRP, multidrug resistance-associated protein.

appeared to be relatively selective MRP2 inhibitors of $E_2$17βG transport (ethacrynic acid, daptomycin, and suramin), with IC_{50} values < 50μM.

The proximity of compounds to one another in Figure 3 indicates similar patterns of effect across the transporter panel (with some bias due to absent MRP2 values). Compounds with similar pharmacological mechanisms of action tended to cluster similarly in Figure 3. For example, kinase inhibitors tended to affect BSEP and MRP4 function, whereas peroxisome proliferator-activated receptor (PPAR) agonists rosiglitazone, pioglitazone, and troglitazone tended to inhibit BSEP and MRP4 while stimulating MRP2 function. Interestingly, rosiglitazone and pioglitazone had no effect on MRP3, whereas troglitazone had an inhibitory effect, thus distinguishing it from rosiglitazone and pioglitazone. Compounds that inhibited BSEP and MRP4, with or without effects to MRP2 or MRP3 function, included the kinase inhibitors, leukotriene inhibitors, endothelin antagonists, and nonsteroidal anti-inflammatory drugs, along with drugs representing other pharmacologic targets.

In an attempt to relate the effect of these benchmark drugs to their clinical outcome, Table 1 was created. Biased toward compounds with some effect on BSEP transport, 121 benchmark compounds are listed in this table. Where available, links to the LiverTox database are provided. This is particularly important given that the column summarizing known clinical outcome was populated subjectively, and the opinion as to what constitutes hepatoxicity (ie, frequency beyond background occurrence) may vary. Known clinical outcomes, pharmacology and pharmacokinetic data were populated for 109 of these compounds as described in the Materials and Methods section. The individual IC_{50} values for all 635 compounds presented here are provided in Supplementary Table 1. This table also contains the annotations provided in Table 1 to allow for further analysis of these data and refinement of the annotations (eg, identify the specific type of hepatotoxicity: cholestasis, hepatocellular, mixed hepatocellular, or steatosis). Where an IC_{50} value of 133μM is listed, this is not an actual IC_{50}. This is the top concentration at which all compounds were tested and implies that the compound either had no effect on transporter function or the effect was insufficient to fit a curve using the regression analysis methods cited in this work.

The AUC parameter was selected to represent exposure. To estimate the C_{ss}, the AUC was divided by dose intervals. AUC values were acquired as described in the Materials and Methods section. To relate the exposure values to the transporter data, C_{ss} concentrations were divided by the IC_{50} values for each of the transporters. A similar means of relating *in vitro* cytochrome p450 (CYP) metabolism data to *in vivo* exposure has been described (Bjornsson *et al.*, 2003). These calculations can be found in Table 1, and the concept is first introduced visually in Figure 4, where the C_{ss}/BSEP IC_{50} ratio is compared with the BSEP IC_{50} value alone. Of the compounds with a C_{ss}/BSEP IC_{50} ratio ≥ 0.1, 95% of them are associated with some incidence of liver injury in humans. In contrast, only 49% of the compounds with a ratio < 0.1 have evidence of liver injury. Further, if a BSEP IC_{50} cutoff value of 25μM was to be used in the absence of exposure, 55 of 70 (79%) annotated compounds were associated with some level of DILI. This suggests that factoring for exposure does improve the prediction of liver injury outcome based on *in vitro* BSEP inhibition data. And, the use of total drug is recommended as an additional safety factor to account for the possibility of accumulation in the liver. An example of this is provided with AMG 009 (Fig. 5), where approximately 24 h postdose, the liver concentration is approximately 27-fold higher than in plasma (Fig. 6). The AUC for AMG 009 in humans where elevated transaminases were observed was 32.8 μg·h/ml (100 mg taken orally twice daily for a C_{ss} estimate of 2.4μM). The IC_{50} values for AMG 009 on BSEP and MRP function were as follows: BSEP (11.5μM), MRP2 (stimulation, followed by inhibition, no IC_{50} generated), MRP3 (1.1μM), and MRP4 (13.5μM). Therefore, the C_{ss}/IC_{50} ratios were BSEP (0.21), MRP3 (2.2), and MRP4 (0.18). Based on total drug, the use of C_{ss} to relate exposure data to *in vitro* potency values for AMG 009, as presented here, would have predicted an adverse outcome in humans. Table 2 summarizes the associations made between transporter interactions relative to exposure and evidence of liver injury in humans. As seen in Table 2, compounds with a transporter C_{ss}/IC_{50} ratio ≥ 0.1 have the strongest association with liver injury in humans (> 80%). As shown in Table 1 and Figure 3, several compounds interfere with one of more of the MRPs with little or no effect on BSEP. Annotation of the liver injury signal observed for these compounds is needed to assess the toxicological relevance of these observations.

Summarized in Table 3 are the percentage of compounds associated with some form of liver injury relative to the total number of compounds with an effect on BSEP and each of the MRPs, meeting select C_{ss}/IC_{50} ratios. As seen in Table 3, when the C_{ss}/BSEP IC_{50} ratio is < 0.1, and the corresponding MRP ratios are < 0.1 or ≥ 0.1, the percentage of compounds with evidence of liver injury varies but is never greater than 63%. In contrast, when the C_{ss}/BSEP IC_{50} ratio is ≥ 0.1, and the corresponding MRP ratios are < 0.1 or ≥ 0.1 (ie, has some effect on any of the MRPs), 100% of the compounds meeting these criteria have some evidence of liver injury, with the exception of MRP4 (BSEP and MRP4 C_{ss}/IC_{50} ≥ 0.1 has 12/13 compounds with evidence of liver injury). However, if the total number of compounds with a C_{ss}/BSEP IC_{50} ratio ≥ 0.1 and have some effect on MRP4 function (ie, a ratio for MRP4 of < 0.1 or ≥ 0.1), then the percentage of compounds with evidence of liver injury goes up to 96% (22/23 compounds). These are important observations as they provide evidence in support of evaluating BSEP and the MRPs to strengthen the correlation with a liver injury outcome. Another observation is with regard to the relationship between BSEP and MRP2 interference. All of the compounds that inhibit BSEP with a C_{ss}/IC_{50} ratio ≥ 0.1 and have some effect on MRP2 (stimulation, stimulation followed by inhibition, or inhibition alone) are associated with

TABLE 1

Transporter Potency Values, C_{ss}/BSEP IC_{50} Ratios, and Clinical Details for 121 Drugs

Compound Name	Human BSEP IC_{50} (μM)	Human MRP2 IC_{50} (μM)	Human MRP3 IC_{50} (μM)	Human MRP4 IC_{50} (μM)	Pharmacology	Acute or Chronic Therapy	Primary Route of Excretion	LiverTox Database Link	Known Effect(s) on Liver	FW	Clinical Dose Levels	Route of Administration	C_{ss}/BSEP Ratio
Mycophenolate mofetil	76	133	133	83	Transplant rejection	Acute	Urinary	http://livertox.nlm.nih.gov/Mycophenolate_Mofetil.htm	Known association with liver injury	433.5	1–1.5 g BID (PO or IV)	PO	49.945
Fusidic acid	10.1	133	133	133	Gram-positive antibiotic	Acute	Biliary	Not curated	Known association with liver injury; not sold within the United States	516.7	1500 mg QD (osteomyelitis)	PO	26.380
Ritonavir	1.74	133	11.1	34	Protease inhibitor for HIV	Chronic	Biliary	http://livertox.nlm.nih.gov/Ritonavir.htm	Known association with liver injury	721.0	600 mg BID	PO	19.796
Pazopanib	10.3	133	133	8.1	Oncology (multityrosine kinase inhibitor)	Acute/chronic	Biliary	Not curated	Black box warning of severe and fatal hepatotoxicity	437.5	200–800 mg QD	PO	9.588
MK-571	3.53	133	9.3	6	Inflammation (leukotriene inhibitor)	Information not found	Information not found	Not curated	Development halted due to liver changes observed during longer term rodent studies	515.1	75–600 mg QD	IV	5.273
Mifepristone	2.02	133	133	133	Abortificient (progesterone and glucocorticoid receptor antagonist); uterine myomas; endometriosis; meningiomas; Cushing's syndrome	Acute/chronic	Biliary	Not curated	No convincing evidence of liver injury	429.6	600 mg QD	PO	3.743
Deferasirox	58.4	133	133	36.5	Iron chelator	Chronic	Biliary	Not curated	Black box warning of severe and fatal hepatotoxicity	373.4	20–40 mg/kg QD	PO	2.363
Pioglitazone	0.4	Stimulation	133	49.5	Diabetes (PPARg)	Chronic	Biliary	http://livertox.nlm.nih.gov/Pioglitazone.htm	Known association with liver injury; liver monitoring recommended	356.4	15–60 mg QD	PO	2.338

TABLE 1—*Continued*

Compound Name	Human BSEP IC_{50} (μM)	Human MRP2 IC_{50} (μM)	Human MRP3 IC_{50} (μM)	Human MRP4 IC_{50} (μM)	Pharmacology	Acute or Chronic Therapy	Primary Route of Excretion	LiverTox Database Link	Known Effect(s) on Liver	FW	Clinical Dose Levels	Route of Administration	C_{ss}/BSEP Ratio
Fenofibrate	15.3	133	133	133	LDL cholesterol lowering (PPARa)	Chronic	Urinary	http://livertox.nlm.nih.gov/Fenofibrate.htm	Known association with liver injury; liver monitoring recommended	360.8	43–130 mg QD	PO	0.973
Tolcapone	36.6	Stimulation	85	16.7	Parkinson's disease (catechol-O-methyl transferase inhibitor)	Chronic	Urinary	http://livertox.nlm.nih.gov/Tolcapone.htm	Known association with liver injury	273.2	100–200 mg TID	PO	0.611
Paclitaxel (aka taxol)	24.4	133	133	133	Tubulin polymerizer	Acute/subchronic	Biliary	Not curated	Known association with liver injury	853.9	135–175 mg/m^2 infusions for 3 or 24 h	IV	0.605
Octreotide acetate	9.34	133	133	133	Acromegaly, carcinoid syndrome, intestinal tumor-associated diarrhea	Chronic	Urinary	Not curated	Known association with liver injury	1019.3	50 μg BID or TID (SC or IV), 50–500 μg TID SC for Sandostatin LAR	SC	0.565
Nelfinavir	11.8	133	133	97	Protease inhibitor	Chronic	Biliary	http://livertox.nlm.nih.gov/Nelfinavir.htm	Known association with liver injury	567.8	750 mg TID or 1250 mg BID	PO	0.549
Posaconazole	8.1	133	133	133	Antifungal (14alpha-demethylase inhibitor)	Acute/chronic	Biliary	http://livertox.nlm.nih.gov/Posaconazole.htm	5%–10% elevated liver enzymes; jaundice and hepatitis appear in product label	700.8	200 mg (5 ml) TID	PO	0.544
Dicloxacillin	56.4	133	133	133	Antibiotic for staphylococcus infection	Acute/chronic	Urinary	http://livertox.nlm.nih.gov/Dicloxacillin.htm	Known association with liver injury	491.0	125–500 mg QID	PO	0.498
Rifapentine	9.91	16.3	80.9	35.9	Pulmonary tuberculosis (antibiotic)	Chronic	Biliary	http://livertox.nlm.nih.gov/Rifapentine.htm	Known association with liver injury	877.0	600 mg twice weekly, then down to 600 mg once weekly	PO	0.474
Cyclosporine A	0.5	14.5	23	133	Transplant rejection (immunosuppressant)	Acute	Biliary	http://livertox.nlm.nih.gov/Cyclosporine.htm	Associated with drug-induced cholestasis	1202.6	20–600 mg/kg	PO	0.406
Lapatinib tosylate	6.49	133	133	133	Oncology (HER2 kinase inhibitor)	Acute	Biliary	http://livertox.nlm.nih.gov/Lapatinib.htm	Black box warning of severe and fatal hepatotoxicity	581.1	1250 mg QD	PO	0.400
Lopinavir	17.3	133	21	47	Protease inhibitor	Chronic	Biliary	http://livertox.nlm.nih.gov/Lopinavir.htm	Known association with liver injury	628.8	400–800 mg QD (formulated with ritonavir, sold as Kaletra)	PO	0.381
Clofibrate	71	133	133	133	Dyslipidemia	Chronic	Urinary	http://livertox.nlm.nih.gov/Clofibrate.htm	Known association with liver injury	242.7	2 g QD	PO	0.381

TABLE 1—*Continued*

Compound Name	Human BSEP IC_{50} (μM)	Human MRP2 IC_{50} (μM)	Human MRP3 IC_{50} (μM)	Human MRP4 IC_{50} (μM)	Pharmacology	Acute or Chronic Therapy	Primary Route of Excretion	LiverTox Database Link	Known Effect(s) on Liver	FW	Clinical Dose Levels	Route of Administration	C_{ss}/BSEP Ratio
Ketoconazole	3.4	133	133	69	Antifungal	Acute	Biliary	http://livertox.nlm.nih.gov/Ketoconazole.htm	10%–15% of patients show elevated liver enzymes; liver monitoring recommended	531.4	200 mg QD	PO	0.381
Troglitazone	3	Stimulation/inhibition	31	61	Diabetes (PPARα and γ)	Chronic	Biliary	http://livertox.nlm.nih.gov/Troglitazone.htm	Withdrawn from market due to liver injury	441.5	200–600 mg QD	PO	0.313
Dipyridamole	4	133	133	8.3	Adjunct therapy to prevent thrombo-embolism following heart valve replacement (platelet inhibitor)	Acute	Biliary	http://livertox.nlm.nih.gov/Dipyridamole.htm	Liver enzyme elevations have been observed; no convincing evidence of liver injury	504.6	75–100 mg QID	PO	0.303
Irbesartan	7.31	Stimulation	21.1	83.2	Hypertension (AT1 subtype angiotensin II antagonist)	Chronic	Biliary	http://livertox.nlm.nih.gov/Irbesartan.htm	Cholestatic jaundice and abnormal liver function tests have been reported	428.5	150–300 mg QD	PO	0.301
Tranilast	41.5	133	133	1.0	Asthma (inhibits the production of interleukin-6)	Chronic	Information not found	Not curated	Known association with liver injury	327.1	80–600 mg QD (found in doi: 10.1002/rcm.2741)	PO	0.286
Indinavir	21.2	133	83	133	Protease inhibitor	Chronic	Biliary	http://livertox.nlm.nih.gov/Indinavir.htm	Known association with liver injury	613.8	800 mg TID	PO	0.266
Bicalutamide	79.6	133	133	133	Prostate carcinoma (antiandrogen)	Chronic	Biliary/urinary (50/50)	http://livertox.nlm.nih.gov/Bicalutamide.htm	Known association with liver injury; liver enzyme monitoring is recommended	430.4	50 mg QD	PO	0.263
Saquinavir	4.9	133	42	59	Protease inhibitor for HIV	Chronic	Biliary	http://livertox.nlm.nih.gov/Saquinavir.htm	Known association with liver injury	670.9	400–1200 mg TID	PO	0.247
Telithromycin	5	133	133	7.1	Ketolide antibiotic	Acute	Biliary	http://livertox.nlm.nih.gov/Telithromycin.htm	Known association with liver injury	812.0	400 mg BID for 5–10 days	PO	0.193

TABLE 1—*Continued*

Compound Name	Human BSEP IC_{50} (μM)	Human MRP2 IC_{50} (μM)	Human MRP3 IC_{50} (μM)	Human MRP4 IC_{50} (μM)	Pharmacology	Acute or Chronic Therapy	Primary Route of Excretion	LiverTox Database Link	Known Effect(s) on Liver	FW	Clinical Dose Levels	Route of Administration	C_{ss}/BSEP Ratio
Imatinib	25.1	133	133	133	Oncology (tyrosine kinase inhibitor)	Acute/ chronic	Biliary	http://livertox.nlm.nih.gov/Imatinib.htm	3%–6% have severe ALT/AST or bilirubin elevations; liver monitoring recommended	493.6	400–600 mg QD	PO	0.176
Docetaxel	41	133	133	133	Antimitotic (tubulin polymerizer)	Acute	Biliary	Not curated	Known association with liver injury	807.9	60–100 mg/m^2 infusion 1 h every 3 weeks	IV	0.160
Sitaxsentan	12.6	Stimulation/ inhibition	45.4	28.4	Pulmonary arterial hypertension (endothelin antagonist)	Chronic	Biliary/ urinary (50/50)	Not curated	Withdrawn from market due to liver injury	454.9	100–300 mg QD	PO	0.158
Rifampicin	25.3	53	69	41.9	Antibiotic	Acute	Biliary	http://livertox.nlm.nih.gov/Rifampin.htm	Known association with liver injury; liver monitoring recommended for some patients	822.9	150–600 mg QD	IV	0.147
Pranlukast	2.97	133	133	2.7	Asthma (leukotriene inhibitor)	Chronic	Biliary	Not curated	Known association with liver injury	481.5	300 QD (found in doi: 10.1046/j.1365-2125.1997.00650.x)	PO	0.122
Rosiglitazone	2.8	Stimulation	133	21	Diabetes (PPARγ)	Chronic	Biliary	http://livertox.nlm.nih.gov/Rosiglitazone.htm	Known association with liver injury; liver monitoring recommended	357.4	2–8 mg QD	PO	0.119
Benzbromarone	17.5	41.6	133	17	Antigout (uricosuric)	Chronic	Biliary	http://livertox.nlm.nih.gov/Benzbromarone.htm	Known association with liver injury; withdrawn from market in 2003	421.9	100–200 mg QD	PO	0.107
Nefazodone	6.11	133	133	71	Antidepressant (5-HT receptor antagonist)	Chronic	Urinary	Not curated	Known association with liver injury; sales discontinued in Canada in 2003	470.0	300–600 mg QD	PO	0.099

TABLE 1—*Continued*

Compound Name	Human BSEP IC_{50} (μM)	Human MRP2 IC_{50} (μM)	Human MRP3 IC_{50} (μM)	Human MRP4 IC_{50} (μM)	Pharmacology	Acute or Chronic Therapy	Primary Route of Excretion	LiverTox Database Link	Known Effect(s) on Liver	FW	Clinical Dose Levels	Route of Administration	C_{ss}/BSEP Ratio
Amprenavir	44.8	133	133	133	HIV (protease inhibitor)	Chronic	Biliary	http://livertox.nlm.nih.gov/Amprenavir_and_Fosamprenavir.htm	Known association with liver injury; liver enzyme monitoring is recommended	505.6	1200 mg BID up to 2800 mg/day	PO	0.097
Praziquantel	67.1	133	133	133	Schistosomiasis and liver fluke infection	Acute	Urinary	http://livertox.nlm.nih.gov/Praziquantel.htm	No convincing evidence of liver injury	312.4	20–25 mg/kg TID	PO	0.094
Megestrol acetate	17.8	133	133	133	Oncology (progesterone derivative)	Chronic	Urinary	Not curated	No convincing evidence of liver injury	384.5	400–800 mg QD	PO	0.084
Olmesartan medoxomil	4.73	Stimulation	2	28.5	Hypertension (AT1 subtype angiotensin II antagonist)	Chronic	Biliary/urinary (50/50)	http://livertox.nlm.nih.gov/Olmesartan.htm	No convincing evidence of liver injury	558.6	20–40 mg QD	PO	0.081
Bosentan	23	Stimulation/inhibition	42	22	Pulmonary arterial hypertension (endothelin antagonist)	Chronic	Biliary	Not curated	Associated with drug-induced cholestasis	551.6	62.5–125 mg BID	PO	0.059
Tolvaptan	9.99	133	133	133	Hypervolemic hyponatremia (vasopressin V2-receptor antagonist)	Acute	Biliary	Not curated	No convincing evidence of liver injury	448.9	15–60 mg QD	PO	0.040
Isotretinoin	37.1	133	133	133.0	Acne (retinoid)	Acute/chronic	Biliary/urinary (50/50)	http://livertox.nlm.nih.gov/Isotretinoin.htm	Liver enzyme elevations have been observed; no convincing evidence of liver injury	300.2	0.5–2 mg/kg QD	PO	0.037
Cisapride monohydrate	22	133	133	133	Gastroesophageal reflux disease (serotonin agonist)	Chronic	Biliary/urinary (50/50)	Not curated	No convincing evidence of liver injury; associated with cardiovascular toxicity	466.0	10–20 mg QID	PO	0.036
Rabeprazole sodium	33.9	133	133	133	Acid reflux (proton pump inhibitor)	Acute/chronic	Urinary	Not curated	No convincing evidence of liver injury	359.4	20–100 mg QD or 60 mg BID	PO	0.036

TABLE 1—*Continued*

Compound Name	Human BSEP IC_{50} (μM)	Human MRP2 IC_{50} (μM)	Human MRP3 IC_{50} (μM)	Human MRP4 IC_{50} (μM)	Pharmacology	Acute or Chronic Therapy	Primary Route of Excretion	LiverTox Database Link	Known Effect(s) on Liver	FW	Clinical Dose Levels	Route of Administration	C_{ss}/BSEP Ratio
Gliquidone	11.6	Stimulation	23.85	6.3	Diabetes (sulfonylurea)	Chronic	Biliary	Not curated	Cholestatic jaundice and abnormal liver function tests have been reported for sulfonylurea therapies	527.6	15–120 QD	PO	0.035
Entacapone	55.6	133	35.1	6.8	Parkinson's (catechol-O-methyltransferase inhibitor)	Chronic	Biliary	http://livertox.nlm.nih.gov/Entacapone.htm	Liver enzyme elevations have been observed; no convincing evidence of liver injury	305.3	200 mg Q3h	PO	0.032
Amiodarone	43	133	133	133	Arrhythmia	Chronic	Biliary	http://livertox.nlm.nih.gov/Amiodarone.htm	Black box warning of severe and fatal hepatotoxicity	645.3	200–1200 mg QD	PO	0.032
Temsirolimus	2.69	17.6	71.9	44	Oncology and immunosuppresion (mTOR inhibitor)	Chronic	Biliary	http://livertox.nlm.nih.gov/Sirolimus.htm#insert	Liver enzyme elevations have been observed; primary metabolite (sirolimus) has known association with liver injury	1030.3	5–20 mg	IV	0.030
Drotaverine	37	133	133	24.5	Antispasmodic (inhibits PDE4)	Acute	Biliary/urinary (50/50)	Not curated	No convincing evidence of liver injury	397.5	40–80 mg TID PO	PO	0.028
Zafirlukast	11.1	58.8	133	12.1	Asthma (leukotriene inhibitor)	Chronic	Biliary	http://livertox.nlm.nih.gov/Zafirlukast.htm	Known association with liver injury; hepatic failure has been reported	575.7	10–20 mg BID	PO	0.027
Indomethacin	42	Stimulation	66	5.8	NSAID	Acute/chronic	Urinary	http://livertox.nlm.nih.gov/Indomethacin.htm	Known association with liver injury	357.8	25–50 mg TID	PO	0.027

TABLE 1—*Continued*

Compound Name	Human BSEP IC_{50} (μM)	Human MRP2 IC_{50} (μM)	Human MRP3 IC_{50} (μM)	Human MRP4 IC_{50} (μM)	Pharmacology	Acute or Chronic Therapy	Primary Route of Excretion	LiverTox Database Link	Known Effect(s) on Liver	FW	Clinical Dose Levels	Route of Administration	C_{ss}/BSEP Ratio
Danazol	18.7	133	133	133	Angioadema, endometriosis, and fibrocystic breast disease (synthetic steroid)	Acute/ chronic	Urinary	Not curated	Black box warning of liver injury with long-term use	337.5	50–400 mg BID	PO	0.024
Febuxostat	42.9	133	133	1.9	Gout (xanthine oxidase inhibitor)	Chronic	Biliary/ urinary (50/50)	http://livertox.nlm.nih.gov/Febuxostat.htm	Known association with liver injury	316.4	40–80 mg QD	PO	0.024
Flupirtine	35.5	133	133	133	Nonnarcotic analgesic	Acute/ chronic	Biliary	Not curated	Known association with liver injury	304.1	100–600 mg QD	PO	0.023
Epalrestat	36.8	84.4	45.8	6.5	Secondary complications due to diabetes (aldose reductase inhibitor)	Chronic	Urinary	Not curated	Liver enzyme elevations have been observed; no convincing evidence of liver injury	319.4	50 mg QD	PO	0.023
Cefpodoxime proxetil	81.7	133	133	133	Broad spectrum antibiotic (cephalosporin class)	Acute	Biliary	http://livertox.nlm.nih.gov/Cephalosporins.htm	Associated with drug-induced cholestasis	557.6	100–400 mg BID	PO	0.022
Glyburide	5	Stimulation	33	10.5	Diabetes (sulfonylurea)	Chronic	Biliary/ urinary (50/50)	http://livertox.nlm.nih.gov/SecondGenerationSulfonylureas.htm	Associated with drug-induced cholestasis	494.0	1.25–25 mg QD	PO	0.022
Gefitinib	10.9	133	133	4.6	Oncology (tyrosine kinase inhibitor)	Acute/ chronic	Biliary	http://livertox.nlm.nih.gov/Gefitinib.htm	Known association with liver injury; liver monitoring recommended	446.9	250–500 mg QD	PO	0.021
Itraconazole	18	133	133	133	Antifungal	Acute	Biliary	http://livertox.nlm.nih.gov/Itraconazole.htm	Known association with liver injury; liver monitoring recommended if taking itraconazole > 1 month	705.6	200 mg QD for 1–2 weeks	PO	0.020
Mibefradil HCL	43.8	133	133	133	Hypertension (Ca^{++} channel blocker)	Chronic	Biliary	Not curated	No convincing evidence of liver injury	495.6	100 mg QD	PO	0.017

TABLE 1—*Continued*

Compound Name	Human BSEP IC_{50} (μM)	Human MRP2 IC_{50} (μM)	Human MRP3 IC_{50} (μM)	Human MRP4 IC_{50} (μM)	Pharmacology	Acute or Chronic Therapy	Primary Route of Excretion	LiverTox Database Link	Known Effect(s) on Liver	FW	Clinical Dose Levels	Route of Administration	C_{ss}/BSEP Ratio
Losartan potassium	8.53	Stimulation/ inhibition	133	34.1	Hypertension and diabetic nephropathy (angiotensin II antagonist)	Chronic	Biliary/ urinary (50/50)	http://livertox.nlm.nih.gov/Losartan.htm	Liver enzyme elevations have been observed; associated with acute liver injury	422.9	50–100 mg QD (with 25 mg used in patients with possible depletion of intravascular volume)	PO	0.012
Everolimus	2	11.3	84	95	Oncology and transplant rejection (mTOR inhibitor)	Chronic	Biliary	http://livertox.nlm.nih.gov/Sirolimus.htm#insert	Liver enzyme elevations have been observed; known association with cholestatic liver injury	958.2	2.5–20 mg QD	PO	0.012
Cetrorelix acetate	1.47	133	133	133	Ovulation inducer (GnRH antagonist)	Acute	Biliary	Not curated	Liver enzyme elevations up to 3× ULN have been reported	1431.1	0.25 mg QD (SC) or a single 3 mg dose (SC) during early to midfollicular phase	SC	0.011
Glimepiride	15.7	Stimulation	6.39	64	Diabetes (sulfonylurea)	Chronic	Urinary	http://livertox.nlm.nih.gov/SecondGenerationSulfonylureas.htm	Associated with drug-induced cholestasis	490.6	1–4 mg QD	PO	0.009
Clofazimine	12.9	10.4	21	44.5	Anti-*Mycobacterium leprae* (lepromatous leprosy)	Acute/ chronic	Biliary	Not curated	No convincing evidence for liver injury; liver enzyme elevations have been observed	473.4	100–300 mg QD	PO	0.007
Acitretin	38.2	Stimulation/ inhibition	133	49.00	Psoriasis (retinoid)	Subchronic	Biliary/ urinary (50/50)	http://livertox.nlm.nih.gov/Acitretin.htm	Black box warning of severe hepatotoxicity; liver enzyme monitoring recommended	326.4	25–50 mg QD	PO	0.007
Drospirenone	16.4	Stimulation/ inhibition	133	37.9	Birth control (synthetic hormone)	Chronic	Biliary/ urinary (50/50)	Not curated	No convincing evidence of liver injury	366.5	3 mg QD	PO (AUC estimated from a range of values listed in Pharmapendium)	0.006

TABLE 1—*Continued*

Compound Name	Human BSEP IC_{50} (μM)	Human MRP2 IC_{50} (μM)	Human MRP3 IC_{50} (μM)	Human MRP4 IC_{50} (μM)	Pharmacology	Acute or Chronic Therapy	Primary Route of Excretion	LiverTox Database Link	Known Effect(s) on Liver	FW	Clinical Dose Levels	Route of Administration	C_{ss}/BSEP Ratio
Valrubicin	24.1	64	76.3	36.8	Oncology (topoisomerase inhibitor)	Acute/subchronic	Urinary	Not curated	No convincing evidence of liver injury; limited systemic exposure due to typical route of administration	723.7	800 mg once per week for 6 weeks	Intravesical instillation	0.005
Donepezil	78	133	133	72.9	Alzheimer's (acetylcholinesterase inhibitor)	Chronic	Urinary	http://livertox.nlm.nih.gov/Donepezil.htm	No convincing evidence of liver injury	379.5	5–10 mg	PO	0.005
Omeprazole	99	133	133	133	Ulcers, GERD, and Zollinger-Ellison syndrome (proton pump inhibitor)	Chronic	Urinary	http://livertox.nlm.nih.gov/Omeprazole.htm	Known association with liver injury; liver enzyme elevations have been observed	345.4	20–40 mg QD (delayed release capsules or IV)	IV	0.005
Dasatinib	13.1	133	133	27.3	Oncology (multikinase inhibitor)	Chronic	Biliary	Not curated	Known association with liver injury	488.0	70–90 mg BID (chronic phase CML) or 100 mg BID (advanced phase CML and Ph^+ ALL)	PO	0.004
Nicardipine	7.87	133	133	88	Hypertension (Ca^{++} channel blocker)	Chronic	Biliary/urinary (50/50)	http://livertox.nlm.nih.gov/Nicardipine.htm	No convincing evidence of liver injury	515.2	60–120 mg QD	PO	0.004
Rifabutin	26.7	49.8	133	15.9	Antimycobacterial (inhibitrs DNA-dependent RNA polymerase in bacteria)	Acute	Urinary	http://livertox.nlm.nih.gov/Rifabutin.htm	Known association with liver injury	847.0	300–900 mg QD	PO	0.004
Telmisartan	16.2	133	60	36	Hypertension (angiotensin II antagonist)	Chronic	Biliary	http://livertox.nlm.nih.gov/Telmisartan.htm	No convincing evidence for liver injury	514.6	20–80 mg QD	PO	0.009
Ezetimibe	56	133	133	133	Hypercholesterolemia (NPC1 inhibitor)	Chronic	Biliary	http://livertox.nlm.nih.gov/Ezetimibe.htm	Liver enzyme elevations have been observed; associated with acute liver injury	409.4	10 mg	PO	0.002
Nifedipine	64	133	133	16.6	Hypertension (Ca^{++} channel blocker)	Chronic	Urinary	http://livertox.nlm.nih.gov/Nifedipine.htm	Known association with liver injury	346.3	30–60 mg QD	PO	0.002

TABLE 1—*Continued*

Compound Name	Human BSEP IC_{50} (μM)	Human MRP2 IC_{50} (μM)	Human MRP3 IC_{50} (μM)	Human MRP4 IC_{50} (μM)	Pharmacology	Acute or Chronic Therapy	Primary Route of Excretion	LiverTox Database Link	Known Effect(s) on Liver	FW	Clinical Dose Levels	Route of Administration	C_{ss}/BSEP Ratio
Primaquine	32.7	133	133	133	Antiprotozoal (vivax malaria)	Acute	Biliary	http://livertox.nlm.nih.gov/Primaquine.htm	No convincing evidence of liver injury	259.3	15 mg QD for 14 days	PO	0.002
Dronedarone hydrochloride	73.9	133	133	133	Cardiac arrhythmia (multichannel blocker)	Chronic	Biliary	Not curated	Known association with liver injury	556.8	400 mg BID	PO	0.002
Fluvastatin	36.1	Stimulation	57	133	Hyperlipidemia (HMG-CoA reductase inhibitor)	Chronic	Biliary	http://livertox.nlm.nih.gov/Fluvastatin.htm	Known association with liver injury; liver monitoring recommended for some patients	411.5	20–80 mg QD	PO	0.002
Doxazosin mesylate	45	133	133	8	Hypertension and benign prostatic hyperplasia (alpha (A1) adranergic receptor inhibitor)	Chronic	Biliary	http://livertox.nlm.nih.gov/Doxazosin.htm	Liver enzyme elevations have been observed; no convincing evidence of liver injury	451.5	1–16 mg QD or 4–8 mg QD extended-release tablets	PO	0.002
Finasteride	28.2	133	133	51	Benign prostate hyperplasia and alopecia (antiandrogen, 5a reductase inhibitor)	Chronic	Biliary	http://livertox.nlm.nih.gov/Finasteride.htm	No convincing evidence of liver injury	372.5	1 mg QD (alopecia); 5 mg QD (benign prostate hyperplasia)	PO	0.00147
Tacrolimus	7.18	40.3	133	133	Transplant rejection (calcineurin inhibitor)	Chronic	Biliary	http://livertox.nlm.nih.gov/Tacrolimus.htm	Liver enzyme elevations have been observed; associated with drug-induced cholestasis	804.0	0.01–0.05 mg/kg/day IV until patient can tolerate PO administration of 0.075 mg/kg/day to 0.26 mg/kg	PO	0.001
Atorvastatin calcium	13	133	14.2	88.5	LDL cholesterol lowering (HMG-CoA reductase inhibitor)	Chronic	Biliary	http://livertox.nlm.nih.gov/Atorvastatin.htm	Known association with liver injury	558.6	10–80 mg QD	PO	0.001
Repaglinide	22	72.3	16.7	53.9	Diabetes type II	Chronic	Biliary	http://livertox.nlm.nih.gov/Repaglinide.htm	Cholestatic jaundice and abnormal liver function tests have been reported	452.6	0.5–4 mg BID, TID, or QID (maximum recommended daily dose is 16 mg)	PO	0.00075

TABLE 1—*Continued*

Compound Name	Human BSEP IC_{50} (μM)	Human MRP2 IC_{50} (μM)	Human MRP3 IC_{50} (μM)	Human MRP4 IC_{50} (μM)	Pharmacology	Acute or Chronic Therapy	Primary Route of Excretion	LiverTox Database Link	Known Effect(s) on Liver	FW	Clinical Dose Levels	Route of Administration	C_{ss}/BSEP Ratio
Adefovir dipivoxil	46	133	133	133	Hepatitis B (reverse transcriptase inhibitor)	Chronic	Urinary	http://livertox.nlm.nih.gov/Adefovir.htm	No convincing evidence of liver injury; liver enzyme elevations are likely due to exacerbation of existing disease	501.5	10 mg QD orally	PO	0.00069
Norethindrone acetate	36	133	133	133	Birth control (antigonadotropic)	Chronic	Information not found	Not curated	PO contraceptives are associated with liver injury; but no convincing evidence for norethindrone alone	340.5	0.35 mg QD	PO	0.00057
Lovastatin	19.3	133	133	133	Hyperlipidemia (HMG-CoA reductase inhibitor)	Chronic	Biliary	http://livertox.nlm.nih.gov/Lovastatin.htm	Liver enzyme elevations have been observed; known association with cholestatic liver injury	404.5	10–80 mg QD	PO	0.00051
Loxapine succinate	77	133	133	133	Schizophrenia	Chronic	Biliary	http://livertox.nlm.nih.gov/Loxapine.htm	Liver enzyme elevations have been observed; associated with acute liver injury	363.1	60–250 mg QD	PO	0.0004
Iloperidone	23.4	133	133	133	Antipsychotic (dopamine and serotonin receptor antagonist)	Chronic	Urinary	Not curated	No convincing evidence of liver injury	426.5	6–12 mg BID	PO	0.00038
Midazolam	41.74	Not tested	Not tested	Not tested	Benzodiazepine (CNS depressant)	Acute	Urinary	http://livertox.nlm.nih.gov/Midazolam.htm	No convincing evidence of liver injury	325.8	0.01–0.04 mg/kg	PO	0.00037
Simvastatin	24.7	133	133	133	LDL cholesterol lowering (HMG-CoA reductase inhibitor)	Chronic	Biliary	http://livertox.nlm.nih.gov/Simvastatin.htm	Known association with liver injury; liver monitoring recommended	418.6	5–80 mg QD	PO	0.00036

TABLE 1—*Continued*

Compound Name	Human BSEP IC_{50} (μM)	Human MRP2 IC_{50} (μM)	Human MRP3 IC_{50} (μM)	Human MRP4 IC_{50} (μM)	Pharmacology	Acute or Chronic Therapy	Primary Route of Excretion	LiverTox Database Link	Known Effect(s) on Liver	FW	Clinical Dose Levels	Route of Administration	C_{ss}/BSEP Ratio
Isradipine	28	133	133	133	Hypertension (Ca^{++} channel blocker)	Chronic	Urinary	http://livertox.nlm.nih.gov/Isradipine.htm	Liver enzyme elevations have been observed; no convincing evidence of liver injury	371.1	2.5–20 mg QD	PO	0.0003
Pimecrolimus	10	133	133	133	Dermatitis (inhibits T-cell activation)	Acute/subchronic	Biliary	Not curated	No convincing evidence of liver injury	810.5	1% cream BID	Topical	0.00019
Nisoldipine	33	133	133	16.5	Hypertension (Ca^{++} channel blocker)	Chronic	Biliary	http://livertox.nlm.nih.gov/Nisoldipine.htm	No convincing evidence of liver injury	388.4	20–60 mg QD	PO	0.00017
Latanoprost	12.9	133	133	133	Glaucoma and ocular hypertension (prostaglandin FP agonist)	Chronic	Urinary	Not curated	No convincing evidence of liver injury	432.6	1 drop (1.5 μg) QD	Intraocular	0.00015
Dutasteride	29.8	133	133	133	Benign prostatic hyperplasia (5-alpha reductase inhibitor)	Chronic	Biliary	http://livertox.nlm.nih.gov/Dutasteride.htm	Liver enzyme elevations have been observed; no convincing evidence of liver injury	528.5	0.5 mg QD	PO	0.00011
Loratadine	12	133	133	133	Antiallergenic (H1 histamine receptor antagonist)	Acute/chronic	Biliary/urinary (50/50)	Not curated	Known association with liver injury	382.9	10 mg QD	PO	0.00010
Reserpine	8.35	68.4	133	133	Antihypertensive and antipsychotic	Chronic	Biliary	http://livertox.nlm.nih.gov/Reserpine.htm	No convincing evidence for liver injury	608.7	0.1–1.0 mg QD (doses up to 40 mg QD have been used)	PO	0.00007
Felodipine	69.7	133	133	133	Hypertension (Ca^{++} channel blocker)	Chronic	Urinary	http://livertox.nlm.nih.gov/Felodipine.htm	Liver enzyme elevations have been observed; no convincing evidence of liver injury	384.3	2.5–10 mg QD	PO	0.00006
Oxybutynin	27.4	133	133	133	Incontinence; over active bladder (anticholinergic)	Chronic	Biliary	Not curated	No convincing evidence of liver injury	357.5	5–30 mg QD	PO	0.00006
Medroxyprogesterone acetate	15.7	133	133	22.5	Birth control; endometriosis; carcinoma (renal, breast, and endometrial)	Acute/chronic	Biliary	http://livertox.nlm.nih.gov/Estrogens.htm	Cholestatic jaundice and abnormal liver function tests have been observed	386.5	5–10 mg QD (PO), 400–1000 mg per week (IM), or 104 mg/0.65 ml every 3 months (SC)	PO	0.00004

TABLE 1—*Continued*

Compound Name	Human BSEP IC_{50} (μM)	Human MRP2 IC_{50} (μM)	Human MRP3 IC_{50} (μM)	Human MRP4 IC_{50} (μM)	Pharmacology	Acute or Chronic Therapy	Primary Route of Excretion	LiverTox Database Link	Known Effect(s) on Liver	FW	Clinical Dose Levels	Route of Administration	C_{ss}/BSEP Ratio
Astemizole	58.8	133	133	133	Antiallergenic (H1 histamine receptor antagonist)	Acute/ chronic	Biliary	Not curated	No convincing evidence of liver injury	458.6	10 mg (serious cardioavascular events at doses as low as 20–30 mg/day)	PO	0.00002
Fluphenazine hydrochloride	87.3	133	133	133	Phenothiazine antipsychotic	Chronic	Biliary	http://livertox.nlm.nih.gov/Fluphenazine.htm	Liver enzyme elevations have been observed; associated with drug-induced cholestasis	437.5	1–40 mg QD or 10 mg injection	PO	0.00001
Budesonide	46	133	54.5	37	Asthma, allergic rhinitis, and Crohn's (corticosteroid)	Chronic	Urinary	Not curated	No convincing evidence of liver injury	430.5	9 mg QD	PO	0.00001
Misoprostol	27.7	133	133	46	Treatment of gastric ulcers, miscarriage, labor induction, and abortifacient (synthetic prostaglandin E1 analogue)	Acute	Urinary	Not curated	No convincing evidence of liver injury as a monotherapy; coadministration of misoprostol with diclofenac is associated with liver injury	382.5	0.2 mg QID	PO	0.00001
Calcitriol	39.7	133	133	133	Hypocalcemia (vitamin D derivative)	Chronic	Biliary	Not curated	No convincing evidence of liver injury	416.6	0.25–0.50 μg QD or solution (1 μg/ml) QD	PO	0.0000016
Bimatoprost	40.4	133	133	133	Glaucoma and ocular hypertension (prostaglandin FP agonist)	Chronic	Urinary	Not curated	Liver enzyme elevations have been observed; no convincing evidence of liver injury	415.6	1 drop QD of 0.03% solution	Intraocular	0.0000002
17α Ethinylestradiol	14	133	133	133	Birth control (synthetic estrogen)	Chronic	Biliary/ urinary (50/50)	Not curated	Associated with drug-induced cholestasis	296.2	0.025 mg QD	PO	Not calculated
Chlordiazepoxide	44.1	133	133	133	Anxiolytic (benzodiazepine)	Acute	Urinary	http://livertox.nlm.nih.gov/Chlordiazepoxide.htm	Known association with liver injury	299.8	5–25 mg QID	PO	Not calculated

TABLE 1—*Continued*

Compound Name	Human BSEP IC_{50} (μM)	Human MRP2 IC_{50} (μM)	Human MRP3 IC_{50} (μM)	Human MRP4 IC_{50} (μM)	Pharmacology	Acute or Chronic Therapy	Primary Route of Excretion	LiverTox Database Link	Known Effect(s) on Liver	FW	Clinical Dose Levels	Route of Administration	C_{ss}/BSEP Ratio
Ciglitazone	37.8	133	133	133	Research substance (PPARg agonist)	Chronic	Biliary	Not curated	Never marketed	333.4	Information not found	Information not found	Not calculated
Cinnarizine	15.7	133	133	133	Motion sickness, antiemetic	Acute/ chronic	Urinary	Not curated	Associated with drug-induced cholestasis	368.2	10–20 mg QD	Information not found	Not calculated
Erythromycin estolate	13	133	133	68.2	Macrolide antibiotic	Acute	Biliary	http://livertox.nlm.nih.gov/Erythromycin.htm	Black box warning of cholestatic liver injury	1055.6	400 mg QID for up to 15 days	PO	Not calculated
GDC-0941 bimesylate	15.2	133	133	2.7	Oncology (PI3kinase inhibitor)	Information not found	Information not found	Not curated	Information not found	513.6	Information not found	Information not found	Not calculated
Mevastatin	27	133	133	133	Hyperlipidemia (HMG-CoA reductase inhibitor)	Chronic	Information not found	Information not found	Never marketed	390.2	Information not found	Information not found	Not calculated
Neratinib	25	133	133	23	Oncology (HER2 and EGFR kinase inhibitor)	Information not found	Information not found	Not curated	Currently undergoing clinical trials; information is minimal	557.0	Information not found	Information not found	Not calculated
Rifaximin	42	15.4	65	30.2	Antibiotic	Acute	Biliary	http://livertox.nlm.nih.gov/Rifaximin.htm	No convincing evidence of liver injury; minimal PO bioavailability	785.9	10–30 mg/kg QD	PO	Not calculated
Staurosporine	18.7	133	133	37.9	Kinase inhibitor (research substance)	Information not found	Information not found	Not curated	Information not found	466.5	Information not found	Information not found	Not calculated
Valinomycin	1.56	133	133	133	Research substance produced by streptomyces bacteria	Information not found	Information not found	Not curated	*In vitro* BSEP interference has been shown by others	1111.3	Information not found	Information not found	Not calculated
Wortmannin	13.6	46.1	133	63.2	Kinase inhibitor (research substance)	Information not found	Information not found	Not curated	Information not found	428.4	Information not found	Information not found	Not calculated

Notes. A list of 109 drugs for which *in vitro* potency values are given for the BSEP and MRP assays. Additional clinical information is provided, including associations with liver injury and exposure data (area under the concentration versus time curve or AUC) in humans. A concentration at steady state or C_{ss} was derived from the AUC values by dividing by the dose interval. The resulting C_{ss} estimates were normalized relative to BSEP IC_{50} values in an attempt to relate exposure in blood to *in vitro* potency concentrations. A supplemental data table is provided that contains all of the information captured in table 1, as well as *in vitro* transporter data for the remaining 500+ drugs.

Abbreviations: ALL, acute lymphoblastic leukemia; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AT1, angiotensin II receptor, type 1; BID, twice daily; CML, chronic myelogenous leukemia; CNS, central nervous system; EGFR, epidermal growth factor receptor; GERD, gastroesophageal reflux disease; HER2, human epidermal growth factor receptor 2; HIV, human immunodeficiency virus; HMG-CoA reductase, 3-hydroxy-3-methyl-glutaryl-CoA reductase; LDL, low density lipoprotein; mTOR, mammalian target of rapamycin; NPC1, Niemann-Pick disease, type C1; PDE4, phosphodiesterase 4; QD, once daily; TID, three times daily; ULN, upper limit of normal; ***sandostatin LAR is the name of the drug product – I cannot find a meaning for LAR, that is just part of the name.

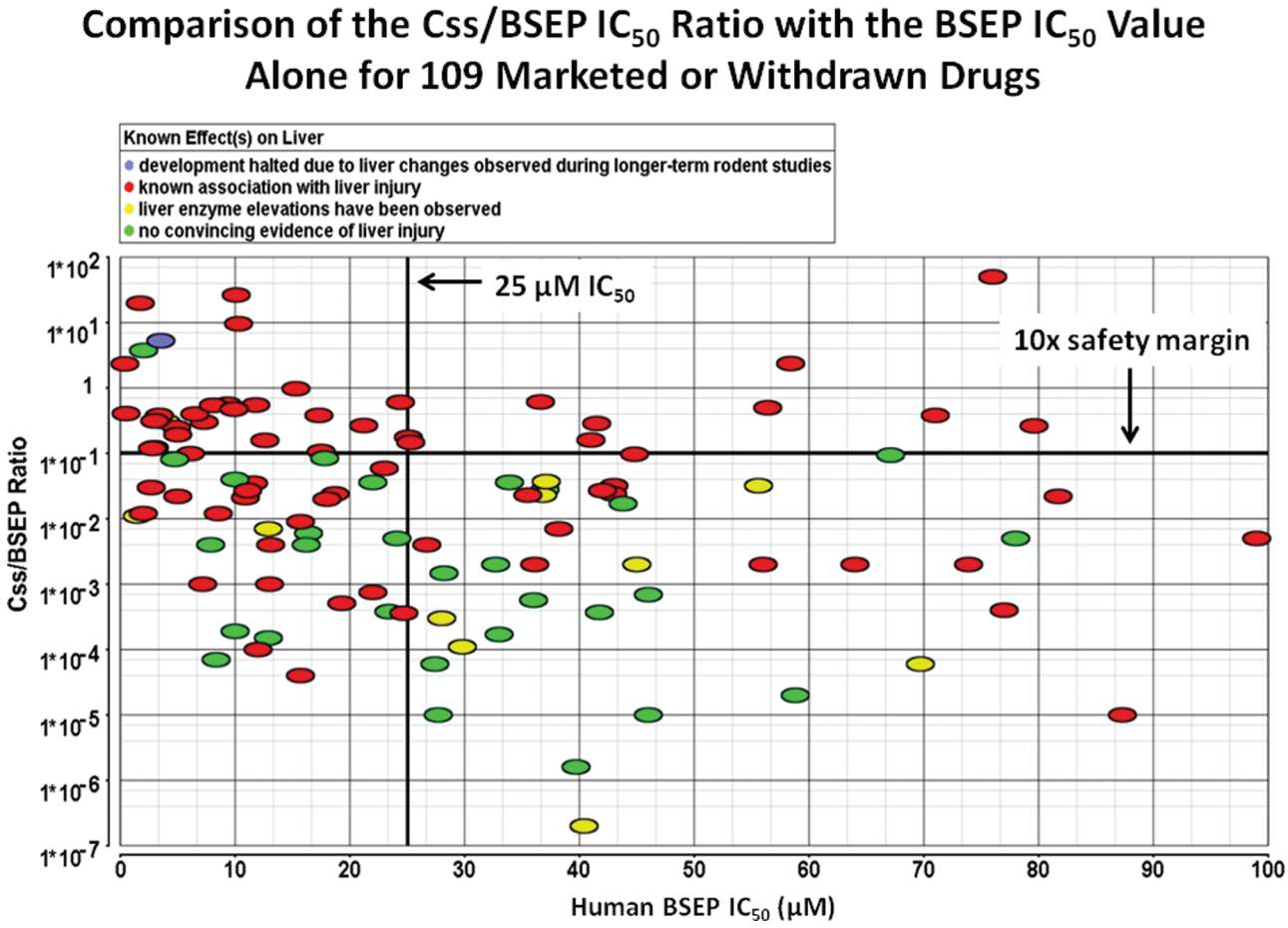

FIG. 4. Dot plot performed in Partek Discovery Suite 6.4, demonstrating how an appreciation for exposure appears to improve the correlation of liver injury in humans with *in vitro* BSEP inhibition. Abbreviation: BSEP, bile salt export pump.

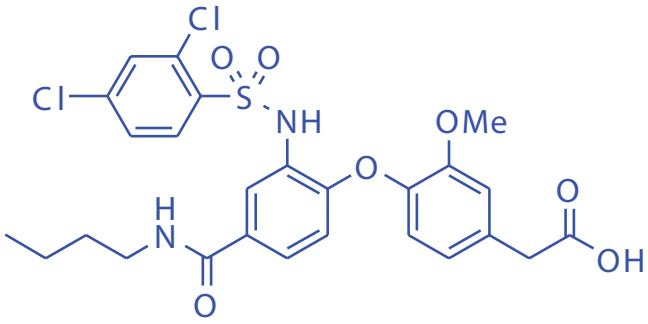

FIG. 5. Chemical structure of AMG 009 (formula weight [FW] = 581.47), a CRTH2/DP dual antagonist being developed for allergic rhinitis and asthma. Due to reversible liver enzyme elevations seen in healthy volunteers, development of this compound was halted. Preclinical models failed to predict the human outcome.

liver injury. Also listed in Table 3 are the identities of the compounds fitting each category of C_{ss}/IC_{50} ratio. In total, 24 compounds populated each of the C_{ss}/IC_{50} ratio categories shown in Table 3, and these 24 compounds represent 14 different drug targets or indications. Multiple compounds for similar targets/indications are listed in Table 4, as well as a summary of the transporters with which they interfered, and these include the PPAR agonists, protease inhibitors, antibiotics, and endothelin antagonists.

DISCUSSION

BSEP inhibition in humans has been implicated as a mechanism of DILI and has been described as a susceptibility factor for DILI based on studies comparing *in vitro* BSEP inhibition data with known clinical outcomes for select benchmark compounds (Dawson *et al.*, 2012; Fattinger *et al.*, 2001; Feng *et al.*, 2009; Funk *et al.*, 2001; Kostrubsky *et al.*, 2003, 2006; Morgan *et al.*, 2010). These association studies are important because neither a reliable biomarker specific for BSEP inhibition nor a toxicologically relevant preclinical model to recapitulate the BSEP-mediated liver injury seen in humans currently exists. The 3 aims of this work were to profile the effect of a large set of benchmark drugs/compounds, representing diverse pharmacological and chemical classes, in a BSEP, MRP2, MRP3, and MRP4 high-throughput *in vitro* screen, to determine if the effect of a

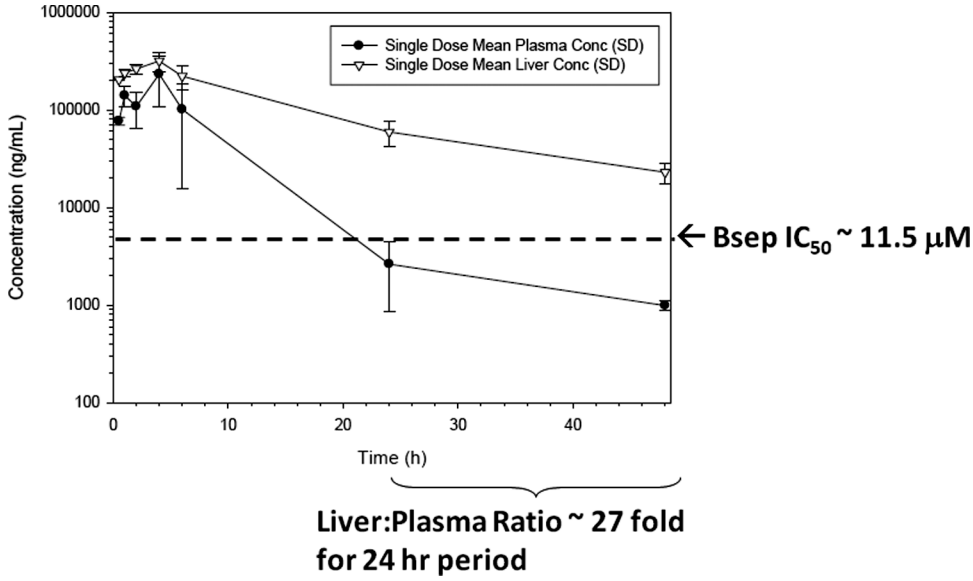

FIG. 6. The concentration of AMG 009 in plasma (closed circles) or liver (open triangles) over a 24 h time course, following a single PO dose. Bars represent SDs. Abbreviations: BSEP, bile salt export pump; conc = concentration.

TABLE 2
Number of Compounds With Evidence of Liver Injury/Total Number of Compounds Fitting Column and Row Criteria (%)

Transporter Assay	C_{ss}/IC_{50} Ratio < 0.01	C_{ss}/IC_{50} Ratio < 0.1	C_{ss}/IC_{50} Ratio ≥ 0.1
BSEP	18/44 (41%)	34/70 (49%)	36/38 (95%)
MRP2	6/9 (67%)	9/13 (69%)	1/1 (100%)
MRP3	7/11 (64%)	17/23 (74%)	5/6 (83%)
MRP4	10/23 (53%)	26/39 (67%)	14/17 (82%)

Notes. The closer exposure values in humans approach *in vitro* potency values in the transporter assays, the stronger the association with liver injury. Conversely, as the exposure values fall further below the *in vitro* potency values, the weaker the association with liver injury.

compound on multiple transporters, including an appreciation for exposure, improved the prediction of liver injury outcome in humans, and to recommend how such a transporter panel could be used to improve therapeutic compound development. These aims also sought to solidify the position of our previous association study between BSEP inhibition and DILI (Morgan *et al.*, 2010).

As shown in Figure 3, the majority of compounds had little or no effect on the 4 transporters evaluated here. The distinct patterns of effect across the transporter panel suggest that the inhibition that is seen is not likely due to compromised membrane integrity but rather actual transporter interaction. The canalicular efflux transporter, MRP2, and the basolateral efflux transporters, MRP3 and MRP4, were selected to compliment BSEP based on their respective roles under cholestatic conditions. MRP2 manages the canalicular excretion of phase 2 conjugated BAs (eg, sulfated or glucuronidated BAs). MRP3 and MRP4, however, manage the elimination of BAs into the blood under cholestatic conditions to help mitigate BA accumulation. Therefore, we hypothesize that compounds that inhibit BSEP and one or more of these other BA transporters may be at an even greater risk of DILI. To test this hypothesis, the effect of compounds on the transporter panel was related to clinical outcomes. These comparisons are summarized in Tables 2–4 and argue that knowledge of the effect of a BSEP inhibitor on an MRP panel does improve the prediction of DILI.

These data also show that this approach cannot discriminate the incidence or severity of DILI, or the idiosyncratic hepatotoxicants from the intrinsic. For example, rosiglitazone and pioglitazone have only rare instances of liver injury, whereas bosentan is more frequent (approximately 11% of the patient population shows transaminase elevations), yet these 3 drugs interact with multiple transporters in this panel. However, the PPAR agonists may not be a good example of BSEP inhibitors and liver injury given the role played by PPARα in BA homeostasis, possibly imparting protection against BA accumulation due to BSEP inhibition (Li *et al.*, 2012; Zollner *et al.*, 2006, 2010). Indeed, Li *et al.* showed that PPARα-null mice fed a diet rich in cholic acid showed severe hepatotoxicity, whereas the wild-type mice fed the same diet showed no evidence of liver injury. This work demonstrates that PPARα activity can ameliorate the hepatocellular accumulation of BAs. Although PPARα is more abundant in liver than PPARγ, it is feasible that PPARγ activity could also participate in BA homeostasis (Rogue *et al.*, 2010). And, although rosiglitazone is a relatively pure PPARγ agonist, pioglitazone and troglitazone do have some PPARα activity. Nevertheless, the data set presented here indicates that other endpoints, *in vitro* or *in vivo*, need to be

TABLE 3
Number of Compounds With Evidence of Liver Injury/Total Number of Compounds Fitting Column and Row Criteria (%)

Transporter Assay	BSEP C_{ss}/IC_{50} Ratio < 0.1	BSEP C_{ss}/IC_{50} Ratio ≥ 0.1	Compounds With BSEP C_{ss}/IC_{50} Ratio ≥ 0.1
MRP2 stimulation or stimulation/inhibition	8/10 (80%)	6/6 (100%)	Sitaxsentan, irbesartan, tolcapone, rosiglitazone, pioglitazone, troglitzone
MRP2 C_{ss}/IC_{50} ratio < 0.1	6/10 (60%)	3/3 (100%)	Benzbromarone, rifampicin, cyclosporine A
MRP2 C_{ss}/IC_{50} ratio ≥ 0.1	None found	1/1 (100%)	Rifapentine
MRP3 C_{ss}/IC_{50} ratio < 0.1	10/16 (63%)	7/7 (100%)	Bosentan, rifampicin, sitaxsentan, saquinavir, indinavir, troglitazone, rifapentine
MRP3 C_{ss}/IC_{50} ratio ≥ 0.1	0/1 (0%)	5/5 (100%)	Irbesartan, lopinavir, tolcapone, ritonavir, [a]MK-571
MRP4 C_{ss}/IC_{50} ratio < 0.1	16/29 (55%)	10/10 (100%)	Nefazodone, pioglitazone, rosiglitazone, troglitazone, sitaxsentan, saquinavir, irbesartan, ketoconazole, nelfinavir, [a]MK-571
MRP4 C_{ss}/IC_{50} ratio ≥ 0.1	2/4 (50%)	12/13 (92%)	Benzbromarone, pranlukast, rifampicin, telithromycin, tranilast, lopinavir, rifapentine, tolcapone, deferasirox, pazopanib, ritonavir, mycophenolate mofetil, [b]dipyridamole

Notes. A multifactorial approach to risk assessment that includes a measurement of BSEP and MRP interference, and accounts for exposure in humans, demonstrates an improved prediction of liver injury.
[a]MK-571 was never marketed due to liver changes observed during longer term rodent studies.
[b]Dipyridamole is not associated with DILI.

included to provide better fidelity as to what compounds are likely to cause blatant liver injury during clinical trials versus those that are associated with a more idiosyncratic-like DILI signal and the compounds that fall somewhere in between. Inclusion of effects to mitochondria, reactive metabolite formation, CYP inhibition, physicochemical properties, and transporter interactions, as well as several other parameters may move predictive safety efforts closer to having such resolution on the early detection of DILI. The data set and annotations provided in this work are a step in this direction, a multifactorial approach to hazard identification.

In the present work, 635 compounds were evaluated for their effect on BSEP (623 for their effect on BSEP and the MRPs), and potency values were derived via nonlinear regression and described as IC_{50} values. For translation to humans, evidence of liver injury and exposure data (AUC values) were annotated for 109 benchmark drugs that showed some effect on BSEP transport. Given that the burden to liver is usually unknown, and the potential for accumulation as described elsewhere (Feng *et al.*, 2009; Hamadeh *et al.*, 2010), and as was demonstrated with AMG 009 in this work, the use of total drug for the purpose of hazard identification would seem appropriate. However, as the methodologies for assessing intracellular free fractions evolve and become more routine, this measurement would likely provide the most accurate assessment of the amount of compound available to interact with efflux transporters.

Figure 4 shows how appreciation for exposure can improve the prediction of adverse outcome (DILI) as it relates to *in vitro* BSEP inhibition. Of the compounds with a BSEP IC_{50} value of 25μM, 79% of them were correctly identified as being associated with DILI. In comparison, of the compounds with a C_{ss}/BSEP IC_{50} ratio ≥ 0.1, 95% of them were correctly identified as having an association with DILI. Although the use of cutoff values, such as 25μM in the case of BSEP, can serve its purpose in early screening strategies to triage compounds, a more accurate risk assessment will account for known or projected exposures. Also illustrated in Figure 4 are compounds with C_{ss}/BSEP IC_{50} ratios < 0.1 with some association with DILI. The lower the exposure value or C_{ss}, in these cases, the less likely BSEP inhibition is a contributing factor to the underlying mechanism of DILI, regardless of the *in vitro* potency value. Of interest, however, are the 2 compounds with a C_{ss}/BSEP IC_{50} ratio ≥ 0.1, but no evidence of liver injury (mifepristone and dipyridamole). In the case of mifepristone, an abortifacient, it is typically administered as a single PO dose of 200 or 600 mg. The acute nature of this dosing regimen probably explains why it is not associated with DILI despite having a C_{ss}/BSEP IC_{50} ratio > 1.0. Orally administered dipyridamole is an adjunct therapy with coumarin to prevent postoperative thromboembolic complications following cardiac valve replacement. For this indication, 75–100 mg are taken 4 times daily, according to the drug label. Although the label does refer to rare reports of liver injury, to the best of our knowledge, no convincing evidence of DILI associated with dipyridamole therapy has been published. This is a good example where the measurement of intracellular free fraction may indicate that the total drug estimate does not accurately reflect how much drug is actually interacting with BSEP and MRP4.

There are some important points to consider with regard to this data set. The membrane vesicle assay is not metabolically competent, so *in vivo* metabolism may render a compound more or less active on a given transporter, such as has been shown for troglitazone (Funk *et al.*, 2001; Masubuchi, 2006). Another consideration is that the MRP2, MRP3, and MRP4 probe substrate

TABLE 4
Compounds Affecting Multiple Transporters and Meeting the C_{ss}/IC_{50} Ratios Listed in Table 3

	Transporters Affected	Pharmacology	Known Effect(s) on Liver
Saquinavir	BSEP, MRP3, MRP4	Protease inhibitors	Known association with liver injury
Indinavir	BSEP, MRP3		Known association with liver injury
Lopinavir	BSEP, MRP3, MRP4		Known association with liver injury
Ritonavir	BSEP, MRP3, MRP4		Known association with liver injury
Nelfinavir	BSEP, MRP4		Known association with liver injury
Rifampicin	BSEP, MRP2, MRP3, MRP4	Antibiotics	Known association with liver injury; liver monitoring recommended for some patients
Rifapentine	BSEP, MRP2, MRP3, MRP4		Known association with liver injury
Telithromycin	BSEP, MRP4		Known association with liver injury
Rosiglitazone	BSEP, MRP2, MRP4	PPAR agonists	Known association with liver injury; liver monitoring recommended
Pioglitazone	BSEP, MRP2, MRP4		Known association with liver injury; liver monitoring recommended
Troglitazone	BSEP, MRP2, MRP3, MRP4		Withdrawn from market due to liver injury
Sitaxsentan	BSEP, MRP2, MRP3, MRP4	Endothelin antagonists	Withdrawn from market due to liver injury
Bosentan	BSEP, MRP3		Associated with drug-induced cholestasis
MK-571	BSEP, [a]MRP2, MRP3, MRP4	Leukotriene inhibitors	Development halted due to liver changes observed during longer term rodent studies
Pranlukast	BSEP, MRP4		Known association with liver injury
Mycophenolate mofetil	BSEP, MRP4	Immunosuppressants	Known association with liver injury
Cyclosporine A	BSEP, MRP2		Associated with drug-induced cholestasis
Tranilast	BSEP, MRP4	Inhibits the production of interleukin-6	Known association with liver injury
Irbesartan	BSEP, MRP2, MRP3	Angiotensin II antagonist	Cholestatic jaundice and abnormal liver function tests have been reported
Tolcapone	BSEP, MRP2, MRP3, MRP4	Catechol-O-methyl transferase inhibitor	Known association with liver injury
Benzbromarone	BSEP, MRP2, MRP4	Uricosuric	Known association with liver injury; withdrawn from market in 2003
Nefazodone	BSEP, MRP4	5-HT receptor antagonist	Known association with liver injury; sales discontinued in Canada in 2003
Deferasirox	BSEP, MRP4	Iron chelator	Black box warning of severe and fatal hepatotoxicity
Pazopanib	BSEP, MRP4	Kinase inhibitor	Black box warning of severe and fatal hepatotoxicity
Dipyridamole	BSEP, MRP4	Platelet inhibitor	No convincing evidence of liver injury
Ketoconazole	BSEP, MRP4	Antifungal	10–15% elevated liver enzymes; liver monitoring recommended

Notes. A listing of the drugs shown to affect BSEP and 1 or more of the MRPs, meeting the C_{ss}/IC_{50} ratios shown in table 3. Also detailed are the specific transporters affected and pertinent clinical information. Of the drugs listed in this table, only dipyridamole has no convincing evidence of liver injury. Although MK-571 also showed no evidence of liver injury in humans during clinical trials, trials were nonetheless halted due to liver-related findings in longer-term rodent studies.

[a]An IC_{50} value for MK-571 could not be generated in the MRP2 assay as presented here due to insufficient effect; however, the literature cites MK-571 as an MRP2 inhibitor.

was an estradiol derivative and not a BA. We attempted to use radiolabeled taurocholate as the reporter substrate for MRP3 and MRP4 but were unsuccessful in achieving a large enough signal to support a screening strategy (data not shown). Based on the data presented here, there is value in knowing whether or not a compound interferes with MRP2, MRP3, and MRP4 function even though the probe substrate is not a BA. Finally, for MRP2, only $E_2$17βG was evaluated, representing a substrate of MRP2 that has 2 or more binding sites (Gerk *et al.*, 2004). A single binding site probe substrate, such as the LTC4 or fluoroprobe 5(6)-carboxy-2′,7′-dichlorofluorescein (CDCF) may provide a different dimension to the understanding of MRP2 interactions. For example, others have shown stimulation of $E_2$17βG transport across MRP2 with indomethacin, but inhibition of CDCF transport (Heredi-Szabo *et al.*, 2008).

In summary, relating IC_{50} values to *in vivo* exposure appears to improve the correlation of BSEP inhibition to liver injury in humans. And, knowledge of the effect of a BSEP inhibitor on MRP2, MRP3, and MRP4 improves the correlation with liver injury relative to BSEP inhibition alone. As a screening strategy, it is recommended that BSEP be the first tier through which compounds are triaged, choosing the ones with as little effect on BSEP as possible, and without detriment to pharmacology, pharmacokinetics, or other toxicity endpoints. If a BSEP inhibitor is to be advanced, it is recommended that effects to MRP2, MRP3, and MRP4 be evaluated, and that one relates these effects to known or projected exposures in humans. As shown here, this practice was associated with close to a 100% correct prediction of DILI. A recommended flow scheme for deploying this transporter panel is illustrated in Figure 7. The data set and annotations

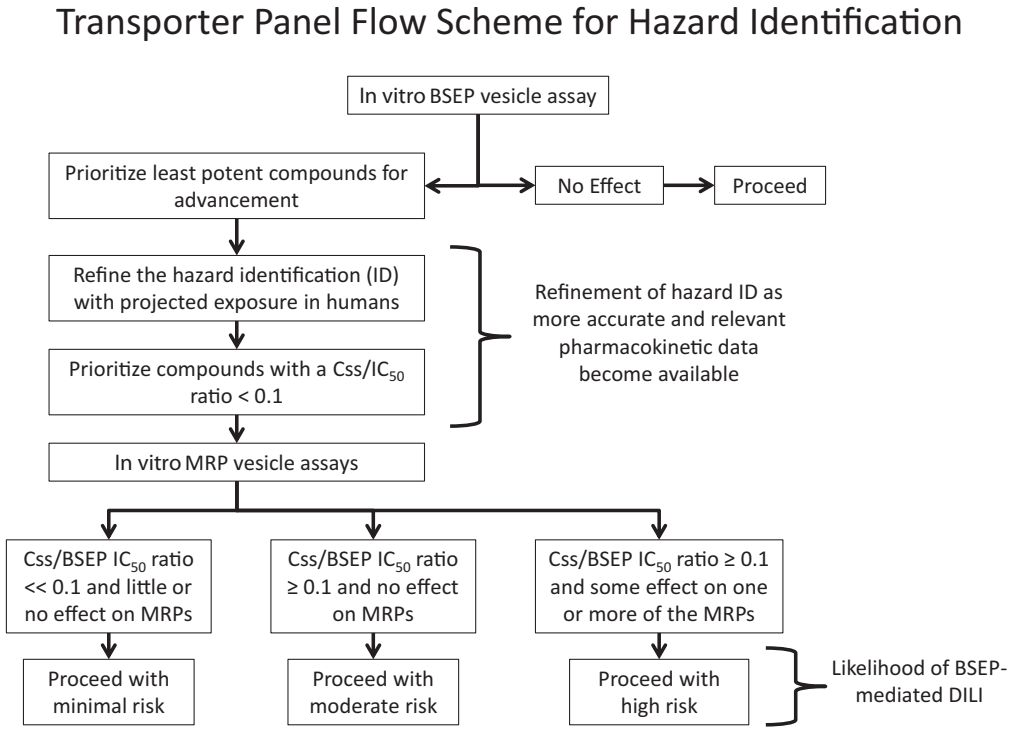

FIG. 7. Flow scheme for deploying a transporter panel during early therapeutic compound development. Abbreviations: BSEP, bile salt export pump; DILI, Drug-induced liver injury; MRP, multidrug resistance-associated protein.

provided here should prove useful in relating the effect of drug candidates in this type of transporter panel to the effect profile of marketed or withdrawn drugs. If the drug candidate has a similar profile to compounds with known associations with liver injury, with consideration of exposure, then this would suggest the drug candidate may have a similar outcome in humans. Finally, until a BSEP/Bsep-specific biomarker is identified, and/or a preclinical animal model that recapitulates the postulated BSEP-mediated liver injury seen in humans, association studies such as the one provided here are the best available tools for preventing or limiting unforeseen DILI via this mechanism.

SUPPLEMENTARY DATA

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

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